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Research Article

All-Trans Retinoic Acid Disrupts Development in *Ex Vivo* Cultured Fetal Rat Testes. I: Altered Seminiferous Cord Maturation and Testicular Cell Fate

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ABSTRACT

Exposure to excess retinoic acid (RA) disrupts the development of the mammalian testicular seminiferous cord. However, the molecular events surrounding RA-driven loss of cord structure have not previously been examined. To investigate the mechanisms associated with this adverse developmental effect, fetal rat testes were isolated on gestational day 15, after testis determination and the initiation of cord development, and cultured in media containing all-trans RA (ATRA; 10^{-8} to 10^{-6} M) or vehicle for 3 days. ATRA exposure resulted in a concentration-dependent decrease in the number of seminiferous cords per testis section and number of germ cells, assessed by histopathology and immunohistochemistry. Following 1 day of culture, genome-wide expression profiling by microarray demonstrated that ATRA exposure altered biological processes related to retinoid metabolism and gonadal sex determination. Real-time RT-PCR analysis confirmed that ATRA enhanced the expression of the key ovarian development gene *Wnt4* and the antitestis gene *Nr0b1* in a concentration-dependent manner. After 3 days of culture, ATRA-treated testes contained both immunohistochemically DMRT1-positive and FOXL2-positive somatic cells, providing evidence of disrupted testicular cell fate maintenance following ATRA exposure. We conclude that exogenous RA disrupts seminiferous cord development in *ex vivo* cultured fetal rat testes, resulting in a reduction in seminiferous cord number, and interferes with maintenance of somatic cell fate by enhancing expression of factors that promote ovarian development.

Key words: fetal testis; retinoic acid; sex determination.

Retinoic acid (RA) signaling is tightly regulated during mammalian gonadal development. Under normal physiological conditions in the XY mouse fetal gonad, *Cyp26b1* is expressed under the control of SOX9 and NR5A1 (steroidogenic factor 1 [SF-1]), leading to inactivation of all-trans RA (ATRA) and a low concentration of ATRA reaching the germ cells (Kashimada *et al.*, 2011). Therefore, *Cyp26b1* is required in the mouse fetal testis to maintain protection of XY germ cells against RA exposure. In wild-type XX gonads, but not XY gonads, initiation of meiosis occurs in fetal germ cells. Deletion or inhibition of CYP26 results in developmentally inappropriate initiation of meiosis in fetal germ cells in the XY gonad (Bowles *et al.*, 2006; Koubova *et al.*, 2006, 2014; Li *et al.*, 2009), and also causes germ cell apoptosis (Hogarth *et al.*, 2015; MacLean *et al.*, 2007). Similarly, stimulation of the RA signaling pathway by exogenous RA during fetal testis development has profound adverse effects on the testis. In rat, mouse, and human fetal testis culture experiments, exposure to ATRA leads to loss of seminiferous cord structure, germ cell

loss, altered germ cell differentiation, altered testosterone biosynthesis, and especially in the mouse, early entry into meiosis (Cupp *et al.*, 1999; Jorgensen *et al.*, 2015; Lambrot *et al.*, 2006; Li and Kim, 2004; Livera *et al.*, 2000, 2001, 2004; Trautmann *et al.*, 2008).

Mammalian gonadal sex determination occurs during early fetal development. In XY individuals, the bipotential gonad is signaled to differentiate into testis as a result of the expression of *Sry* and subsequent induction of *Sox9*. In the XX gonad, the lack of *Sry* expression leads to the onset of the ovary development program that is driven largely by WNT4/β-catenin signaling (Brennan and Capel, 2004). Expression of *Sry*, the initial switch for this critical divergence in developmental pathways, occurs between embryonic days 10.5–12.5 in mice (Koopman *et al.*, 1990). Recent evidence indicates that maintenance of gonadal sex after initial sex determination is dependent on ongoing downstream signaling by factors including SOX9, β-catenin, DMRT1, and FOXL2 (Minkina *et al.*, 2014; Nicol and Yao, 2015; Uhlenhaut *et al.*, 2009). A gene that may contribute to maintenance of gonadal sex is the RA metabolizing enzyme, *Cyp26b1* (Nicol and Yao, 2015), which is a direct target of *Sry* and *Sox9* regulation (Li *et al.*, 2014), indicating a link between control of RA signaling and maintenance of testis cell fate.

Because control of RA signaling is so critical for gonad development, it follows that exposure to compounds that disrupt local RA concentrations, metabolism, transport, or RA signaling in the developing gonad could result in testicular toxicity. Despite previous reports that ATRA disrupts development of seminiferous cords, the mechanistic events associated with this effect have not been thoroughly characterized. Further, RA signaling has not been widely considered as a potential mechanism of developmental testicular toxicity. Given the tight regulation of RA signaling by CYP26B1, under the control of *Sry* and *Sox9*, we hypothesized that RA produces disorganized seminiferous cords in the rat fetal testis by disrupting normal developmental signaling processes required for maintenance of cell fate in the testis. To test this hypothesis, for the first time, we studied the effect of ATRA on genome-wide expression in an *ex vivo* rat fetal testis culture system. Gene expression evidence for disruption of sex determination was corroborated by histology and immunohistochemistry.

MATERIALS AND METHODS

Animals

All procedures involving animals were approved by the Brown University Institutional Animal Care and Use Committee, and were designed and performed in accordance with U.S. Public Health Service policy. Timed pregnant SAS Sprague Dawley rats (strain code 400) were obtained from Charles River and euthanized on gestational day (GD) 15 by inhalation of isoflurane. Fetuses were euthanized by decapitation. The weight of each fetus was recorded, and sex was determined by visualization of gonads under a stereomicroscope.

Testis cultures

Testes obtained from GD 15 male rats were dissected completely from the mesonephros, to eliminate mesonephros-derived retinoids and migrating cells, and cultured on Millicell-CM cell culture inserts (EMD-Millipore, Billerica, Massachusetts) in Falcon 24-well cell culture plates (Corning Life Sciences, Tewksbury, Massachusetts), on 480 μl of cell culture media. Media consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco/Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 20 μg/ml gentamicin, 1X penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively) (Gibco), 50 μg/ml Albumax II (Gibco), 200 mg/ml bovine serum albumin (Sigma Aldrich, St Louis, Missouri), 1X ITS liquid media supplement (Sigma Aldrich), and 50 μg/ml sodium L-ascorbate (Sigma Aldrich), based on previously published conditions for fetal gonad cultures (Devine *et al.*, 2009; Dutta *et al.*, 2016; Grive *et al.*, 2014). One testis from each fetus was placed on a Millicell membrane and cultured on media containing 10^{-8}, 10^{-7}, or 10^{-6} M ATRA (Sigma Aldrich), and the contralateral testis was cultured on media containing only 1:4000 dimethyl sulfoxide (DMSO) vehicle, creating a paired design with the fetus as the sampling unit. Fetuses from the same litter were not used in the same combination of treatment group and endpoint. Treatment concentrations and experimental design were based on those used in the FeTA assay experiments reported by Habert *et al.* (2014). Media was prepared fresh from ATRA stock daily and changed every 24 h. ATRA stock solution (0.01 M) was prepared by dissolving preweighed ATRA powder in pure DMSO in a light-protected vial under a fume hood, and dispensing aliquots into amber glass and/or aluminum foil wrapped vials to protect from light, followed by storage at −20°C. All aliquots were used within 14 days of preparation. At all steps, samples and plates were handled under low light conditions and/or yellow light and protected from direct exposure to fluorescent light. Testes were collected after 24 h, snap frozen in liquid nitrogen, and stored at −80°C for isolation of RNA (6 replicates per concentration), or were collected after 3 d, fixed in modified Davidson's solution for 15 min, then transferred to 70% ethanol and stored at 4°C until processing for histology and immunohistochemistry (6–7 replicates per concentration). Gene expression analysis was performed after 24 h to capture early changes affecting signaling pathways prior to the appearance of tissue-level changes on day 3.

Histology and immunohistochemistry

Fixed tissue samples obtained after 3 days of culture (6–7 per treatment group, plus paired vehicle-treated samples) were processed by hand through a series of graded ethanols, 3 changes of xylene, and 4 changes of paraffin wax. Samples were embedded in paraffin and re-embedded on end to allow for transverse sections to be obtained with consistent orientation. Sections were cut at a thickness of 5 μm at approximately 150, 300, 450, and 600 μm depths in each block so that histological scoring would include representative sections from multiple locations in the testis. Cut sections were deparaffinized in xylene and rehydrated through 2 changes of 100% ethanol, followed by 95% ethanol, 70% ethanol, and water. For purposes of general histology, sections were stained with hematoxylin and eosin (H&E). Seminiferous cord number was counted on H&E-stained slides using an Olympus BH-2 light microscope at 100×–400× total magnification.

Immunohistochemical labeling was performed for ACTA2, which is expressed around seminiferous cords and commonly called smooth muscle actin; DDX4, a germ cell marker also referred to as VASA; DMRT1, a Sertoli cell- and gonocyte-expressed developmental transcription factor; and FOXL2, a granulosa cell-expressed transcription factor. Staining was performed according to the following methods, using the antibody concentrations reported in Table 1. Slides were deparaffinized in xylenes and rehydrated through a series of graded ethanols. For nuclear antigens (DMRT1, FOXL2), antigen retrieval was

Table 1. Antibodies Used for Immunohistochemistry

Antibody Type	Species	Target	Manufacturer	Product no.	Dilution
primary	rabbit	DDX4	Abcam	ab13840	1: 2000
primary	mouse	DMRT1	Santa Cruz	sc-377167	1: 500
primary	goat	FOXL2	Abcam	ab5069	1: 2000
primary	mouse	ACTA2	Sigma	A2547	1: 4000
secondary	goat	mouse IgG	Sigma	B0529	1: 750
secondary	goat	rabbit IgG	Sigma	B8895	1: 1500
secondary	rabbit	goat IgG	Thermo	31732	1: 1000

performed by heating slides in 10 mM citrate buffer, pH 6.0, in a vegetable steamer for 20 min, followed by 20 min at room temperature. Slides were subsequently permeabilized for 5 min in 0.1% sodium citrate with 0.1% Triton X-100. For nonnuclear antigens (DDX4, ACTA2), antigen retrieval timing was 10 min in steamer, 10 min at room temperature, and the permeabilization step was omitted. To block endogenous peroxidase, sections were incubated in 3% hydrogen peroxide, diluted in methanol, for 60 min. Endogenous avidin and biotin were blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, California), according to the manufacturer's instructions. To block nonspecific antigen binding, sections were incubated for 40 min in a blocking buffer consisting of phosphate buffered saline with 8% serum from the species in which the secondary antibody was produced and 1% BSA. Sections were incubated with primary antibody diluted in blocking buffer, or with blocking buffer alone as a negative control, overnight at 4°C in a humidified chamber, followed by secondary antibody for 1 h at room temperature. Avidin/biotin peroxidase complex was bound using the Vector Laboratories ABC Elite kit, and peroxidase detection was performed using the DAB Peroxidase Substrate Kit (Vector Laboratories) 3, 3'-diaminobenzidine peroxidase substrate, per manufacturer's instructions. Adult Fisher rat ovary used as positive control for FOXL2 IHC was generously provided by Dr Jennifer Sanders, Brown University. Adult Sprague Dawley rat testis used as positive control for DMRT1 IHC was obtained from a previous study. All images of histological sections were obtained from slides scanned at 40× magnification using an Aperio ScanScope CS (Aperio Digital Pathology/Leica Biosystems, Buffalo Grove, Illinois). Scanned slides were manually annotated to determine section area and the number of DMRT1-, DDX4-, or FOXL2-positive cells in Aperio ImageScope software. DDX4-positive cells were only counted if the cytoplasm was labeled and the nucleus was visible in the plane of section. For DMRT1, only somatic cells were counted.

RNA extraction

Snap-frozen fetal testes ($n = 6$ replicates per concentration), obtained after 1 day culture, were homogenized using approximately 100–200 mg stainless steel beads (Next Advance, Averill Park, New York), shaken in a Disruptor Genie (Scientific Industries) for 2 min at maximum speed. RNA was isolated and purified using the DNA/RNA AllPrep kit (Qiagen, Valencia, California), according to the manufacturer's protocol, including the optional on-column DNase digestion.

Microarray analysis

RNA samples extracted from testes that were cultured in media containing 10^{-6} M ATRA (high concentration, $n = 6$) and paired vehicle samples were analyzed using the Affymetrix Clariom D microarray (rat) assay/rat transcriptome array (RTA) 1.0 GeneChips (Affymetrix, Santa Clara, California) in the Brown University Genomics Core, according to the manufacturer's protocols. 100 ng of total RNA per sample was converted to cDNA using the WT Amplification Kit (Affymetrix). cDNA was converted and amplified in an overnight *in vitro* transcription (IVT) reaction and purified. A 15 μg purified IVT cRNA was reverse transcribed and labeled with deoxyuridine triphosphate. A 5.5 μg purified ss-cDNA was fragmented to an average size of 75 bp using UDG and APE1. 5.15 μg fragmented ss-cDNA was end-labeled with biotin using TdT enzyme and hybridized for 16 h at 45°C and 60 rpm to RTA 1.0 GeneChips (Affymetrix). Arrays were washed and stained on an Affymetrix FS450 Fluidics Station, and arrays were visualized using the Affymetrix 3000 7 G Scanner. Raw data were processed in Affymetrix Gene Chip Command Console software to produce .cel files and Affymetrix Expression Console for .chp files using both the gene-level and alt-splice analyses based on the Signal Space Transformation-Robust Multi-Chip Analysis workflow. Microarray data were deposited in the NCBI Gene Expression Omnibus database (accession number GSE101634).

Real-time RT-PCR

cDNA was prepared from RNA extracted after 1 day of culture ($n = 6$ testes per treatment), using the Qiagen RT^2 First Strand Kit (Qiagen), with 150 ng input total RNA from each sample. An 1 μl cDNA (approximately 1.35 ng input RNA) per well was used for real-time PCR using the SYBR Green (ROX) Mastermix and RT^2 QPCR Primer Assays (SA Biosciences/Qiagen), on a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, California). Cycling parameters for real-time PCR reactions were an initial denaturation at 95°C for 10 min, followed by 40 cycles of a 2-step amplification (95°C for 15 s, 60°C for 1 min). The instrument default dissociation curve was performed following amplification.

Real-time RT-PCR was used to quantify expression of genes following 1 day of culture ($n = 6$ per treatment group) that were identified by ATRA microarray analysis, including retinol-binding protein 1 (*Rbp1*), nuclear receptor, subfamily 0, GROUP B, member 1 (DAX-1) (*Nr0b1*), Wnt family member 4 (*Wnt4*). Other targets were germ cell markers previously reported to be affected by RA exposure, including DEAD-box helicase 4 (*Ddx4*) and POU class 5 homeobox 1 (OCT-3/4) (*Pou5f1*), and a Leydig cell gene required for testosterone biosynthesis, steroidogenic acute regulatory protein (*Star*). Housekeeping genes used for real-time RT-PCR analysis were beta-2 macroglobulin (*B2m*) and lactate dehydrogenase A (*Ldha*). Each real-time PCR plate included no-reverse transcriptase negative controls created from pooled RNA samples, as well as no-template (water) negative controls. The SA Biosciences rat genomic DNA negative control primer set was also run for all samples. The amplicon size and specificity of each primer set was confirmed by purifying PCR products from a trial run of each primer assay using the QIAquick PCR Purification Kit (Qiagen), and running the product on an agarose gel. The purified products were used to create a 7-point standard curve consisting of 10^8 through 10^2 copies per reaction, and a standard curve was run on each plate. Starting quantity for each sample was determined using the standard curve method, and samples were normalized to the geometric mean value of the housekeeping genes.

Data analysis and statistics

Data were summarized using Excel, and statistical hypothesis testing and graphing were performed using GraphPad Prism 7 (GraphPad Software, San Diego, California). Real-time RT-PCR data, seminiferous cord counts, and cell counts were analyzed using 2-tailed paired *t* tests. Data are presented in charts as mean ± SEM. Microarray data were analyzed by both the gene-level and alternative splicing (alt-splice) analysis within Affymetrix Transcriptome Analysis Console, using false discovery rate (FDR) to adjust for multiple testing (Benjamini and Hochberg, 1995). Functional analysis was performed in gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005) using the raw data obtained from gene-level analysis, and in Ingenuity Pathway Analysis software (IPA, Qiagen) using the fold-change values for all significant genes (gene-level analysis, FDR $p < .05$, $|\text{fold change}| \geq 1.5$) as the input for IPA core analysis. Microarray Venn diagram was drawn using eulerAPE (Micallef and Rodgers, 2014). Microarray data were deposited in the NCBI Gene Expression Omnibus database (accession number GSE101634).

RESULTS

ATRA Exposure Diminished Seminiferous Cord Structure

Following 3 day *in vitro* exposure of GD 15 testes, ATRA caused a loss of seminiferous cord structure, characterized by germ cells present outside of cords, cords that were poorly defined or had weakly visible basement membranes, or complete loss of cords (Figure 1). In contrast, vehicle-treated fetal rat testes displayed organized seminiferous cords. ACTA2 was present in all testis cultures, and was strongly expressed in the tunica and around seminiferous cord basement membranes. Following 3 days of culture, vehicle control samples showed significant development of seminiferous cords relative to preculture controls (Supplementary Figure 1), accompanied by increased expression of ACTA2 surrounding cords. In 10^{-6} M ATRA-treated cultures, the expression of ACTA2 was mostly contained in an area near the bottom (membrane-facing) side of the culture. The number of seminiferous cords visible per section decreased with increasing concentration of ATRA, and the reduced cord number was statistically significant at the highest concentration (Figure 2).

Exposure to ATRA Accelerated Germ Cell Loss

To determine the effect of RA on survival of prospermatogonia, also called gonocytes, immunohistochemical staining for DDX4 was performed and DDX4-positive cells were quantified. Germ cell count per unit area decreased with increasing ATRA concentration, with this effect being significant at the 10^{-6} M ATRA concentration (Figure 3). DDX4-positive germ cells were present in all treatment groups, including a small number of germ cells in samples exposed to the highest concentration of ATRA, despite the absence of seminiferous cords.

ATRA-Induced Canonical RA Signaling Genes and Influenced Expression of Genes Critical for the Maintenance of Gonadal Somatic Cell Fate

Genome-wide expression profiling was conducted to identify genes and pathways in *ex vivo* cultured testes that were disrupted following 1 day of exposure to 10^{-6} M ATRA. Expression data obtained from Affymetrix RTA 1.0 microarrays was analyzed using both the gene-level and alternative splicing (alt-splice) analyses in Affymetrix Transcriptome Analysis Console. Gene-level analysis identified 389 significantly altered transcript clusters (genes) (FDR $p < .05$, $|\text{fold change}| \geq 1.5$) in testes cultured in 10^{-6} M ATRA, relative to vehicle (Figure 4, Supplementary Table 1). In the alt-splice analysis, which identifies both differentially regulated genes and alternatively spliced transcript regions, 332 transcript clusters were identified as significantly differentially regulated, using the same significance and fold-change cutoffs (Supplementary Table 2). Significant transcript cluster lists obtained from each analysis overlapped by 56.7% (261 genes) (Figure 4A), with 128 unique transcript clusters reaching significance only in the gene-level analysis and 71 significant transcript clusters unique to the alt-splice analysis. Alt-splice analysis identified 18 Junctions or probe selection regions (PSRs) within 16 transcript clusters with evidence of alternative splicing (Supplementary Table 3). In total 13 of those 16 transcript clusters overlapped with significant genes identified by both the gene-level and alt-splice analysis, while 3 PSRs with alternate splicing events belonged to transcript clusters that were not significantly differentially regulated. Notably, a PSR within the *Lrat* transcript cluster had a significant 5' alternative splicing site in exon 1. *Lrat* was also the most up-regulated gene in the analysis (Figs. 4B and 4C).

A total of 10 gene ontology (GO) biological process terms were significantly enriched in ATRA-treated samples according to GSEA (Table 2). Most significant GO terms were related to hormone metabolism, including *fat soluble vitamin metabolic process*, *retinol metabolic process*, *cellular hormone metabolic process*, *long chain fatty acid metabolic process*, and *terpenoid metabolic process*; or development, including *adrenal gland development*, *sex determination*, and *regulation of chondrocyte differentiation*. The enriched terms also included *phospholipase C activating G protein-coupled receptor signaling pathway* and *positive regulation of transcription from polymerase II promoter in response to stress*. These results indicated 2 major categories within the gene expression response: metabolic responses to RA; and altered expression of genes involved in developmental signaling pathways, including sex determination. Of the significantly differentially regulated genes within GO terms identified by GSEA (Table 2), 6 genes related to RA signaling, retinoid metabolism, and retinoid transport (*Lrat*, *Rbp1*, *Cyp26a1*, *Stra6*, *Dhrs3*, and *Rdh10*) appeared under 3 enriched terms, and a seventh, *Aldh1a1*, appeared under 2. After considering genes related to retinoid metabolism and signaling, the only other significant genes that fell under 2 or more significant GSEA terms were *Wnt4* and *Nr0b1*, the latter of which codes for the nuclear receptor DAX-1. Both genes are involved in gonadal sex determination. Notably, the GSEA enrichment score for the *sex determination* gene set is also influenced by genes that did not individually meet significance cutoffs, including *Sry* (1.3-fold, $p = .016$, FDR $p = .249$), *Wt1* (1.69-fold, $p = .002$, FDR $p = .094$), *Insrr* (1.15-fold, $p = .001$, FDR $p = 0.089$), *Sox9* (1.42-fold, $p = .014$, FDR $p = .239$), *Amh* (−1.32-fold, $p = .018$, FDR $p = .257$), *Cited2* (−1.23, $p = .042$, FDR $p = .360$), and *Six4* (−1.21-fold, $p = .015$, FDR $p = .245$) (Supplementary Table 4). Other genes related to reproductive system development, but not listed under significantly over-represented terms in the GSEA analysis, were significantly up- or down-regulated. The follistatin-like gene, *Fstl3*, was among the most highly up-regulated genes in the microarray analysis (21.53-fold, FDR $p = .010$). *Efhd1* (5.33-fold, FDR $p = .009$), *Mmd2* (2.11-fold, FDR $p = .013$), *Ptgds* (2.09-fold, FDR $p = .049$), and *Sel1l3* (−8.62-fold, FDR $p = .009$) were significantly altered. Finally, 2 genes involved in PDGF signaling, which has a role in testis cord development, *Pdgfa* (−1.59-fold, DFR $p = .052$)

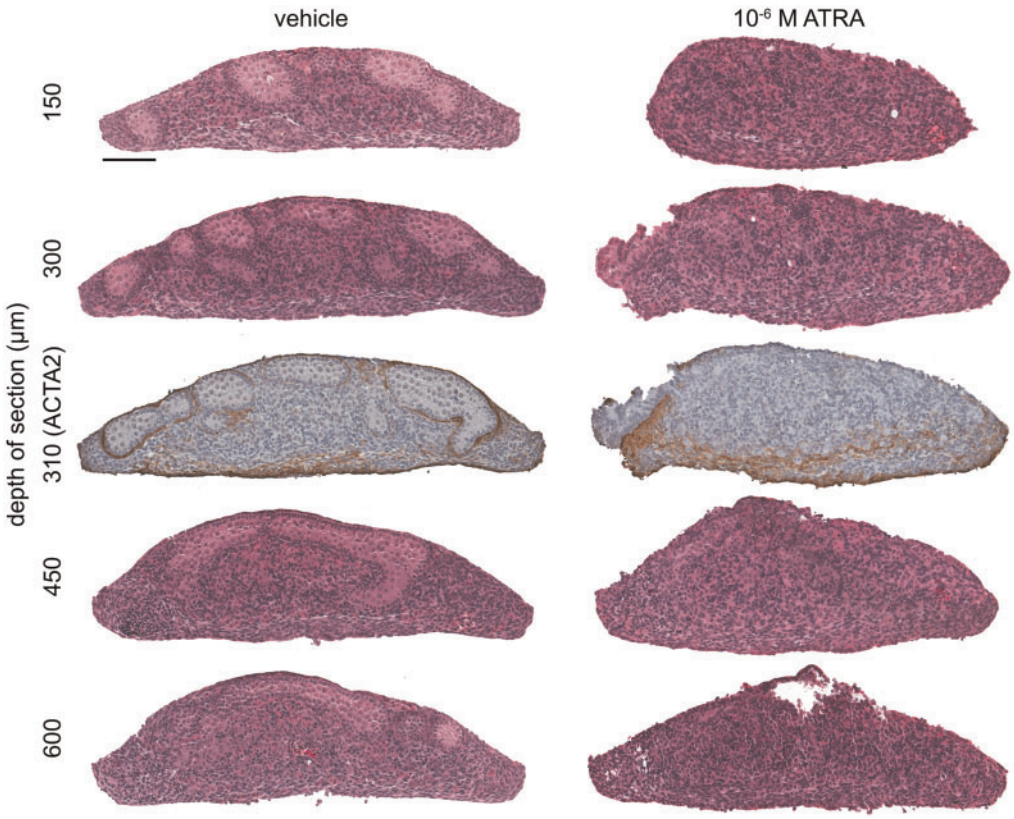

Figure 1. ATRA exposure disrupted seminiferous cord structure in cultured rat fetal testes. Histological sections from paired rat fetal testes showed differential seminiferous cord structure following 3-day culture in vehicle or 10^{-6} M ATRA. H&E-stained sections were taken from each block at 4 depths. Vehicle sample had evidence of seminiferous cord development in all sections. The paired sample, treated with 10^{-6} ATRA, showed limited seminiferous cord development at any depth. A central section was stained immunohistochemically for ACTA2. ACTA2 expression confirmed more advanced cord development in vehicle samples, with ACTA2 localized to basement membrane regions of most cords. Scale bar = 100 µm.

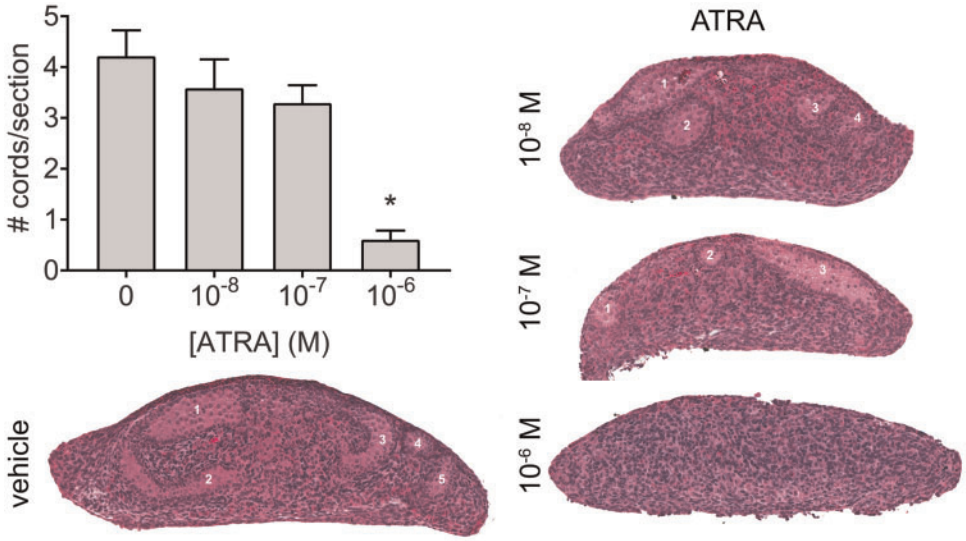

Figure 2. ATRA exposure decreased seminiferous cord number in cultured rat fetal testes. Seminiferous cords were counted on H&E-stained sections taken at approximately 150, 300, 450, and 600 µm from each block. 10^{-6} M ATRA decreased the number of distinct seminiferous cords per section significantly. *$p < .05$ by paired t test (n = 6–7 testes/group). Representative images were chosen with approximately the average number of seminiferous cords for each treatment. White numerals indicate cords counted on each section.

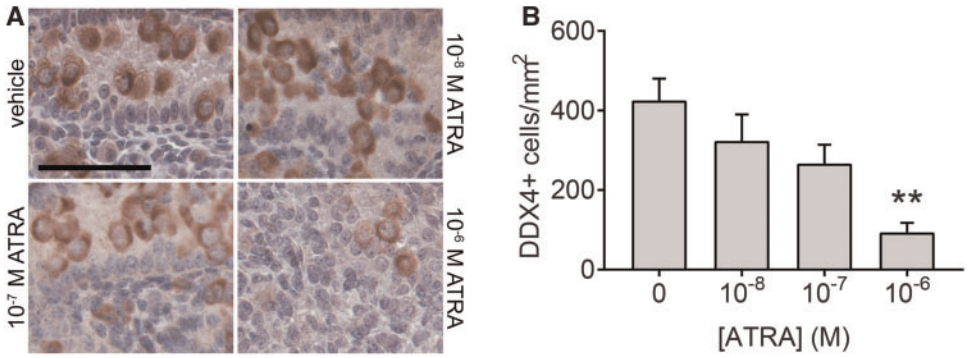

Figure 3. ATRA exposure resulted in germ cell loss. A, DDX4 IHC-positive germ cells were present in vehicle cultures, predominantly within seminiferous cords. Germ cells were present in all ATRA-treated samples, but in 10^{-6} M ATRA cultures, germ cells were reduced in number and were largely present outside of seminiferous cords. Scale bar = 60 μm. B, DDX4-positive germ cell number was decreased by 10^{-6} M ATRA following 3-day culture. $^{**}p < .01$, $n = 6$ testes/group.

and *Pdgfrb* (−1.84-fold, FDR $p = .097$), approached statistical significance.

Results obtained from IPA also indicated enrichment of gene expression related to reproductive development and RA signaling and metabolism (Supplementary Tables 5–7). Top canonical pathways identified by IPA included retinoic acid receptor (RAR) activation, as well as Wnt/β-catenin signaling, which relates directly to ovary development (Supplemental Table 5). Upstream regulators predicted by IPA included tretinoin (a synonym of ATRA), retinoid X receptor alpha, and RAR alpha (Supplementary Table 6, Supplementary Figure 2). To summarize the effects of ATRA on gonadogenesis, genes falling under the terms *gonadogenesis*, *formation of testis*, and *development of female reproductive system* were selected from the numerous reproduction-related diseases or biological functions determined to be overrepresented by IPA analysis (Supplementary Table 7), and were used to construct a network (Figure 4D).

Genes that were selected for further analysis by real-time PCR included 2 germ cell markers, *Ddx4* and *Pou5f1*, and a Leydig cell marker required for testosterone biosynthesis, *Star*, as well as the RA receptor target, *Rbp1*, and the sex determination genes, *Wnt4* and *Nr0b1*, which were selected from the GSEA results (Figure 5). ATRA exposure increased *Rbp1* expression in a concentration-dependent manner, confirming activation of RA signaling. *Nr0b1* and *Wnt4* expression also increased in a concentration-dependent manner, suggesting direct or indirect regulation by RA signaling. *Ddx4* and *Pou5f1* mRNA levels were decreased significantly only at the highest dose of ATRA, consistent with decreased germ cell number (Figure 3), but with an apparent, nonsignificant, increase at lower concentrations. When the germ cell markers were analyzed as the ratio of *Pou5f1* to *Ddx4* mRNA, there were no significant differences across the concentration range, suggesting that there was no change in average expression of the pluripotency factor *Pou5f1* across germ cells. *Star* expression was not consistently significantly altered by ATRA across the concentration range.

FOXL2 and DMRT1 Expression in ATRA-Exposed Testes

To examine potential effects of ATRA on testicular somatic cell fate, cultured testes were stained for the developmentally expressed transcription factors, DMRT1 and FOXL2, which are typically expressed in testis and ovary, respectively (Figure 6). DMRT1 was expressed in Sertoli cells and few germ cells in all samples. ATRA exposure caused dispersal of DMRT1-positive cells throughout the cultured testis, but the number of DMRT1-positive cells per unit area in testis sections was not reduced at any ATRA treatment level, relative to paired vehicle. Morphology of DMRT1-positive cells was difficult to discern in ATRA-treated samples because of loss of cord structure and dispersion of multiple cell types. However, the DMRT1-positive cells in high-dose ATRA-treated samples did not appear to be germ cells, based on morphology, and a small number of DMRT1-positive cells had identifiable Sertoli cell-like nuclear morphology and localization in remnants of seminiferous cords. Cultured testes also contained FOXL2-positive cells. Few FOXL2-positive cells were present in vehicle-treated testes, and the number of FOXL2-expressing cells increased with increasing concentration of ATRA, significant at the 2 highest concentrations. Especially in 10^{-7} and 10^{-6} M ATRA-treated testes, FOXL2-positive cells were dispersed throughout the sections in a pattern similar to that of DMRT1-positive cells.

DISCUSSION

This study is the first to our knowledge to demonstrate that exposure of *ex vivo* cultured fetal testes to exogenous ATRA, in addition to disrupting seminiferous cord structure, disrupts expression of regulatory factors required for maintenance of testicular cell fate. In mammals, RA signaling plays a significant role in fetal ovary development and germ cell meiosis (Bowles *et al.*, 2006) and also controls postnatal testis processes such as spermatogonial differentiation, meiosis initiation, and spermiation (Busada and Geyer, 2016; Hogarth *et al.*, 2015; Huang and Marshall, 1983; Koubova *et al.*, 2006). Therefore, control of RA signaling in the fetal testis is critical for normal testis development (Koubova *et al.*, 2006; Li *et al.*, 2009; MacLean *et al.*, 2007). The testis development pathway is established in XY individuals when *Sry* expression activates expression of downstream protestis genes, including the Sertoli-cell-expressed *Amh* and *Sox9* (Brennan and Capel, 2004). However, gonadal fate remains plastic beyond the brief window during which *Sry* is expressed in the XY gonad. *Sox9* and other downstream signaling factors are required for continued commitment to testis development; in the absence of *Sry* expression, ovary development is promoted by downstream signaling factors including *Wnt4* and *Ctnnb1* (Brennan and Capel, 2004; Minkina *et al.*, 2014; Nicol and Yao, 2015; Uhlenhaut *et al.*, 2009). Sex determination in the mouse fetal gonad is sufficiently plastic that overactivation of Wnt/β-catenin signaling in the Sertoli cell leads to disruption of Sertoli cell fate maintenance and loss of seminiferous cord structure, consistent with the results of this study (Chang *et al.*, 2008; Li *et al.*, 2017). Further, a known link exists between ATRA

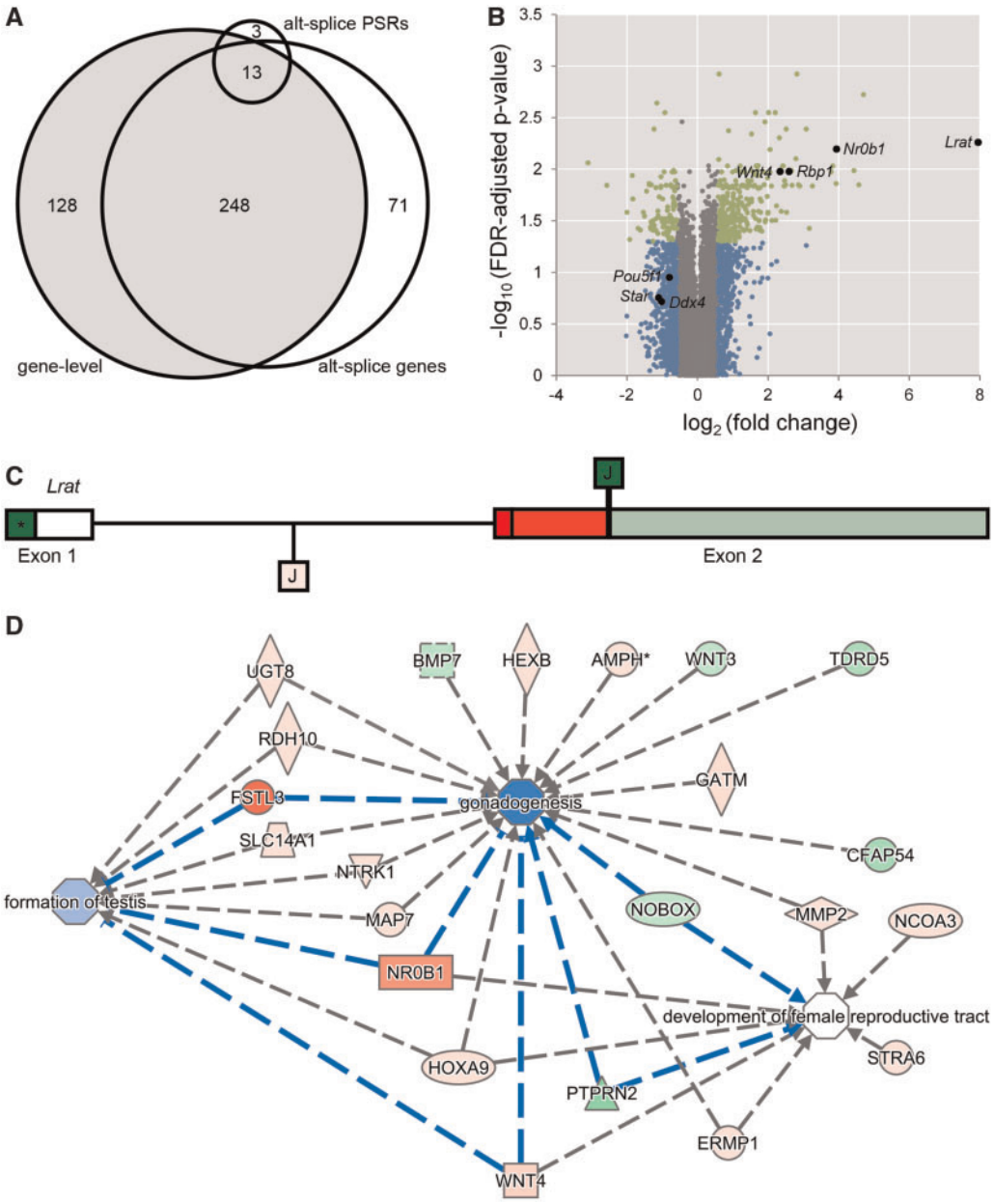

Figure 4. Changes in expression of genes involved in RA signaling and sex determination. A, Gene-level microarray analysis identified 389 probes altered by 10^{-6} M ATRA (gray circle). Alternative splicing (alt-splice) analysis identified 332 significantly differentially regulated genes, 261 of which were in common with the gene-level analysis. Only 16 PSRs met the significance criteria for alternative splicing (small circle), of which 13 were in differentially regulated genes ($n = 6$ testes/group). B, Significant genes identified by gene-level analysis (green, FDR $p < 0.05$, |fold change| ≥ 1.5) included *Wnt4*, *Nr0b1*, *Rbp1*, and *Lrat*. Germ and Leydig cell markers *Ddx4*, *Pou5f1*, and *Star* were down-regulated, but did not meet the FDR *p*-value cutoff. C, *Lrat*, the most upregulated gene in the analysis, also had a significant alternative splicing event, consisting of an alternative 5' splicing site in exon 1. J—splicing junction, green—PSR downregulated, red—PSR upregulated, *FDR $p < .05$. D, Selected "diseases or bio functions" from IPA, illustrating how changes in gene expression related to gonad differentiation. Genes are colored by direction of regulation in 10^{-6} M ATRA versus vehicle (red—upregulated, green—downregulated). Blue indicates predicted inhibition of gonadogenesis and formation of testis. Numerous changes also relate to development of female reproductive tract. For interpretation of references to color, the full-color version of the figure is available online.

metabolism and the genes that are involved sex determination and maintenance of sex-specific cell fate: expression of the RA metabolizing enzyme encoding gene, *Cyp26b1*, is induced in the testis by *Sry* and *Sox9*, and is antagonized by *Foxl2* (Kashimada *et al.*, 2011; Li *et al.*, 2014).

On GD 15, at the beginning of culture in this study, testis determination had occurred, and seminiferous cords had already formed (Supplementary Figure 1). This is consistent with the timing of endothelial cell migration from the mesonephros into the testis between GD 10 and 12, which contributes to seminiferous cord formation in the mouse (Combes *et al.*, 2009). Although exposure to RA earlier in gestation alters migration of cells from the mesonephros (Li and Kim, 2004), the later gestational timing in our study and the use of testes that were separated from the mesonephros allowed the influence of exogenous RA on testis development to be observed in the absence of additional migrating cells or retinoids from the mesonephros. Therefore, our results indicated that ATRA exposure was sufficient in *ex vivo* culture to disrupt existing seminiferous cord organization and to alter the expression of factors required

Table 2. Significant GO Biological Processes in GSEA of ATRA Microarray Data

GO Biological Process	GO ID	Significant Genes	NES or Fold Change	FDR *q*-value
Fat soluble vitamin metabolic process	GO: 0006775		2.11	0.032
		Lrat	248.55	0.006
		Rbp1	5.09	0.011
		Cyp26a1	3.80	0.031
Adrenal gland development	GO: 0030325		2.11	0.016
		Fstl3	21.53	0.010
		Nr0b1	15.44	0.006
		Wnt4	6.07	0.011
		Dkk3	3.75	0.003
		Stra6	2.10	0.028
Phospholipase C activating G protein-coupled receptor signaling pathway	GO: 0007200		2.06	0.026
		Tgm2	5.69	0.004
		Oprk1	5.52	0.013
		Gna14	2.20	0.020
Retinol metabolic process	GO: 0042572		2.03	0.028
		Lrat	—	—
		Dhrs3	25.93	0.002
		Rdh10	1.86	0.042
		Aldh1a1	−2.52	0.038
Sex determination	GO: 0007530		1.95	0.048
		Nr0b1	—	—
		Wnt4	—	—
Cellular hormone metabolic process	GO: 0034754		1.95	0.042
		Lrat	—	—
		Dhrs3	—	—
		Cyp26a1	—	—
		Wnt4	—	—
		Rbp1	—	—
		Rdh10	—	—
Positive regulation of transcription from RNA polymerase II promoter in response to stress	GO: 0036003		1.91	0.046
		Mt3	7.24	0.014
Long chain fatty acid metabolic process	GO: 0001676		1.90	0.045
		Acot1	2.02	0.019
		Slc27a6	1.92	0.042
Terpenoid metabolic process	GO: 0006721		1.90	0.044
		Lrat	—	—
		Dhrs3	—	—
		Rbp1	—	—
		Cyp26a1	—	—
		Stra6	—	—
		Rdh10	—	—
		Gpc1	−2.49	0.014
		Aldh1a1	—	—
Regulation of chondrocyte differentiation	GO: 0032330		1.89	0.045
		Rarb	4.60	0.003
		Sox6	1.71	0.032

All gene sets enriched in ATRA with $q < 0.05$ are listed. Significant genes are genes from the significant gene sets that had |fold change ≥ 1.5| and $q < 0.05$ in gene-level microarray analysis.

Abbreviations: FDR *q*-value, FDR-adjusted significance from GSEA or gene-level microarray analysis; fold change, linear fold change (ATRA-vehicle) from gene-level microarray analysis; GO, gene ontology, NES, normalized enrichment score from GSEA.

for maintenance of testicular somatic cell fate, including induction of *Wnt4* and *Nr0b1* and expression of the granulosa cell marker FOXL2. Notably, this did not result in a complete transition to an ovary phenotype or total loss of testicular signaling factors. Sertoli cells remained in ATRA-treated samples and expressed DMRT1, a transcription factor necessary for Sertoli cell development. However, DMRT1-positive Sertoli cells were dispersed throughout sections and failed to maintain cord structure as in paired vehicle-treated testes.

Wnt4 gene expression was induced following 1 day of culture in this study. Given the known role of *Wnt4* in fetal ovary development (Boyer *et al.*, 2010; Liu *et al.*, 2010) and the link between increased *Wnt4* expression and dosage-sensitive sex reversal (Jordan *et al.*, 2001), induction of *Wnt4* appeared to be a key event in the disruption of testis development observed in our study. In mouse genetic studies, *Wnt4* deficiency in females results in partial female-to-male sex reversal (Heikkila *et al.*, 2005), while overexpression in males antagonizes testis development (Jordan *et al.*, 2003). Wnt/β-catenin signaling occurs through stabilization of β-catenin; consequently, the loss of β-catenin in late gestation (17.5 dpc) in the mouse also disrupts sex development (Nicol and Yao, 2015). Sertoli cell overexpression of the β-catenin gene *Ctnnb1* in male mice disrupts seminiferous cord structure (Chang *et al.*, 2008) and induces FOXL2 expression in Sertoli cells (Li *et al.*, 2017). A similar increase in FOXL2-expressing cells was observed following ATRA exposure in this study, and it is reasonable to hypothesize that this occurred indirectly, through enhanced Wnt/β-catenin signaling, rather than direct activation of the *Foxl2* gene by RARs. However, this hypothesis remains to be tested. In addition to a concentration-dependent increase in *Wnt4*, ATRA exposure led to enhanced expression of *Nr0b1* (Figure 5). This is notable because expression of *Nr0b1* is controlled by *Wnt4* in the fetal ovary (Mizusaki *et al.*, 2003). *Nr0b1* is a complex regulator of gonad development. Its gene product, DAX-1, is referred to as an antitestis gene, and it is known to antagonize the critical testis developmental transcription factor SF-1 (Crawford *et al.*, 1998; Goodfellow and Camerino, 1999; Swain *et al.*, 1998; Zazopoulos *et al.*, 1997). However, DAX-1 is also required for testis development. Mutations in *Nr0b1* result in hypogonadotropic hypogonadism, DAX-1 can act as a coactivator for SF-1, and *Nr0b1* knockout mice have dysgenic testes characterized by enlarged seminiferous cords, germ cell death, and loss of peritubular

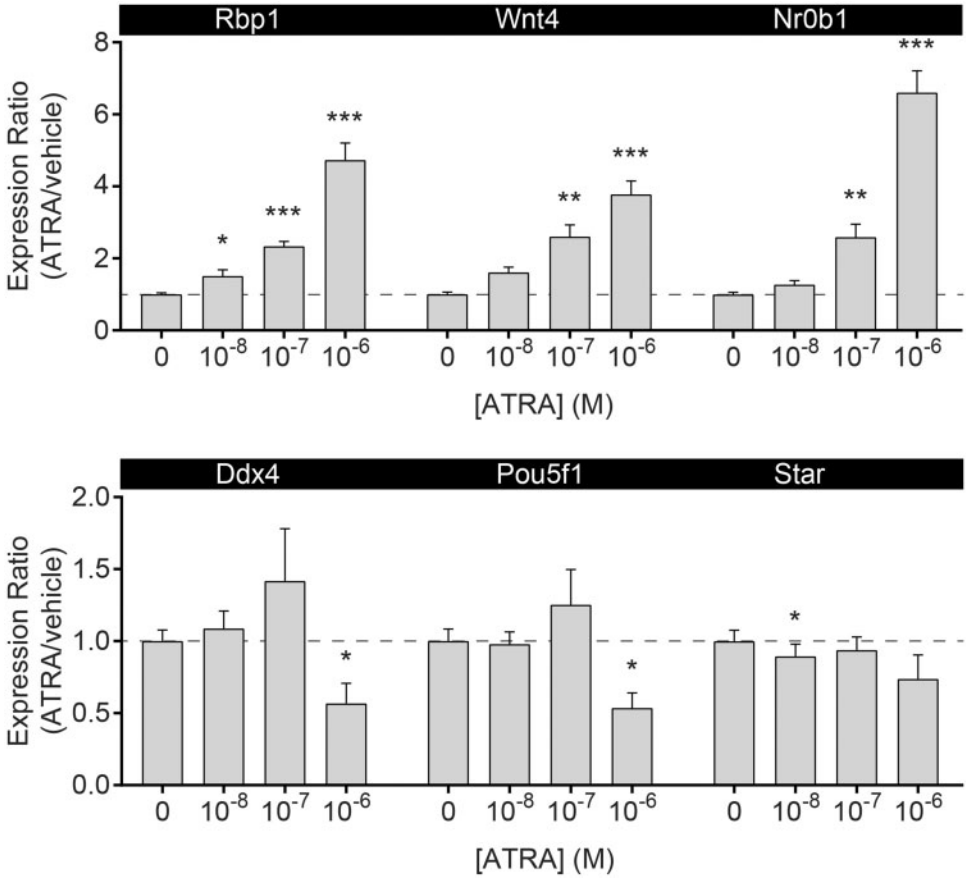

Figure 5. Influence of RA on expression of genes involved in sex determination was concentration-dependent. ATRA-enhanced expression of the RAR target gene *Rbp1* and the gonad development-related genes *Wnt4* and *Nr0b1* in a concentration-dependent manner. Steady-state level of germ cell-expressed genes, *Ddx4* and *Pou5f1*, was significantly reduced at the highest dose of ATRA. There was no consistent association between ATRA concentration and the Leydig cell functional marker, *Star*. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ by paired *t* test ($n = 6$ testes/group).

myoid cells (Meeks *et al.*, 2003a,b; Muscatelli *et al.*, 1994; Xu *et al.*, 2009; Zanaria *et al.*, 1994). *Nr0b1*-overexpressing XY mice exhibit dysgenic testes with partial sex reversal and expression of FOXL2, similar to this study, as well as down-regulation of *Sox9* (Ludbrook *et al.*, 2012).

Genome-wide profiling in this study indicated a broad effect of ATRA on expression of genes with less clearly defined roles in sex determination (Figure 4, Supplementary Table 1). ATRA exposure led to induction of the follistatin-like gene *Fstl3*, which may also be a direct result of *Wnt4* induction, as follistatin expression is controlled by *Wnt4* (Yao *et al.*, 2004). ATRA exposure also induced *Efhd1*, *Ptgds*, and *Mmd2*, and significantly down-regulated *Sel1l3*, 4 genes that were previously identified as candidates for maintenance of sex determination in a study of β-catenin/*Sox9* double-knockout mice (Nicol and Yao, 2015). Other genes involved in the classical model of sex determination that were altered at near-significant levels ($p < .05$ but FDR $p > .05$) included *Insrr*, *Wt1*, *Sox9*, *Six4*, *Sry*, *Amh*, and *Cited2* (Supplementary Table 4) (Brennan and Capel, 2004). It is also notable that 2 genes involved in PDGF signaling, *Pdgfa* (−1.59-fold, FDR $p = .052$) and *Pdgfrb* (−1.84-fold, FDR $p = .097$), approached statistical significance, because PDGF signaling is required for seminiferous cord development (Brennan *et al.*, 2003). There are several possible links between RA signaling and key sex determination-related genes. *Pdgfa* and *Pdgfrb* are candidate RAR targets; *Wnt4* has not been previously characterized as an RAR target gene, but several *Wnt* genes, including *Wnt1*, *Wnt3a*, and *Wnt8d*, are known or suspected RAR targets (Balmer and Blomhoff, 2002). Additionally, suspected regulation of *Sox9* by RARs (Sekiya *et al.*, 2000) is a possible upstream connection between RA signaling and sex determination. Notably, the microarray results in this study also included induction of retinoid signaling and metabolism genes, including induction of *Lrat*, *Rbp1*, *Cyp26a1*, *Stra6*, *Dhrs3*, and *Rdh10*, confirming robust canonical RA signaling activity in this model, similar to previous reports of RA effects on early postnatal germ cells in rats and mice (Wang and Culty, 2007; Zhou *et al.*, 2008).

The presence of dispersed DMRT1- and FOXL2-positive cells in ATRA-treated testes (Figure 6) is evidence of a functional disruption of testis development following ATRA exposure, including disassociation of Sertoli cells, reduced seminiferous cord number, and gain of an ovary-like FOXL2-positive phenotype in some gonadal somatic cells. However, retention of DMRT1 expression in Sertoli cells indicates that Sertoli cells did not generally dedifferentiate or transdifferentiate to ovary-like cells. This is consistent with the literature on DMRT1- and FOXL2-deficient genetic models. In the early postnatal mouse testis, DMRT1 knockout combined with exogenous retinol induces a sex reversal phenotype in which Sertoli cells dedifferentiate and develop into FOXL2-expressing granulosa-like cells (Minkina *et al.*, 2014). In the adult ovary, loss of functional FOXL2 leads to gonadal sex reversal in which seminiferous cord structures with a basement membrane and Sertoli-like cells are present (Uhlenhaut *et al.*, 2009).

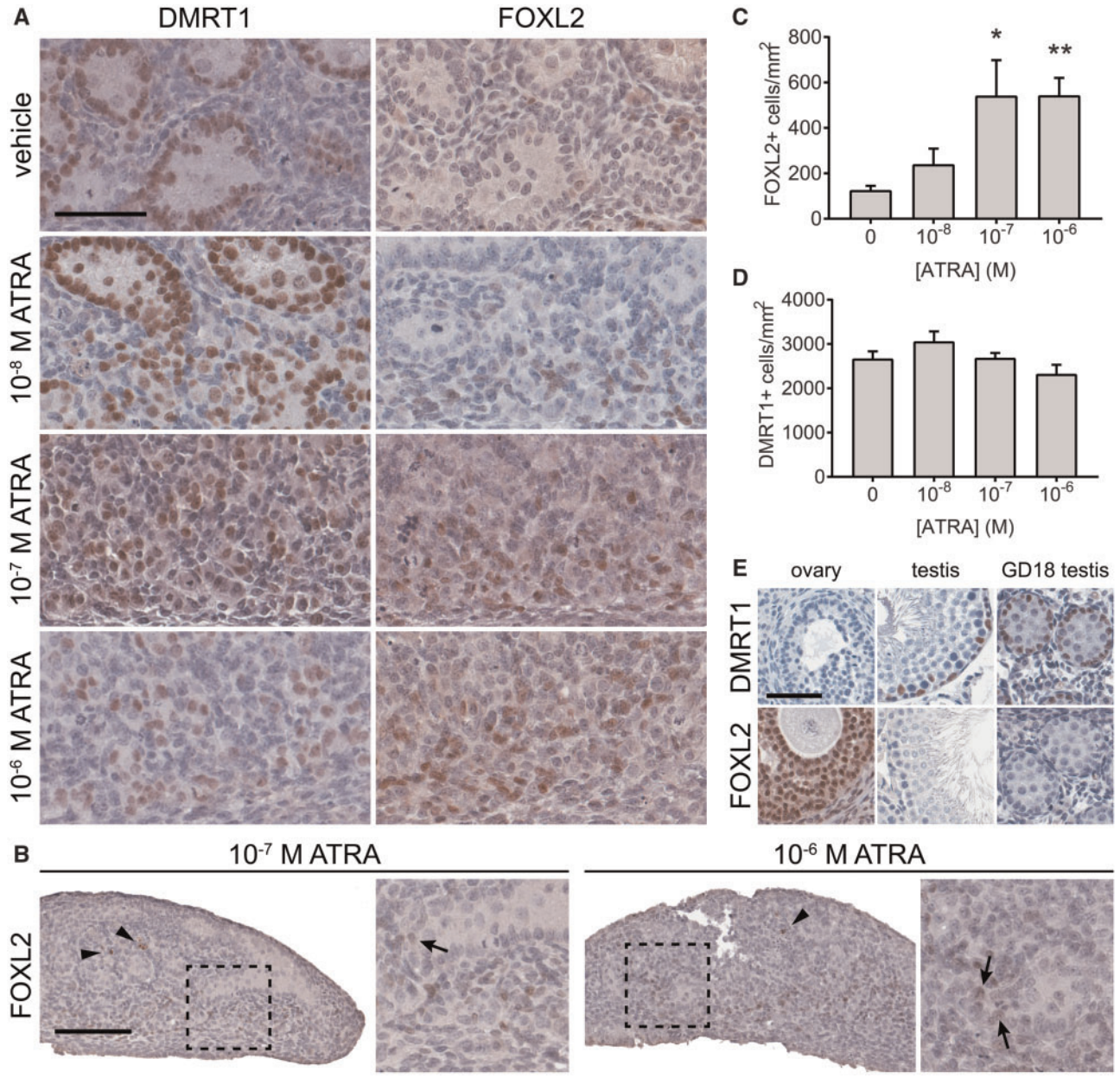

Figure 6. ATRA exposure resulted in dispersion of Sertoli cells and induction of FOXL2. A, In vehicle-treated fetal testis cultures, Sertoli cells and some germ cells expressed DMRT1; samples contained very few FOXL2-positive cells. Following 3-day culture with ATRA, DMRT1-positive cells were scattered throughout the section, and there were a greater number of strongly FOXL2-positive cells. B, FOXL2-positive cells were dispersed throughout ATRA-treated sections, with many cells appearing in the lower (membrane-facing) half of the section. Rarely, FOXL2-positive cells were localized in cords, with morphology and localization similar to Sertoli cells (arrows). Dashed lines identify inset areas. Arrowheads indicate nonspecific staining in endothelial cells, excluded from counts. Scale bar = 50 μm. C, The number of FOXL2-positive cells was significantly increased in testes cultured with 10^{-7} and 10^{-6} M ATRA. $^{*}p < .05$, $^{**}p < .01$ by paired *t* test. D, The number of DMRT1-positive cells was not significantly altered at any ATRA treatment level ($n = 6$–7 testes/group in (C) and (D). E, Adult ovary and testis and GD 18 control testis samples showed appropriate expression of FOXL2 and DMRT1: Adult rat ovary expressed FOXL2 in granulosa and some theca cells, but did not express DMRT1. Adult rat testis expressed DMRT1 in Sertoli cells and spermatogonia. GD 18 rat testis expressed DMRT1 in Sertoli cells. No FOXL2 expression was detected in adult or GD 18 rat testis. Scale bars in (A) and (E) = 60 μm. Scale bar in (B) = 120 μm. The full-color image is available online.

Expression of *Foxl2*, as well as *Wnt4*, is required for ovary determination in XX mice (Ottolenghi *et al.*, 2007).

Germ cells were also adversely affected by ATRA treatment, consistent with previous reports of RA administration in rats and mice (Cupp *et al.*, 1999; Li and Kim, 2004; Livera *et al.*, 2000; Trautmann *et al.*, 2008) and CYP26-deficient mouse models (Hogarth *et al.*, 2015; Li *et al.*, 2009; MacLean *et al.*, 2007). The loss of germ cells following 3-day ATRA exposure in this study (Figure 3) was corroborated by decreased expression of *Ddx4* and *Pou5f1* by 10^{-6} M ATRA treatment after 1 d of culture (Figure 5). Germ cells that survived ATRA exposure were often found outside of cords (Figure 3). A previous report indicated that ATRA decreases the proportion of germ cells expressing the *Pou5f1* gene product OCT-3/4 in cultured human fetal testis (Jorgensen *et al.*, 2015), but in this study, the ratio of *Pou5f1* and *Ddx4* expression was similar at each dose, indicating no measurable effect of ATRA on average *Pou5f1* expression across germ cells. Notably, in the mouse, RA has a well-established

role in induction of meiosis in the fetal gonad (Bowles *et al.*, 2006; Koubova *et al.*, 2006, 2014; Li *et al.*, 2009; MacLean *et al.*, 2007; Trautmann *et al.*, 2008). However, the literature on RA exposure models in the fetal rat testis does not include evidence of early induction of meiosis (Cupp *et al.*, 1999; Li and Kim, 2004; Livera *et al.*, 2000). In this study, we found minimal evidence of meiosis induction following ATRA exposure. For instance, *Stra8* was weakly induced according to the microarray data, but did not reach statistical significance (1.96-fold, FDR $p = 0.201$). This lack of evidence for meiosis induction by RA may have resulted from germ cell death interfering with detection of meiosis initiation. However, given the consistency with the published rat literature, this may be evidence of a species difference between rats and mice. This should be investigated further in a future study.

In addition to gene expression, we investigated alternative splicing events as possible regulators of ATRA effects on germ cells, because germ cell developmental mechanisms in spermatogenesis involve high rates of alternative splicing (Elliott and Grellscheid, 2006). However, we found only a small amount of evidence for alternative splicing as a regulatory mechanism controlling response to exogenous ATRA (Figure 4, Supplementary Table 3). We did find evidence for alternative splicing of the first exon of *Lrat* (Figure 4, Supplementary Table 3), which is consistent with human data (Zolfaghari and Ross, 2004). Not only was *Lrat* alternatively spliced, but it was the most up-regulated gene in the transcriptome-wide analysis (Figure 4), and was represented in 4 of the 10 significantly enriched GO terms in the GSEA analysis, including *retinol metabolic process* (Table 2). Further, despite significant effects of ATRA on Sertoli and germ cells, we did not observe altered Leydig cell function, using *Star* expression as a marker (Figs. 4 and 5). *Star* expression was investigated by real-time RT-PCR because down-regulation of *Star* transcription would be consistent with *Nr0b1* up-regulation (Zazopoulos *et al.*, 1997) and with previous reports of decreased testosterone levels in fetal testes cultured with ATRA (Livera *et al.*, 2000, 2004). However, *Star* expression was not significantly altered, nor was the expression of other major Leydig cell functional markers, including *Cyp11a1* (−1.18-fold, FDR $p = 0.55$), *Cyp17a1* (−1.74-fold, FDR $p = .15642$), *Hsd3b1* (1.08-fold, FDR $p = .66$), or *Hsd17b1* (1.16-fold, FDR $p = .33$), according to microarray data.

In this study, exposure of *ex vivo* cultured rat fetal testes to exogenous ATRA reduced the number of seminiferous cords present per testis section, consistent with previous reports of disorganized testis cords following ATRA exposure (Cupp *et al.*, 1999; Jorgensen *et al.*, 2015; Livera *et al.*, 2000, 2001). The effect of ATRA on seminiferous cord number was concentration-dependent (Figure 2), and the expression of ACTA2 in the area around seminiferous cord basement membranes was lost in ATRA-treated cultures (Figure 1). ACTA2 is a marker of peritubular myoid cell differentiation, which is controlled by Sertoli cells in the fetal testis (Rebourcet *et al.*, 2014). This disorganized phenotype may have resulted from the effects of ATRA on Sertoli cell differentiation. Seminiferous cord formation is a critical step in testis development, and requires migration of endothelial cells, assembly of Sertoli cells around germ cells, and recruitment of peritubular myoid cells (Brennan and Capel, 2004; Cool *et al.*, 2008). This study provides novel evidence that exogenous retinoids can disrupt seminiferous cord development and alter the expression of genes and proteins involved in maintenance of testicular cell fate, even when exposure begins after gonad determination and the initiation of seminiferous cord development. This is likely to be of broad interest in reproductive toxicology: in addition to retinoids, this pathway could be sensitive to perturbation by compounds that alter RA metabolism, such as some imidazole fungicides (Gomaa *et al.*, 2011; Nelson *et al.*, 2013), and compounds that might alter RA signaling through crosstalk, such as phthalates (Bhattacharya *et al.*, 2005; Dufour *et al.*, 2003). This interaction between RA and phthalates is addressed in our companion study. Future studies will aim to assess the cell type-specific gene expression response to ATRA, including the mechanism and consequences of *Wnt4* induction following exposure for ATRA. Additionally, this study did not address the influence of RA on development of the testicular vasculature, a critical developmental process that takes place in concert with seminiferous cord development. This should be assessed in a future study.

SUPPLEMENTARY DATA

Supplementary data are available at the Dryad Digital Repository: https://doi.org/10.5061/dryad.12jj3sv.

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