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The anti-androgenic fungicide triticonazole induces region-specific transcriptional changes in the developing rat perineum and phallus

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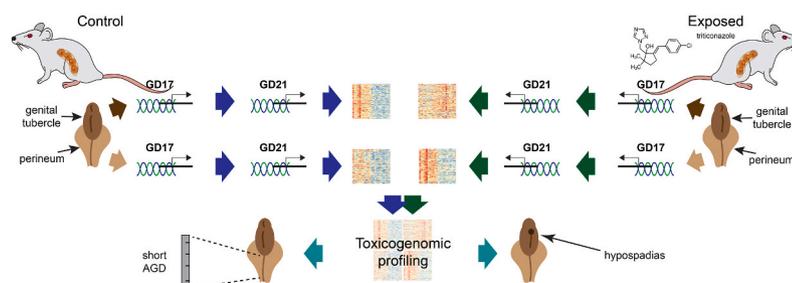
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HIGHLIGHTS

- The azole fungicide triticonazole has anti-androgenic properties.
- Triticonazole alters the transcriptome of fetal male rat external genitalia.
- Expression changes in external genitalia include both AR and ER target genes.
- Phallus and perineum have distinct transcriptional landscapes during development.

GRAPHICAL ABSTRACT



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ABSTRACT

Intrauterine exposure to endocrine disrupting chemicals can interfere with male reproductive development. This can lead to male reproductive disorders such as hypospadias, cryptorchidism and reduced fertility, as well as shorter anogenital distance (AGD) – a biomarker for incomplete androgen-dependent fetal masculinization. However, it remains challenging to predict adverse *in vivo* outcomes based on *in vitro* effect patterns for many chemicals. This is a challenge for modern toxicology, which aims to reduce animal testing for chemical safety assessments. To enable the transition towards higher reliance on alternative test methods, we need to better map underlying mechanisms leading to adverse effects. Herein, we have analyzed the transcriptome of the perineum and phallus of male fetal rats and defined the impacts of exposure to an anti-androgenic fungicide, triticonazole. Previously we have shown that developmental exposure to triticonazole can induce short male AGD, but without a marked effect on the transcriptome of the fetal testes. In contrast, we report here significant changes to the transcriptional landscape of the perineum and phallus, including regional differences between these adjacent tissues. This highlights the importance of analyzing the correct tissue when characterizing mechanisms of complex *in vivo* effect outcomes. Our results provide a rich resource for the spatiotemporal gene networks that are involved in the development of male external genitalia, and that can be disrupted upon exposure to chemicals

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that prevent normal masculinization of the perineum and phallus. Such data will be critical in the development of novel alternative test methods to determine the endocrine disrupting potential of existing and emerging chemicals.

1. Introduction

Male reproductive disorders are common and incidence rates are increasing. These disorders include hypospadias and cryptorchidism, reduced fertility and testis cancers (Skakkebaek et al., 2016). Since the development of male primary and secondary sex characteristics depends on androgens synthesized by the fetal testes, a common origin of disease, the testicular dysgenesis syndrome (TDS) hypothesis was proposed (Sharpe and Skakkebaek, 2008; Skakkebaek et al., 2001). However, in addition to androgen signaling being perturbed at the level of the testes, it can also impact peripheral tissues directly. This can occur by different mechanisms, for instance by directly blocking nuclear receptor activation, or by sourcing androgen precursor steroids from tissues other than the testes via an alternative, backdoor pathway as for instance is the case in wallabies (Renfree et al., 1996) and humans (O'Shaughnessy et al., 2019).

Endocrine disrupting chemicals (EDCs) can cause male reproductive disorders by either disrupting testis development or interfering with testicular androgen synthesis. However, when considering adverse reproductive outcomes such as hypospadias, it is important to consider that anti-androgenic EDCs can also induce male reproductive disorders by directly affecting the development of target tissues and not necessarily secondary to disrupted testis development. During male development, differentiation of androgen-dependent tissues require the activation of the androgen receptor (AR). Although testicular testosterone is a ligand for the AR in many organs and tissues, AR activation requires the more potent ligand dihydrotestosterone (DHT), which is synthesized locally by the enzyme 5α -reductase, not least in tissues such as the external genitalia, the perineum, and the developing prostate (Azzouni et al., 2012; Davey and Grossmann, 2016; Imperato-McGinley et al., 1992). Thus, EDCs with a capacity to either inhibit 5α -reductase or block AR activity can perturb the masculinization process without directly affecting the testes.

When considering reproductive toxicity studies using intact animals, knowing the exact underlying mechanism of action causing adverse effect outcomes is not necessarily essential in that the adversity is observable regardless. However, there is a need from both an ethical and practical standpoint to develop alternative test methods and strategies for chemical toxicity evaluations. For this, however, the mechanisms of effects must be properly mapped out in order to employ the correct methodologies and measuring the correct parameters. This also holds for male reproductive toxicity despite decades of research and the identification of several key signaling pathways that, when perturbed, can cause adverse outcomes (Svingen et al., 2022). Due to their extreme sensitivity to hormonal perturbations, the external genitalia and perineum (the region between the anus and phallus) are obvious tissues to focus on for the identification of target pathways. Hypospadias, a malformation of the phallus, is among the most common birth defects in newborn boys (Baskin, 2000; Nassar et al., 2007), whereas the anogenital distance (AGD) measured between anus and external genitalia is a sensitive morphological biomarker for compromised masculinization during fetal life (Schwartz et al., 2019a).

We recently reported that fetal exposure to the azole fungicide triticonazole induced a short AGD in male rat offspring (Draskau et al., 2019). *In vitro*, triticonazole was shown to be a strong AR antagonist and weak steroidogenesis inhibitor, suggesting that the driving mechanisms behind a short AGD was not caused by reduced testosterone output from the testes, but rather perturbed AR signaling in the target tissue; the perineum. Indeed, transcriptional profiling of the fetal testes from these exposed animals revealed very little changes (Draskau et al., 2021).

Thus, to explore further how triticonazole, and possibly many other EDCs, potentially disrupt male reproductive development through various mechanisms in different target tissues, we aimed to perform transcriptional analyses of both the perineum and developing phallus in the same animals. With this approach we not only establish how male reproductive disorders can arise by local chemical-molecule interactions in target tissues, but potentially also point towards alternative mechanisms that can be involved in disease development, an area that has been highlighted as important for future assessment of endocrine disrupting potentials (Martyniuk et al., 2022; OECD, 2012).

2. Materials and methods

2.1. Chemicals

Triticonazole (CAS No. 131983-72-7) was purchased from Abcam (>95% pure, #ab143728, lot no. GR3232419-3(N/A), Cambridge, UK). Corn oil (Sigma-Aldrich, #C867-2.5 L, Denmark) was used as both vehicle and control substance.

2.2. Animal study

The *in vivo* rat toxicity study was previously described in (Draskau et al., 2019). Briefly, time-mated Sprague-Dawley rats (CrI:CD (SD), 9 weeks old) (Charles River Laboratories, Sulzfeld, Germany, via SCANBUR, Karlslunde, Denmark) were delivered to the Technical University of Denmark (DTU) animal facilities on gestational day (GD) 3. The day following overnight mating was termed GD1. On GD4, dams were weighted and assigned to control and treatment groups (12 dams/group) with similar body weight (bw) distributions. Animals were housed in pairs (until GD17) or individually (GD17-21) under standard conditions with 12 h light/dark cycles in ScanTainers (Ventilated Cabinets from SCANBUR, Karlslunde, Denmark) with 50–60 air changes per hour at $22\text{ }^{\circ}\text{C} \pm 1$ and humidity kept at $55\% \pm 5$. High Temperature polysulfone cages with wood chip bedding, nesting material and a wooden shelter (Tapvei, Denmark). Animals were fed Altromin 1314 (Altromin GmbH, Lage, Germany, soy and alfalfa-free) and tap water was freely available in polysulfone bottles (84-ACBT0702SU Tecniplast, VA, Italy). Exposure was initiated at GD7 and continued until necropsy on GD17 or 21. Rats were weighed and gavaged each morning with vehicle (corn oil) or triticonazole (450 mg/kg bw/day) with a dosing volume of 2 ml/kg bw. The dose of 450 mg/kg bw/day triticonazole was based on literature studies and selected as this was the highest achievable dose not expected to induce any toxicity in the pregnant rat dams.

2.3. Necropsy

Dams were exposed 1 h \pm 15 min before decapitation under CO_2/O_2 -anesthesia on GD17 or 21. Uteri were collected, weighed, and the number of live fetuses, location in uterus, implantations, and resorptions were registered. Bw and any anomalies of the fetuses were recorded. AGD was measured using a stereomicroscope with a micrometer eyepiece by an experienced technician blinded with respect to treatment groups. Fetal testes and the perineum including the genital tubercle (GT) were harvested under a stereomicroscope, placed in RNAlater (Thermo Fisher Scientific) and stored at $-80\text{ }^{\circ}\text{C}$ until further processing. All animal procedures were performed in accordance with relevant regulations and guidelines. The animal experiment had ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553).

2.4. RNA extraction

Fine dissection of the perineum and GT from GD17 and GD21 male fetuses was carried out under a stereomicroscope, then immediately subjected to RNA extraction. The GT was separated from the surrounding tissue and the perineum was isolated from the same fetal males

and processed as individual samples, as previously described (Schwartz et al., 2019b). Total RNA was extracted (n = 11–12 fetuses/group) using an RNeasy Micro Kit (Qiagen, Germany) according to manufacturer’s instructions, including on-column DNase I digestion. RNA quantity and quality were assessed using a Nanodrop-1000 spectrophotometer and a 2100 Bioanalyzer Instrument (Agilent Technologies, CA, USA) following

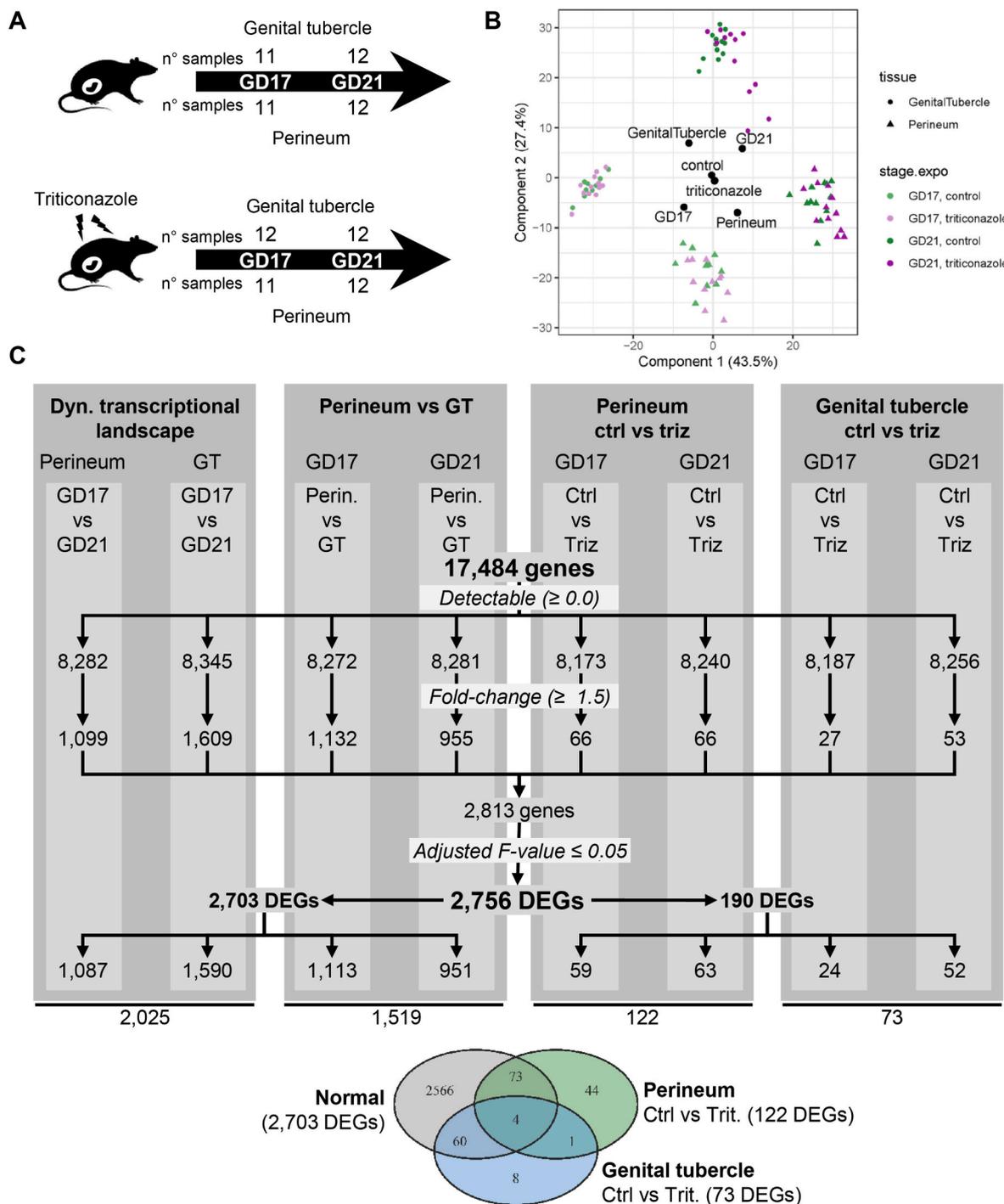


Fig. 1. Differentially expressed genes (DEGs) in controls and triticonazole (triz)-exposed fetal perineum (perin.) and genital tubercle (GT). A) We analyzed the transcriptome of the developing perineum and GT at gestational day (GD) 17 and GD21 in triz-exposed rats vs control rats. B) The two first dimensions of a principal component analysis (explaining 43.5% and 27.4% of variances) on the resulting gene expression dataset clearly discriminated tissues and developmental stages. C) DEGs with a fold change ≥ 1.5 were analyzed for significance using adjusted F-value ≤ 0.05 . BRB-profiling detected 2756 DEGs. The listed numbers of DEGs refer to unique genes within each analysis, so that genes shared between groups are not counted repeatedly (i.e. for the GT the 73 DEGs represent 73 unique genes amongst the 76 (24 + 52) DEGs observed between control and exposed tissues at GD17 and GD21). DEGs are listed for controls at GD21 compared to controls at GD17 in perineum and GT (temporal and spatial changes) and in exposure groups compared to control at GD17 and at GD21 in perineum and GT (toxicological footprint). The Venn Diagram displays the number of DEGs shared between groups. N = 11–12/group.

manufacturer's instructions. Only samples with an RNA integrity number (RIN)-score >7 were included for RNA sequencing.

2.5. Bulk RNA barcoding and sequencing (BRB-seq)

Sample preparation for the BRB-seq experiment (Alpern et al., 2019) was performed at the Research Institute for Environmental and Occupational Health (Irset, Rennes, France) as previously described (Pham et al., 2020; Saury et al., 2018). Briefly, a first step of reverse transcription and template switching reactions were performed using 4 μ L total RNA at 2.5 ng/ μ L. Then cDNA was purified and double-strand (ds) cDNAs were generated by PCR. The sequencing libraries were built by tagmentation using 50 ng of ds cDNA with the Illumina Nextera XT Kit (Illumina, #FC-131-1024) following the manufacturer's recommendations. The IntegraGen Company (<https://integragen.com/fr/>) sequenced the resulting library on a NovaSeq sequencer following Illumina's instructions. Image analysis and base calling were performed using RTA 2.7.7 and bcl2fastq 2.17.1.14. Adapter dimer reads were removed using DimerRemover (<https://sourceforge.net/projects/dimerremover/>).

2.6. Data preprocessing and quality control

Quality control and preprocessing of data were performed by

SciLicium (Rennes, France). The first read contains 16 bases that must have a quality score higher than 10. The first 6 bp correspond to a unique sample-specific barcode and the following 10 bp to a unique molecular identifier (UMI). The second reads were aligned to the rat reference transcriptome from the UCSC website (release rn6, downloaded August 2020) using BWA version 0.7.4.4 with the parameter "-l 24". Reads mapping to several positions in the genome were filtered out from the analysis. The pipeline is described in (Giacosa et al., 2021). After quality control and data preprocessing, a gene count matrix was generated by counting the number of unique UMIs associated with each gene (lines) for each sample (columns). The UMI matrix was further normalized with the regularized log (rlog) transformation package implemented in the DeSeq2 package (Love et al., 2014). Raw and pre-processed data were deposited at the GEO repository under the accession number GSE199234 (Edgar et al., 2002). The resulting transcriptomic signatures were also deposited at the TOXsign repository under the accession number TSP1267 (<https://toxsign.genouest.org/>) (Darde et al., 2018).

2.7. Differential gene expression analysis

The following statistical comparisons were made in the AMEN suite of tools (Chalmel and Primig, 2008): GD17 perineum controls vs GD21 perineum controls, GD17 phallus controls vs GD21 phallus controls,

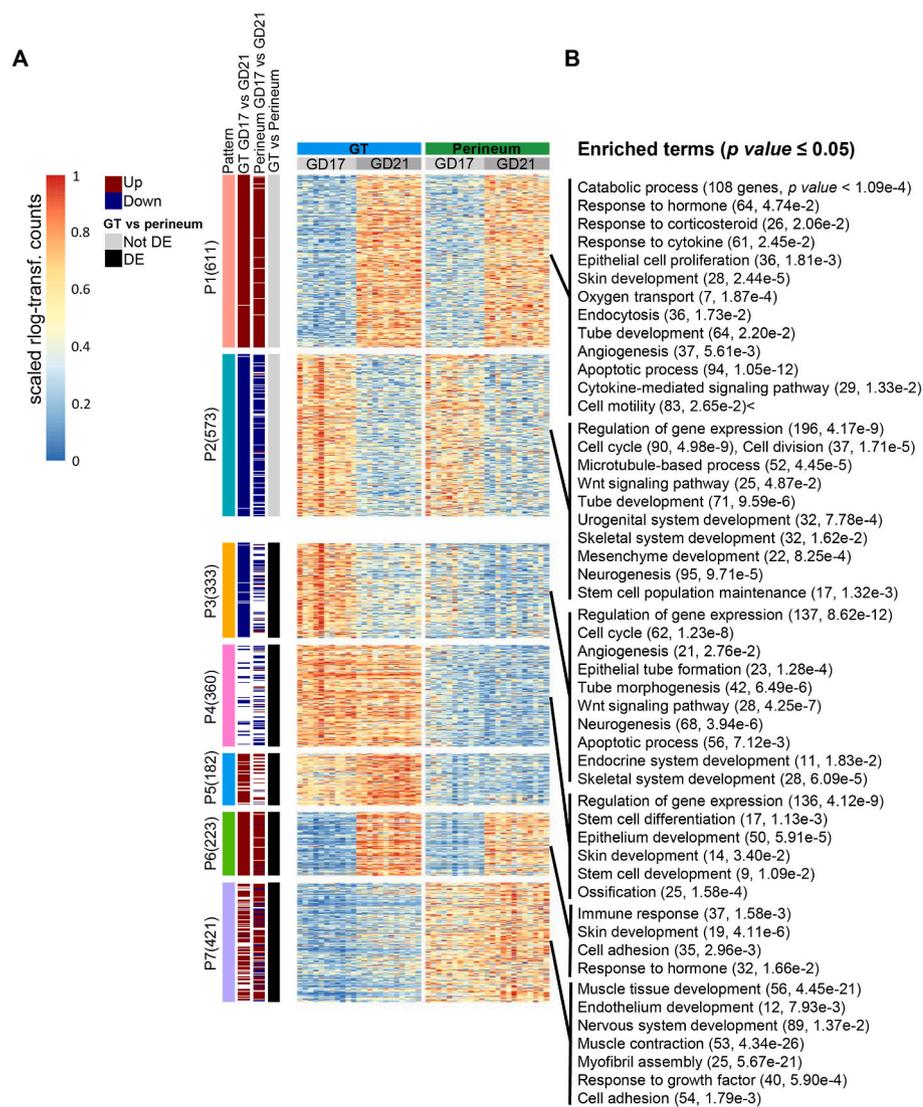


Fig. 2. The dynamic transcriptional landscape in the developing perineum and genital tubercle (GT). **A**) Heatmap representation of differentially expressed (DE) genes (DEGs) in rat perineum and GT from gestational day (GD) 17 to GD21 of development and between tissues. Overall, BRB-profiling detected 2703 DEGs that could be divided into seven expression patterns P1-7 (the number of DEGs in each pattern is listed inside brackets). Red and blue colored bars indicate up (red) or down (blue) regulated genes for GT and perineum when comparing the transcriptomes at GD17 vs GD21. **B**) Significantly enriched biological process terms (Supplementary Table S2) are given followed by the total number of genes associated with the term and the p values. $N = 11-12$ samples/group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GD17 phallus controls vs GD17 perineum controls, GD21 phallus controls vs GD21 perineum controls, GD17 triticonazole-exposed samples vs GD17 controls, and GD21 triticonazole-exposed samples vs GD21 controls (Fig. 1C). Briefly, genes showing an expression signal higher than a given background cutoff (corresponding to the overall median of the rlog-transformed UMI dataset, 0.0) and at least a 1.5-fold change between the group expression values were selected. To define a set of 2756 genes with significant statistical changes across comparisons, the linear models for microarray data (LIMMA) package was used (F-value adjusted with the false discovery rate (FDR) method, $p \leq 0.05$) (Smyth, 2004).

2.8. Functional analysis

A Gene Ontology enrichment analysis was performed with AMEN (Chalmel and Primig, 2008). A specific annotation term was considered enriched in a gene cluster when the FDR-adjusted p-value was < 0.01 (Fisher's exact probability).

3. Results

3.1. Temporal changes to the transcriptomes of rat perineum and GT between GD17 and GD21

We analyzed the transcriptome of the developing perineum and GT during fetal life, at GD17 and GD21 in both triticonazole-exposed and unexposed control rats (Fig. 1A). The two first dimensions of a principal component analysis (explaining 43.5% and 27.4% of variances) on the resulting gene expression dataset clearly discriminated tissues and developmental stages, but independently of their exposure (Fig. 1B). We next identified a set of 2703 statistically significantly differentially expressed genes (DEGs) comparing dynamic transcriptional variations between developmental stages (GD17 vs GD21) in perineum (1087 DEGs) and in GT (1590 DEGs), and when comparing perineum and GT at GD17 (1113 DEGs) and GD21 (951 DEGs) from control animals (Fig. 1C). Overall, a set of 2025 genes constituted the temporal changes during late gestation in perineum and GT (when comparing their transcriptomes at GD17 and GD21), whereas 1519 genes made up the spatial differences at GD17 and GD21 (when comparing these two tissues, the perineum and GT) (Fig. 1C).

The 2703 DEGs from the above comparisons were subsequently partitioned into seven expression patterns (named P1–P7, Fig. 2A, Supplementary Table S1) and functional analysis was performed to associate the expression patterns to biological processes (Fig. 2B, Supplementary Table S2). Pattern P1 (611 DEGs) showed increased expression from GD17 to GD21 in both tissues and was associated with genes involved in catabolic processes (108 DEGs, p value $< 1.09e-4$), response to hormones (64 DEGs, p value $< 4.74e-2$), tube development (64 DEGs, p value $< 2.20e-2$), apoptotic process (94 DEGs, p value $< 1.05e-12$) and others. P2 (573 DEGs) showed decreased expression from GD17 to GD21 in both tissues and was associated with terms such as regulation of gene expression (196 DEGs, p value $< 4.17e-9$), neurogenesis (95 DEGs, p value $< 9.71e-5$), and cell cycle (90, p value $< 4.98e-9$).

Patterns 3–7 (P3–P7) displayed distinct expression profiles between GT and perineum. Briefly, P3 (333 DEGs), P4 (360 DEGs) and P5 (182 DEGs) comprised genes with high expression in GT at GD17 and/or GD21 as compared to perineum. P3 and P4 both included genes associated with regulation of gene expression (137 and 136 DEGs, p values $< 8.62e-12$ and $< 4.12e-9$, respectively), whereas pattern P5 did not associate with any biological process terms. Similar to P1, P6 (223 DEGs) contained genes showing an increased expression profile from GD17 to GD21 in both tissues, but with slightly lower expression in perineum. Similarly to P1, P6 was associated with genes involved in response to hormones (32 DEGs, p value $< 1.66e-2$) and skin development (19 DEGs, p value $< 4.11e-6$). Finally, P7 (421 DEGs) corresponded

to genes associated with a higher expression in perineum than in GT at GD17 and GD21 and was associated uniquely with genes involved in muscle tissue development (56 DEGs, p value $< 4.45e-21$), endothelium development (12 DEGs, p value $< 7.93e-3$), nervous system development (89 DEGs, p value $< 1.37e-2$), muscle contraction (53 DEGs, p value $< 4.34e-26$), myofibril assembly (25 DEGs, p value $< 5.67e-21$), and response to growth factor (40 DEGs, p value $< 5.90e-4$). Overall, these 2703 genes define the temporal and spatial transcriptional landscape of normal perineum and GT in the rat at late gestation.

3.2. Triticonazole exposure induced significant transcriptional changes in the rat perineum and GT during late gestation

We identified a set of 190 DEGs associated with significant changes when comparing transcriptional changes in control perineum at GD17 (59 DEGs) and GD21 (63 DEGs) with those from triticonazole-exposed animals, as well as when comparing control GT at GD17 (24 DEGs) and GD21 (52 DEGs) with those from triticonazole-exposed animals (Fig. 1C). The 122 DEGs in the perineum were subsequently partitioned into four expression patterns denoted Q1–Q4 (Fig. 3, Supplementary Table S1). The patterns Q1 (11 DEGs) and Q2 (45 DEGs) showed increased transcript levels at GD17 and GD21 after triticonazole exposure, whereas patterns Q3 (52 DEGs) and Q4 (14 DEGs) showed decreased transcript levels at GD17 and GD21 after triticonazole exposure. We then performed a functional analysis to associate the resulting expression patterns to biological processes (Fig. 3, Supplementary Table S2). Patterns Q1 and Q2 were associated with genes involved in muscle structure development (14 DEGs, p value $< 1.16e-4$), muscle contraction (10 DEGs, p value $< 1.60e-4$), and regulation of calcium-mediated signaling (6 DEGs, p value $< 3.17e-2$), while patterns Q3 and Q4 did not associate with any biological process terms. Patterns Q2 and Q3 both contained known target genes for AR (*Rbfox1*, *Actr3*, *Cpsf7*, *Klhl5*, and *Srek1*) and ER (*Cidec*, *Lpl*, *Bmpr2*, *Elk3*, and *Kdm2a*) (Fig. 3).

The 73 identified DEGs in the GT were partitioned into six expression patterns, denoted R1–R6 (Fig. 4, Supplementary Table S1). Pattern R1 (8 DEGs) showed increased transcript levels after triticonazole exposure compared to controls at GD17. Patterns R2 (6 DEGs) and R3 (13 DEGs) displayed increased transcript levels after triticonazole exposure compared to controls at GD21. Pattern R4 showed decreased transcript levels after triticonazole exposure compared to controls at GD17, whereas patterns R5 (17 DEGs) and R6 (15 DEGs) showed decreased transcript levels at GD21 after triticonazole exposure. Neither of the patterns were associated with any significantly enriched biological process terms after functional analysis (Fig. 4, Supplementary Table S2). However, the DEGs included genes associated with hypospadias in humans (Fig. 4, Table 1) and patterns R1, 2, 4, and 6 all contained AR and ER target genes (Fig. 4, Table 1).

4. Discussion

Intrauterine exposure to EDCs can cause a range of male reproductive disorders, many by incomplete androgen action during critical developmental windows. Androgen signaling is not the only critical signaling pathway, however, and the underlying mechanisms of disease development are complex. This is evident by the fact that it remains difficult to predict *in vivo* adverse effect outcomes based on *in vitro* effects, including well-defined androgen-sensitive phenotypes such as hypospadias or short male AGD (Svingen et al., 2022). This is challenging when it comes to developing chemical testing strategies that do not rely on animal toxicity studies. To be able to rely on alternative test method data to predict *in vivo* effect outcomes, we must ensure that the correct tests are used and interpreted correctly. And for this to be possible, we must first ensure we have identified the key mechanisms of action in the right tissues at the right life stages and use this knowledge to develop and employ the correct methods for testing purposes.

In this study, we have focused on characterizing key molecular

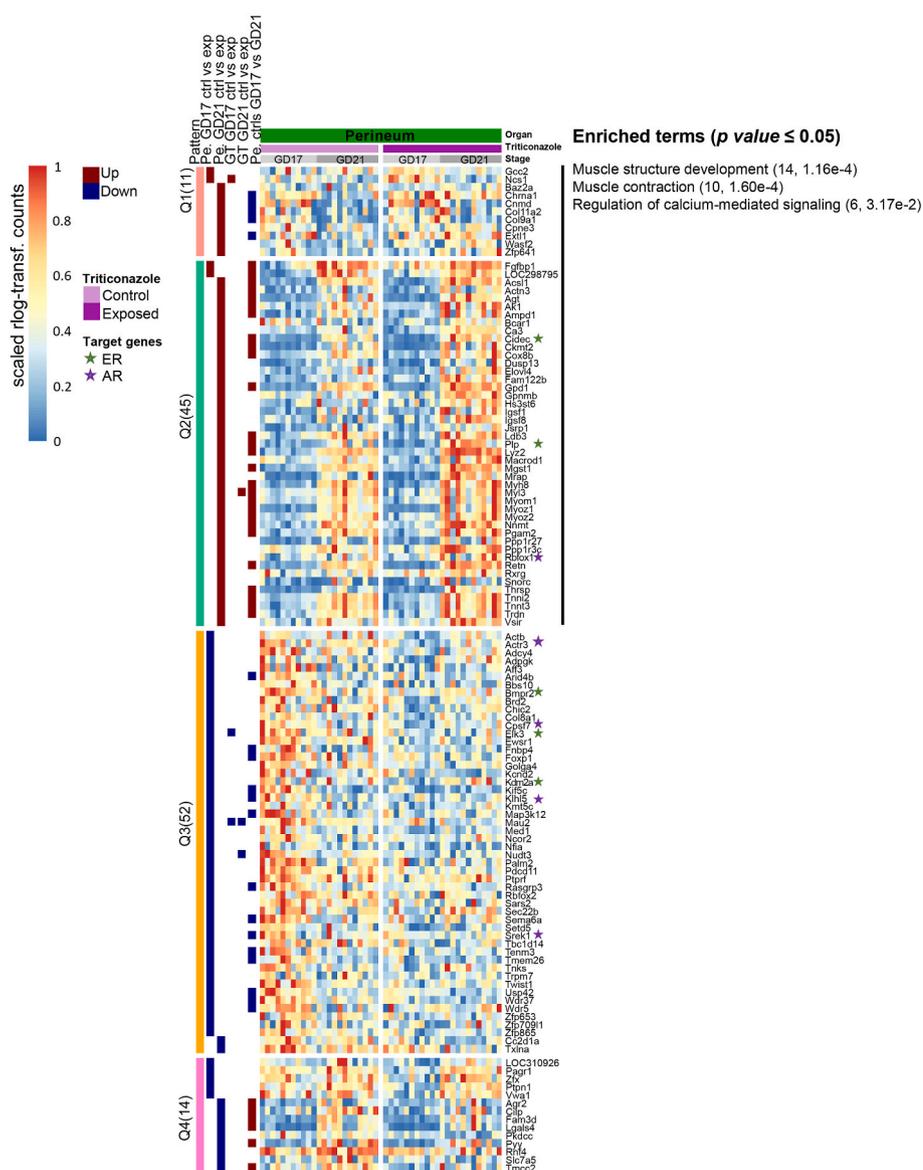


Fig. 3. Transcriptional changes in the developing perineum after triticonazole exposure. Heatmap representation of differentially expressed (DE) genes (DEGs) in rat perineum at gestational day (GD) 17 and GD21. Overall, BRB-profiling detected 122 DEGs that could be divided into four expression patterns Q1-4 (the number of DEGs in each pattern is listed inside brackets). Significantly enriched biological process terms (Supplementary Table S2) are given followed by the total number of genes associated with the term and the p values. $N = 11-12$ samples/group. Pe. = perineum, GT = genital tubercle, ctrl = control, exp = exposed, AR = androgen receptor, ER = estrogen receptor.

changes in two steroid hormone sensitive tissues, the perineum and developing phallus (the male GT). By performing transcriptome analyses of these tissues at late gestational stages, GD17 and GD21, we have mapped clear differences in expression for a large number of genes in rats. This data set is in itself a valuable resource that can be used to map species differences and similarities. Firstly to compare to mouse transcriptional landscapes for which much data already exist (Amato and Yao, 2021; Tarulli et al., 2022), and later to compare to human tissues as they become available; but also to decipher, in more detail, the involvement of other signaling pathways for development of reproductive disorders typically attributed to steroid hormone signaling. We have not analyzed these data to their full potential herein, as the focus of this study was on transcriptional changes that have been induced by *in utero* exposure to the anti-androgenic fungicide triticonazole. Notably, however, the expression data for the control tissues highlight the fact that the perineum and GT are truly distinct tissues at late gestational development, with both similar and dissimilar expression patterns.

We have previously shown that *in utero* exposure to triticonazole from GD7 onwards results in a shorter male AGD in rats (Draskau et al., 2019). The same study also determined that triticonazole was a potent AR antagonist *in vitro* and, at the same time, did not significantly affect testosterone biosynthesis *in vivo*. Hence, we predicted that triticonazole

would exert its anti-androgenic effects in peripheral androgen-sensitive tissues and not necessarily affect testis development or function. We have shown, in a second study, that indeed, the fetal rat testis transcriptome was almost unaffected by *in utero* triticonazole exposure (Draskau et al., 2021). Analyzing the external genitalia of the same animals would therefore be of great interest, as it allows for a multi-organ comparison and a more holistic approach to characterize the mechanism involved in the development of male reproductive disorders caused by exposure to EDCs.

With respect to animals exposed to the azole fungicide triticonazole, BRB-seq revealed 122 DEGs between control and exposed male perineal tissues that could be clustered into four patterns, Q1-4. Patterns Q1 and Q2 included genes related to muscle structure development, contraction, and regulation of calcium-mediated signaling. In these patterns, gene transcripts were increased at GD21 in response to gestational exposure to triticonazole. This corroborates the fact that the development of the perineum, and in particular the muscle cells, is sensitive to disruption of androgen action. This has also been shown in rat fetuses exposed to the 5 α -reductase inhibitor finasteride, albeit the perineum and phallus were not separated for transcriptome analysis, but rather sequenced as one tissue (Schwartz et al., 2019b). Herein, we identified five DEGs related to ER signaling as well as five DEGs related to AR signaling. This suggests

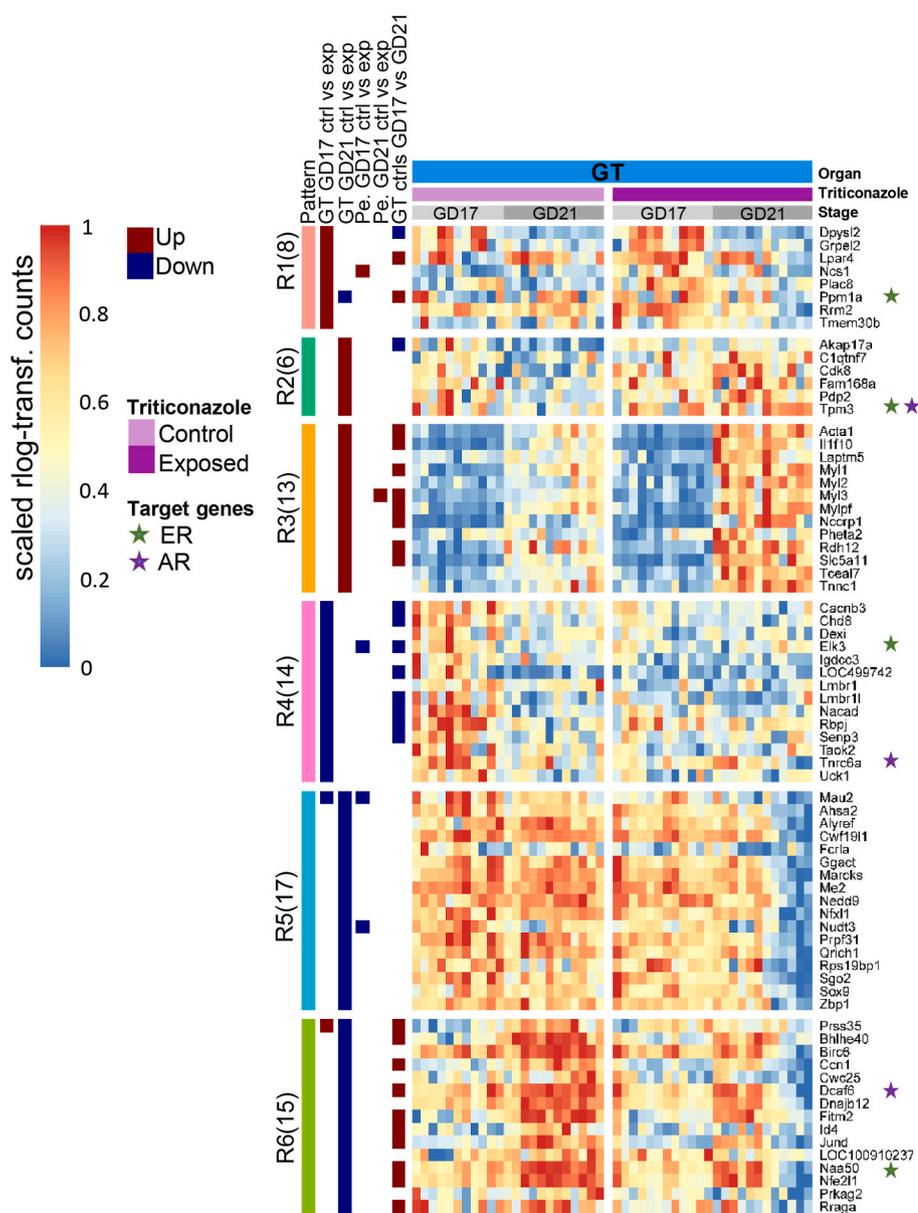


Fig. 4. Transcriptional changes in the developing genital tubercle (GT) after triticonazole exposure. Heatmap representation of differentially expressed (DE) genes (DEGs) in rat GT at gestational day (GD) 17 and GD21. Overall, BRB-profiling detected 73 DEGs that could be divided into six expression patterns R1-6 (the number of DEGs in each pattern is listed inside brackets). N = 11–12 samples/group. Pe. = perineum, ctrl = control, exp = exposed, AR = androgen receptor, ER = estrogen receptor.

that AR signaling in the perineum may not be functioning independently of ER signaling and in agreement with a postulated dual targeting of androgen and estrogen signaling in external genitalia (Govers et al., 2019; Mattiske and Pask, 2021).

In a previous study, finasteride exposure was shown to affect estrogen signaling in male fetal rat perineum and genitalia, pushing these tissues towards the female transcriptional phenotype (Schwartz et al., 2019b). Perhaps also in the perineum, as suggested for phallus development (Mattiske and Pask, 2021), the balance between androgens and estrogens, or their respective receptors, may be key to sex-specific differentiation of these tissues. This is further supported by the observation that, amongst the 73 DEGs we identified in the GT of triticonazole-exposed rats, four were ER target genes and three AR target genes. Among the DEGs identified in these analyses were several genes with known associations with hypospadias in humans, including SOX9 and its direct target gene NEDD9 (Sreenivasan et al., 2017; Yang et al., 2019). We also saw a significant increase in ACTA1 which, together with LMOD3, are essential for correct craniofacial patterning, palate fusion and urethral closure (Avasthi et al., 2019). TNNC1 represents another interesting DEG in our dataset. It is a known target of GATA4 which,

when mutated, is associated with hypospadias in humans (Yu et al., 2011). Finally, MAU2 was downregulated in both phallus and the perineum, which is of interest in that it is an essential component of the cohesion complex along with ATRX, both of which are implicated in hypospadias (Piché et al., 2019; Stevenson, 2000). Taken together, these genes demonstrate the value of this screen in identifying genes involved in urogenital patterning. Furthermore, they highlight the relevance of these data to humans, by identifying DEGs as a result of EDC exposure that are directly related to human disease states.

In regulatory toxicology, knowing the exact mechanisms for how chemicals induce adverse health effects holds great value. As modern toxicology moves away from assessing chemicals using intact animals and towards relying more on non-animal test data, it is imperative that we employ test strategies that adequately cover all the key events that may cause disease in intact organisms, including in humans. As our study has clearly highlighted, however, transcriptomics approaches require careful considerations about what tissues are analyzed and at what developmental stages. For instance, male animals displaying a short AGD (Draskau et al., 2019) and a marked transcriptional footprint in the perineum and GT following *in utero* exposure to triticonazole (this

Table 1

Estrogen receptor (ER) and androgen receptor (AR) target genes and hypospadias associated genes identified in gestational day (GD) 17 or 21 rat genital tubercle differentially expressed following triticonazole exposure compared to vehicle-treated controls. Arrows and shading indicate that the gene was either upregulated or downregulated in triticonazole-exposed samples relative to controls.

ER target genes	AR target genes	Hypospadias associated genes, humans	Triticonazole exposure, rats
<i>Elk3</i>			↓
<i>Naa50</i>			↓
<i>Ppm1a</i>			↑
<i>Tpm3</i>	<i>Tpm3</i>		↑
	<i>Tnrc6a</i>		↓
	<i>Dcaf6</i>		↓
		<i>SOX9</i>	↓
		<i>NEDD9</i>	↓
		<i>ACTA1</i>	↑
		<i>TNNC1</i>	↑
		<i>MAU2</i>	↓

study), did not display significant transcriptional changes in the testes themselves (Draskau et al., 2021). This illustrates the importance of analyzing the correct tissue when characterizing mechanisms of complex *in vivo* effect outcomes in toxicity studies and to take this into account in predictive toxicology. In addition, apart from considering the predictive potential of alternative test methods, the human relevance of animal data is strongly emphasized in chemical risk assessment. In this study, we found several genes associated with hypospadias in humans that, upon further validation, can be used to infer causality to human disease.

5. Concluding remarks

We have shown that developmental exposure to the anti-androgenic fungicide triticonazole causes marked changes to the transcriptome of external genitalia of male rat fetuses. At the morphological level, these same rat fetuses have previously been reported to display a short AGD, but little transcriptional changes to the fetal testicles. Thus, EDCs can clearly induce morphological changes in hormone-sensitive tissues by acting directly on the target tissues, a fact that must be considered when elaborating non-animal test strategies for chemical toxicity. We have also shown a complex effect pattern at the transcriptional level that strongly suggests that so-called anti-androgenic chemicals disrupt not only androgen-specific signaling pathways, but also disrupt other relevant pathways, including estrogen signaling. Again, something that must be considered for alternative test strategies. Finally, our data provide a deeper understanding of the impacts of EDCs on male urogenital development and provide a novel knowledgebase for further development and refinement of novel alternative test strategies for EDC toxicity, including rat-to-human similarities in factors implicated in hypospadias.

Author contributions

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editing **Aurélie Lardenois:** Methodology, Software, Formal analysis, Data curation, Writing – review & editing **Andrew Pask:** Investigation, Writing – review & editing **Frédéric Chalmel:** Methodology, Software, Formal analysis, Visualization, Data curation, Writing – review & editing **Terje Svingen:** Conceptualization, Methodology, Visualization, Supervision, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are included as supplementary files and deposited online in the GEO repository (accession number GSE199234) and TOXsIgN repository (accession number TSP1267).

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Appendix A. Supplementary data

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