REPRODUCTIVE TOXICOLOGY

Environmental exposures, fetal testis development and function: phthalates and beyondHui Li and Daniel J Spade¹*Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island, USA**Correspondence should be addressed to D J Spade; Email: daniel_spade@brown.edu*

This paper forms part of a special section on Reproductive toxicology. The guest editors for this section were Dr Adam Watkins (The University of Nottingham, UK) and Dr Aileen Keating (Iowa State University, IA, USA).

Abstract

Fetal development of the mammalian testis relies on a series of interrelated cellular processes: commitment of somatic progenitor cells to Sertoli and Leydig cell fate, migration of endothelial cells and Sertoli cells, differentiation of germ cells, deposition of the basement membrane, and establishment of cell–cell contacts, including Sertoli–Sertoli and Sertoli–germ cell contacts. These processes are orchestrated by intracellular, endocrine, and paracrine signaling processes. Because of this complexity, testis development can be disrupted by a variety of environmental toxicants. The toxicity of phthalic acid esters (phthalates) on the fetal testis has been the subject of extensive research for two decades, and phthalates have become an archetypal fetal testis toxicant. Phthalates disrupt the seminiferous cord formation and maturation, Sertoli cell function, biosynthesis of testosterone in Leydig cells, and impair germ cell survival and development, producing characteristic multinucleated germ cells. However, the mechanisms responsible for these effects are not fully understood. This review describes current knowledge of the adverse effects of phthalates on the fetal testis and their associated windows of sensitivity, and compares and contrasts the mechanisms by which toxicants of current interest, bisphenol A and its replacements, analgesics, and perfluorinated alkyl substances, alter testicular developmental processes. Working toward a better understanding of the molecular mechanisms responsible for phthalate toxicity will be critical for understanding the long-term impacts of environmental chemicals and pharmaceuticals on human reproductive health.

*Reproduction (2021) 162 F147–F167***Introduction**

Mammalian fetal testis development relies on a remarkably intricate series of interrelated cellular division, migration, and differentiation processes, which are orchestrated by multiple endocrine, paracrine, and intracellular signaling pathways. As a result, the fetal testis is susceptible to injury resulting from exposure to a variety of environmental chemicals and pharmaceuticals. The bipotential gonad consists of somatic cells – a supporting cell lineage and a steroidogenic cell lineage, germ cells that have migrated from the allantois, and yolk sac-derived macrophages. In XY mice, the testis developmental program is initiated by transient expression of *Sry* on an embryonic day 11.5 (e11.5, also called gestational day 11 or GD 11) (Koopman *et al.* 1990), which leads to a persistent gene expression program that promotes the commitment of the supporting cell lineage to Sertoli cell fate and the eventual development of the testis (Lin & Capel 2015). Sertoli cells continue to express Sertoli cell identity

genes, including *Sox9*, *Pdgfra*, and *Nr0b1* (DAX1), throughout life (Brennan & Capel 2004).

The commitment of undifferentiated supporting cells leads to the formation of seminiferous cords, the predecessors of the seminiferous tubules, comprised of Sertoli cells surrounding fetal germ cells (Nel-Themaat *et al.* 2011). Peritubular myoid cells (PTMCs) are recruited to the cords, and a basement membrane is established from collagens and laminins secreted by Sertoli cells and PTMCs (elOuali *et al.* 1991, Virtanen *et al.* 1997). The Sertoli cell population is mitotically active throughout gestation, leading to elongation of cords. Germ cells, termed 'prospermatogonia,' meanwhile, mitotically expand prior to a quiescent period from e16.5 in the mouse (GD 18 in the rat) until birth, during when they differentiate and begin to display characteristics of spermatogonia (Culty 2013). Surrounding the cords is an interstitial compartment, containing Leydig cells, macrophages, connective tissue, and vasculature. The development of the testicular vasculature and seminiferous cords is simultaneous, promoted by VEGF

secreted by macrophages, and PDGF from somatic cells at the testis–mesonephros boundary (Coveney *et al.* 2008, DeFalco *et al.* 2014). Fetal Leydig cells differentiate from the steroidogenic cell lineage between e11.5 and e13.5 in the mouse (Stevant *et al.* 2018). Once committed, fetal Leydig cells secrete testosterone and INSL3, which are responsible for masculinization of the reproductive tract and AMH, which signals for regression of the Müllerian duct (Behringer 1995, Emmen *et al.* 2000).

The testis is susceptible to disruption by numerous toxicants, including pharmaceuticals, industrial and agricultural chemicals, and other environmental toxicants, such as heavy metals. Male reproductive toxicants are often classified by their target cell type. Germ cell toxicants produce direct germ cell death, which is a significant adverse effect, as the primary function of the testis is to maintain and support germ cells and produce mature spermatozoa. Germ cells, which are frequently dividing at many developmental stages and during spermatogenesis, can be directly targeted by, for example, DNA damage-inducing alkylating agents (Boekelheide 2005). Sertoli cell toxicants can also cause germ cell death indirectly, often through damage to the Sertoli cell cytoskeleton, which is the mechanism of the *n*-hexane metabolite, 2,5-hexanedione (Boekelheide 1988, Allard & Boekelheide 1996). Apart from directly injuring germ cells or Sertoli cells, endocrine disruption represents a common mode of male reproductive toxicity. Endocrine-disrupting chemicals mimic hormones by binding to their receptors, inhibit the binding of endogenous hormones to their receptors, or alter the metabolic processes that control the quantity of hormone in circulation. Estrogenic compounds, such as diethylstilbestrol, and antiandrogens, such as vinclozolin, are well-characterized male reproductive toxicants in both the adult and fetal testis (Fielden *et al.* 2002, Hotchkiss *et al.* 2004, Gray *et al.* 2005, N'Tumba-Byn *et al.* 2012).

In the human health context, exposure to male reproductive toxicants and endocrine disruptors contributes to male factor infertility and other adverse health outcomes. The most straightforward examples are toxicant exposures during adulthood that result in disrupted spermatogenesis, potentially leading to infertility due to oligozoospermia, which can be transient or persistent. A classic example of this was discovered when a cohort of agricultural workers suffered irreversible infertility as a result of exposure to the nematocide, 1,2-dibromo-3-chloropropane (Whorton *et al.* 1977). As opposed to occupational exposure to testicular toxicants, understanding the long-term impact of gestational exposure to male reproductive toxicants presents a greater challenge for several reasons. First, typical levels of human exposure to environmental toxicants in the general population are much lower than the levels experienced by workers in industrial or occupational settings. Secondly, the latency

of effects makes it difficult to test for causality between human exposures and outcomes and it is costly and time-consuming to design experiments in animals to test for later-life effects. Finally, testicular development is complex and still incompletely understood, which makes the discovery of toxicity mechanisms targeting fetal testis development a challenge. In spite of these difficulties, it has been hypothesized that environmental factors are contributing to increasing rates of adverse male reproductive health outcomes worldwide. These adverse outcomes, including infertility, testicular cancer, and congenital defects of the male reproductive tract, are hypothesized to share a common origin in disrupted fetal development, a phenomenon termed 'testicular dysgenesis syndrome' (Skakkebaek *et al.* 2001). For example, a report from a Danish cancer registry shows that testicular cancer rates have more than tripled between 1944 and 2009, rising to a rate of approximately 10 cases per 100,000 (Andersson *et al.* 2016). A recent meta-analysis of global sperm counts, similarly, showed a significant negative trend over time from 1973 to 2011, regardless of whether the populations were known to be fertile or unselected for fertility (Levine *et al.* 2017). Declining sperm count may contribute to infertility, which is experienced by approximately 12% of couples attempting to achieve pregnancy (Mehta *et al.* 2016). Hypospadias and cryptorchid testis, meanwhile, are two of the most commonly diagnosed birth defects in males, and there is some evidence that rates are increasing (Toppari *et al.* 2001). Despite this evidence of increases in TDS outcomes, the causality of environmental exposures has not been definitively supported.

Phthalic acid esters (phthalates) are an environmental exposure of concern for male reproductive health, which provide an excellent illustration of how susceptible many of the cellular and biochemical processes in testis development are to disruption (Fig. 1) and which may provide a model for TDS. Phthalates disrupt spatial patterning of cells during testis development, causing a histological phenotype termed 'testicular dysgenesis'. The combination of antiandrogenic effects and dysgenesis in animals results in immediate and later-life defects in testicular function and male reproductive tract development, a phenomenon termed 'phthalate syndrome,' which has been proposed as a model of TDS in humans. Despite this profound toxicity, the mechanisms underlying the response still remain unclear. Phthalate toxicity is likely the result of multiple injuries to the fetal Sertoli and Leydig cells. It may be initiated by disruption to androgen signaling, INSL3 signaling, and/or additional nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) and retinoic acid receptors (RARs). The goals of this review are to demonstrate the sensitivity of fetal testis development to environmental insults by reviewing the current knowledge of phthalate toxicity mechanisms and the remaining knowledge gaps, review trends in

Figure 1 Normal rodent testis development and effects of fetal phthalate exposure in rats and mice. Fetal testis development, depicted in the top two panels, begins with the determination of the indifferent gonad on embryonic day 11.5 (e11.5) in the mouse or gestational day (GD) 12 in the rat. Supporting cells commit to Sertoli cell (SC) fate, which initiates the formation of seminiferous cords. Angiogenesis occurs simultaneously, relying on endothelial cells that migrate from the mesonephros, and steroidogenic cells commit to Leydig cell (LC) fate. Seminiferous cord maturation continues with recruitment of peritubular myoid cells (PTMCs), deposition of basement membrane (BM), and proliferation of SCs, giving rise to longer, convoluted cords. Germ cells (GCs), which are termed 'prospmatogonia,' proliferate until e16.5 (mouse) or GD 18 (rat), at which point they enter a quiescent phase and differentiate, taking on some characteristics of spermatogonia, reviewed in (Culty 2009). LCs produce testosterone, INSL3, and anti-Müllerian hormone throughout the fetal period. The critical window during which these hormones are responsible for masculinization of the male reproductive tract, termed the 'masculinization programming window' (MPW), spans approximately GD 15–17 in the rat (Welsh et al. 2008). Phthalate toxicity, as depicted in the lower two panels, has been well characterized in the fetal rat testis, in which the different phases of testis development create unique windows of sensitivity for phthalates. Only in rats, gestational phthalate exposure reduces testosterone (illustrated here as orange particles emanating from Leydig cells), regardless of the exposure timing. However, exposure during the MPW (early exposure, above) leads to the most significant adverse outcomes on masculinization of the reproductive tract, such as undescended testis and hypospadias. Similarly, dysgenesis of seminiferous cords is reportedly greatest when exposure occurs during the MPW, leading to incomplete or ruptured cords, ectopic GCs and SCs, and clustering of LCs (Lara et al. 2017, van den Driesche et al. 2017). This early exposure window is also associated with increased germ cell death in the fetal mouse (Lehrai et al. 2009). Late gestational phthalate exposure, defined as exposure beginning after the GC quiescent period on GD 18 in the rat, results in inhibition of SC proliferation, induces multinucleated germ cells (MNGs), germ cell clustering, dilated cords, and thickening of the BM (Boekelheide et al. 2009, Spade et al. 2015). The exposure that continues throughout the early and late windows results in a combination of these effects, though with less prevalent dysgenic areas (Lara et al. 2017). All of these effects may contribute to reduced later-life fertility following exposure to male reproductive toxicants during fetal development.

the recent experimental literature (2010–2020) on fetal testis toxicant effects from rodent and human tissue models, and summarize evidence that disrupted fetal testis development leads to impaired later-life male reproductive function.

Search methodology, results, and scope of review

We identified manuscripts for this review by searching PubMed between August 10, 2020, and April 16, 2021 (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). To determine the overall volume of published literature on fetal testis toxicants, we performed an initial search for '(toxic OR toxicant OR toxicity) AND (fetal OR gestational) AND (testis OR testicular),' which returned 1745 results published since 1964, with a significant increase in publication rate during the 1990s leading to an average of 50.5 papers per year since 2010 (excluding the partial year 2021). Based on our initial search, we determined that the classes of compounds most frequently investigated for fetal testicular toxicity in recent years were phthalates, bisphenol A, analgesics and/or NSAIDs, and perfluoroalkyl substances (PFAS). Thus, in subsequent searches, we added the terms '(phthalate OR phthalates),' '('bisphenol A' OR BPA OR bisphenols); '(analgesic OR NSAID)'; and '(PFAS OR 'perfluorinated alkyl substances' OR PFOA OR PFOS).' We limited the searches by automatically excluding any papers indexed as 'review' or 'critical review' on PubMed. Subsequently, we manually reviewed the results and excluded any additional review or commentary articles, epidemiology publications, articles in which the exposure window did not include gestation, articles that used only cell culture models, articles that did not include experimental data on the class of compounds in question, and articles for which the full text could not be retrieved.

Our searches returned 389 results pertaining to phthalates, spanning the years 1982 to 2021, almost all published from 2000 to the present, and 154, 94, and 18 results pertaining to bisphenol A, analgesics, and PFAS, respectively, comprising 31.0, 14.9, 4.7, and 2.8% of results since 2010, respectively (Supplementary Tables 1, 2 and 3). After manual exclusion, the totals were 99, 47, 12, and 8 papers per class, respectively. We did not seek to comprehensively review the 166 papers that met our search and exclusion criteria. Rather, based on the prevalence of papers in these categories, we focused this review on the current knowledge and knowledge gaps in the fetal testis toxicity of phthalates, and we reviewed the literature on bisphenol A, analgesic drugs, and perfluorinated compounds to make comparisons about known mechanisms of fetal testis toxicity. Given space restraints, we did not attempt to review the epidemiology literature on fetal testis toxicants, the kinetics of most compounds, or the reproductive toxic effects on non-testis organs, such as the prostate or placenta. Where

possible, we have cited prior reviews that address those topics.

Phthalates

Phthalates are the most widely studied class of fetal male reproductive toxicants. Despite intense interest in fetal testis toxicity of phthalates for two decades, the mechanisms remain incompletely understood. Several recent papers describe the major knowledge gap in phthalate toxicity research by applying the adverse outcome pathway (AOP) paradigm, a conceptual framework that links toxicants to adverse (toxic) outcomes through a 'molecular initiating event (MIE)' and subsequent 'key events,' usually cellular processes that mediate the toxic response (Ankley *et al.* 2010, OECD 2018). Hypothesized AOPs to describe phthalates' antiandrogenic mode of action illustrate that there is no known MIE for phthalate toxicity in the fetal testis (Howdeshell *et al.* 2015, Conley *et al.* 2018, Arzuaga *et al.* 2019, Clewell *et al.* 2020, Gray *et al.* 2020). In spite of this knowledge gap, it is well known that phthalates alter testicular morphology, survival, and differentiation of germ cells in all species studied to date and disrupt endocrine functions in the rat fetal testis. This illustrates both the unique susceptibility of the fetal testis to environmental toxicants and the remaining work that will be required to understand this susceptibility in its entirety.

Reduced testosterone biosynthesis

Phthalate diesters are metabolized to toxicologically active monoesters by intestinal and liver lipases (Albro 1986) and are transported across the placenta (Struve *et al.* 2009). Synthesis of testosterone in fetal Leydig cells is diminished by phthalates, especially in the rat (Gray *et al.* 2000, Parks *et al.* 2000, Mylchreest *et al.* 2002), but phthalates do not antagonize the androgen receptor (Parks *et al.* 2000, Stroheker *et al.* 2005). All ortho-phthalic acid esters in which the major aliphatic side chains are between four and six carbons in length reduce testosterone in this way, as do some with longer aliphatic or cyclic side chains; the lowest *in vivo* dose at which a phthalate has been reported to significantly reduce testosterone is 33 mg/kg/day dipentyl phthalate (DPeP) (Table 1) (Furr *et al.* 2014). The loss of testosterone leads to reduced masculinization of the male reproductive tract and loss of masculine secondary sex characteristics in rats. This phenomenon, termed 'phthalate syndrome,' results in hypospadias, undescended testis, retained nipples, and decreased anogenital distance (AGD) (Foster 2006). The testis has an abdominal descent phase driven by INSL3 and a scrotal descent phase driven by testosterone. Both of these signals are reduced by phthalate exposure, impairing testis descent (Shono *et al.* 2000, Wilson *et al.* 2004). This has been considered a

Table 1 Phthalate effects reported in mouse, rat, and human fetal testis models. The table provides a comprehensive list of reported effects of phthalates on mouse fetal testis and in human fetal testis tissue culture and xenograft models and selected reports on rat fetal testis.

Species/Reference	Compound	Model	Antiandrogenic effects*			Seminiferous cord effects*					DYG
			LC#†	LC GPE	MAS	MNG	GC#‡	SC#	SC GPE	d _{cond}	
Mouse											
	Gaido <i>et al.</i> (2007)	IU									
	van den Driesche <i>et al.</i> (2012b)	IU		NC (500)							↑ (250)
	Heger <i>et al.</i> (2012)	IU			NC (500)						
	Saffarini <i>et al.</i> (2012)	IU									
	Ungewitter <i>et al.</i> (2017)	IU		↓ (5)	↑ (250)	↑ (500)	NC (250)		↑↓ (5)		
	Gaido <i>et al.</i> (2007)	IU									
	Gaido <i>et al.</i> (2007)	IU		↑↓ (500)							
	Muczynski <i>et al.</i> (2012)	IU									
	Lehraiki <i>et al.</i> (2009)	EV		↑↓ (200 μM)		↑ (200 μM)	↓ (720)		↑↓ (500)		
	Muczynski <i>et al.</i> (2012)	EV									
	Heger <i>et al.</i> (2012)	XG		NC (500)*		↑ (250)	↓ (10 μM)	NC (500)			
	Human										
	Habert <i>et al.</i> (2014)	EV									
	Lambrot <i>et al.</i> (2009)	EV		NC (100 μM)							
	Muczynski <i>et al.</i> (2012)	EV									
	Mitchell <i>et al.</i> (2012)	XG									
Rat											
	Spade <i>et al.</i> (2018)	IU									
	Wilson <i>et al.</i> (2004)	IU									
	Spade <i>et al.</i> (2018)	IU									
	Mahood <i>et al.</i> (2005)	IU									
	Mahood <i>et al.</i> (2007)	IU									
	Mylchreest <i>et al.</i> (2002)	IU									
	Wilson <i>et al.</i> (2004)	IU									
	Johnson <i>et al.</i> (2007)	IU									
	Barlow <i>et al.</i> (2003)	IU									
	Shultz <i>et al.</i> (2001)	IU									
	Kuhl <i>et al.</i> (2007)	IU									
	Thompson <i>et al.</i> (2005)	IU									
	van den Driesche <i>et al.</i> (2012a)	IU									
	van den Driesche <i>et al.</i> (2012b)	IU									
	Lara <i>et al.</i> (2017)	IU									
	Boekelheide <i>et al.</i> (2009)	IU									
	Kleymenova <i>et al.</i> (2005)	IU									
	Spade <i>et al.</i> (2015)	IU									
	Spade <i>et al.</i> (2018)	IU									

(Continued)

Table 1 Continued.

Species/Reference	Compound	Model	Antiandrogenic effects [‡]			Seminiferous cord effects [‡]				
			T	LC ^{††}	LC GPE	MAS	MNG	GC ^{††}	SC [‡]	SC GPE
Parks <i>et al.</i> (2000)	DEHP	IU	↓ (750)	↑ (750)			↑ (750)			
Wilson <i>et al.</i> (2004)	DEHP	IU	↓ (1000)			↓ (750)				
Borch <i>et al.</i> (2006)	DEHP	IU	↓ (300)		↓ (100)		↓ (300)			
Spade <i>et al.</i> (2018)	DEHP	IU	↓ (300)				↑ (300)			
Furr <i>et al.</i> (2014)	DEHP	IU	↓							
Hannas <i>et al.</i> (2012)	many ^{‡‡}	IU	↓							
Shono <i>et al.</i> (2000)	MBP	IU	↓			↓ (1000)				
Howdeshell <i>et al.</i> (2015)	mixture [‡]	IU	↓ (215)***			↓ (65)				
Heger <i>et al.</i> (2012)	MBP	EV			↓ (250)					
Habert <i>et al.</i> (2014)	MEHP	EV	↓ (10 µM)							
Heger <i>et al.</i> (2012)	DBP	XGt	↓ (500)		↓ (500)		↑ (250)	NC (500)		
Mitchell <i>et al.</i> (2012)	DBP	XG	NC (500)****		↓ (500)	↓ (500)				

Dysgenesis is classified separately from Leydig cell and cord effects because it involves both compartments and is associated with exposure during the masculinization programming window. †The lowest dose or concentration at which an effect was reported is presented in parentheses (mg/kg/day unless otherwise noted). Some changes listed are approximations of holistic change such as directional changes in 'Leydig cell' or 'Sertoli cell' genes in transcriptomic studies; †refers to LC numbers and to effects explicitly described as clustering or as Leydig cell hyperplasia; ‡refers to GC number or apoptosis; ‡Exposure consisted of a mixture of BBP, DBP, DEHP, diisobutyl phthalate (DiBP), and DEHP. ‡Paper reports dose-response data for many phthalates. No specific effect doses are listed in the table. *One of five genes decreased significantly. **Reported with $P=0.051$. ***Calculated based on the ED₅₀ as a percent of the maximum mixture dose. ****Reported with $P=0.062$. †, increase; ‡, dose-response was non-monotonic; ↓, decrease; BBP, benzyl butyl phthalate; DBP, di-n-butyl phthalate; DEHP, di-(2-ethylhexyl) phthalate; DEHP, dipentyl phthalate; DYG, dysgenesis, explicitly identified in the manuscript as such and including non-intact seminiferous cords, ectopic cells, or regions lacking discernable seminiferous cord structure; EV, ex vivo; GPE, gene and/or protein expression; IU, in utero; LC, Leydig cell; MAS, masculinization, including anogenital distance, testis descent, or weights of accessory sex organs; MBP, mono-n-butyl phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MNG, Sertoli cell; NC, no change; SC, Sertoli cell; T, testosterone; XG, xenograft.

model for 'testicular dysgenesis syndrome' in humans, which consists of undescended testes, hypospadias, decreased male fertility, and increased testicular cancer risk (Skakkebaek *et al.* 2001).

The mechanism through which phthalates decrease testicular testosterone is not understood. Phthalates bind and activate PPARs in other tissues, but there is no evidence that phthalates enhance the expression of PPARα target genes in the fetal testis (Hannas *et al.* 2012). The loss of testicular testosterone occurs in part through decreased expression of genes and proteins involved in steroid hormone biosynthesis, including *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Hsd17b1*, *Scarb1*, and *Star* (Shultz *et al.* 2001, Barlow *et al.* 2003, Johnson *et al.* 2007, Hannas *et al.* 2012). Some of the phthalate-driven gene expression changes are dependent on inhibition of CEBPβ and possibly SF-1 (Borch *et al.* 2006, Kuhl *et al.* 2007) or prolonged expression of COUP-TFII (van den Driesche *et al.* 2012b). However, phthalates also cause a rapid decline in testosterone within 1 h of a single *in utero* phthalate exposure (Thompson *et al.* 2005), suggesting that a fairly rapid biochemical mechanism initiates this effect. Phthalate-driven decreases in testosterone also coincide with changes in Leydig cell biology, including Leydig cell clustering, hyperplasia, and intra-seminiferous cord fetal Leydig cells (Mylchreest *et al.* 2002, Mahood *et al.* 2005).

Species differences

Although the effect of phthalates on testosterone biosynthesis is well characterized in the fetal rat, there are differences in the reported responses of rat, mouse, nonhuman primate, and human fetal testes to phthalates, especially with regard to testosterone suppression (Johnson *et al.* 2012, Habert *et al.* 2014) (Table 1). Several *in utero* exposure studies have reported that the mouse fetal testis is insensitive to the antiandrogenic effects of certain phthalates that are known to reduce testosterone in the rat, specifically di-n-butyl phthalate (DBP), mono-n-butyl phthalate (MBP), and mono-(2-ethylhexyl) phthalate (MEHP) at doses as high as 1500 mg/kg/day DBP (Gaido *et al.* 2007, Heger *et al.* 2012, van den Driesche *et al.* 2012b, Ungewitter *et al.* 2017). However, those same phthalates induce multinucleated germ cells (MNGs), increase seminiferous cord diameter, and alter Sertoli cell gene expression in mice, as they do in rats (Gaido *et al.* 2007, Saffarini *et al.* 2012, Ungewitter *et al.* 2017). MNGs have not been quantified in as many publications as effects on testosterone, but they have been induced significantly at doses as low as 100 mg/kg/day DBP in the rat (Boekelheide *et al.* 2009) and 250 mg/kg/day in mouse fetal testis xenografts (Heger *et al.* 2012) and have been observed but not quantified at doses as low as 5 mg/kg/day in the mouse (Ungewitter *et al.* 2017). This distinction between what we will term 'antiandrogenic effects' and 'seminiferous cord effects'

of phthalates has been supported by *ex vivo* mouse testis culture experiments, in which phthalate exposure has been reported to reduce germ cell number or increase MNGs, while effects on testosterone were equivocal in one experiment. Specifically, MEHP exposure with or without LH stimulation led to increased testosterone in e 13.5 testis cultures. In e 18.5 cultures, MEHP exposure with no LH stimulation increased testosterone, but with LH or cAMP stimulation, MEHP reduced testosterone (Lehraiki *et al.* 2009, Muczynski *et al.* 2012). Likewise, in a xenotransplant experiment, DBP increased MNGs in mouse fetal testes grafted into mouse hosts but had no effect on testosterone or host anogenital distance, a secondary sex characteristic used as a measure of testosterone-driven developmental masculinization (Heger *et al.* 2012). Few nonhuman primate studies of phthalate toxicity have been reported. Exposure of fetal marmosets to 500 mg/kg/day MBP resulted in sporadic germ cell clustering but no other effects on testicular histology and no changes in testosterone (McKinnell *et al.* 2009), while the same dose suppressed testosterone biosynthesis and induced Leydig cell hyperplasia in neonatal marmosets (Hallmark *et al.* 2007).

Similar to the mouse and nonhuman primate, the human fetal testis is insensitive to the testosterone-reducing effect of phthalates. This has been demonstrated by three human fetal testis xenotransplant experiments under slightly different experimental conditions (Heger *et al.* 2012, Mitchell *et al.* 2012, Spade *et al.* 2014) (Table 1). First, human fetal testis tissue subcutaneously xenografted into immunosuppressed mice showed no significant reduction in testosterone or secondary sex characteristics following exposure to DBP or MBP, with concurrent hCG stimulation (Mitchell *et al.* 2012). In a separate experiment, the same research group has shown that DBP exposure results in germ cell clustering, altered germ cell-Sertoli cell contacts, and induction of MNGs in xenografted human fetal testis tissue (van den Driesche *et al.* 2015). Secondly, in a study of human fetal testes xenografted into the renal subcapsular space of immunosuppressed rats, DBP caused an increase in MNGs but no change in expression of genes required for steroidogenesis (Heger *et al.* 2012). Finally, we used a model in which human fetal testis tissue was grafted into the renal subcapsular space of an hCG-stimulated, castrate, athymic nude mouse host to test whether suppression of testosterone was related to phthalate exposure or sufficient to cause induction of MNGs. We found that DBP did not reduce testosterone, and that the reduction of testosterone caused by the CYP17A1 inhibitor, abiraterone, did not induce MNGs, further demonstrating that testosterone reduction and histopathology caused by phthalates are independent effects (Spade *et al.* 2014). This lack of phthalate effects on testosterone biosynthesis has also been corroborated by evidence from *ex vivo* human fetal testis culture experiments (Lambrot *et al.* 2009, Muczynski *et al.* 2012,

Habert *et al.* 2014). To date, there has been no direct evidence from any experimental model that phthalates reduce testosterone biosynthesis in human fetal testis tissue. There are some notable limitations to these models. Human fetal testis exposure experiments often have smaller sample sizes than *in utero* rodent exposure models, and the gestational age range is limited to tissue from pre-viable fetal losses. Ideally, these experiments should be replicated further with varied exposure timing and dose. It is impossible to rule out the possibility that some phthalate compound at some exposure level would have an antiandrogenic effect at some gestational time point. However, the combination of xenograft and *ex vivo* culture models performed by different research groups, under different conditions, combined with the comparison of DBP with abiraterone as a positive control antiandrogen, gives high confidence that the human fetal testis is either quantitatively less sensitive or categorically insensitive to the antiandrogenic effects of phthalates.

This species difference raises the question of how to interpret the antiandrogenic effects of phthalates on the rat fetal testis in the context of human health. At least two factors are important to consider: the linkage between antiandrogenic and seminiferous cord effects of phthalates in the rat, and the epidemiologic evidence of androgen-related phthalate effects in humans. In the human and mouse studies that found the fetal testis to be insensitive to phthalate antiandrogenic effects, the phthalates nonetheless caused adverse effects on the development of the testis, including changes in seminiferous cord development, Sertoli cell function, germ cell death, and multinucleation of germ cells, which are described below. In other words, in all experimental systems that have been tested, phthalate exposures result in adverse seminiferous cord effects. This is likely to mean that the most directly relevant endpoints for human health are the seminiferous cord effects. On the other hand, antiandrogenic effects in the rat are clearly linked to seminiferous cord effects. We have demonstrated that in the rat model, there is a high degree of concordance of effects within a particular phthalate molecule, between the antiandrogenic effects and histological alterations in the seminiferous cord. In other words, phthalates, such as di-(2-ethylhexyl) phthalate (DEHP), DBP, benzyl butyl phthalate (BBP), and DPeP, cause both a reduction in testosterone and altered germ cell and seminiferous cord morphology in the fetal rat testis, while shorter chain phthalates, such as dimethyl phthalate (DMP) and diethyl phthalate (DEP), para-phthalic acid diesters, such as dioctyl terephthalate (DOTP), and a brominated phthalate cause neither effect (Spade *et al.* 2018). This suggests that phthalate toxicity is organized by upstream regulatory mechanisms that are consistent across species. This is an unproven hypothesis, but a similar dose-response for antiandrogenic and seminiferous cord effects of phthalates in the rat would indicate that reduced fetal testosterone is a biomarker

of phthalate effect that is relevant to human health risk assessment.

A second point is that some male reproductive system outcomes related to inhibition of androgen signaling are associated with maternal urinary phthalate metabolites or other measures of gestational phthalate exposure in epidemiological studies of humans. These findings include associations between human maternal urinary phthalate metabolites and anogenital distance, cryptorchid testis, and hypospadias (Bornehag *et al.* 2015, Radke *et al.* 2018). Some of these associations have been supported by critical reviews, such as the relationship between gestational DEHP exposure and reduced anogenital distance (Dorman *et al.* 2018). This is difficult to reconcile with the lack of evidence for this effect in the experimental literature. However, it is notable that this species difference only applies to fetal exposures, as postnatal phthalate exposures are antiandrogenic in humans (Albert & Jegou 2014).

Testicular dysgenesis maldevelopment of testicular structures

The fetal testis alterations caused by phthalates have been referred to as 'testicular dysgenesis,' not to be confused with testicular dysgenesis syndrome. Testicular dysgenesis consists of malformed tubules, Leydig cell clustering, intratubular Leydig cells, germ cell death, and induction of MNGs (Mahood *et al.* 2006, 2007) (Fig. 1). Testicular dysgenesis may be most severe when treatment is initiated after cord formation and during the masculinization programming window (MPW) (van den Driesche *et al.* 2012a, Lara *et al.* 2017), but there are several, potentially distinct, windows of sensitivity for histopathologic effects of phthalates. Phthalates impair Sertoli cell proliferation when treatment begins on GD 12, leading to a reduced number of seminiferous cord cross-sections and increased cross-sectional diameter in testicular sections taken during later gestation. This likely indicates impairment of the processes required for testis cords to elongate and convolute. Phthalate exposure also alters the cytoskeleton of Sertoli cells, leading to a loss of contact between Sertoli and germ cells, which may contribute to the phenotype of germ cell clustering (Kleymenova *et al.* 2005, Boekelheide *et al.* 2009). In later gestation, only from GD 18 onward in the rat, phthalate exposure leads to the production of MNGs, not through nuclear division without cytokinesis but presumably through fusion or collapse of germ cell clones that are connected by intercellular bridges (Ferrara *et al.* 2006, Spade *et al.* 2015). This window of sensitivity corresponds with the quiescent period of germ cell development (Culty 2013). Additionally, phthalate effects on PTMCs may contribute to testicular dysgenesis, as phthalate exposure leads to thickening

of the seminiferous cord basement membrane, assessed in late gestation (Veeramachaneni & Klinefelter 2014).

Possible molecular targets

Given that the histopathological effects of phthalates are consistent across species and do not rely on testosterone levels, a critical question is what upstream molecular initiating events could be responsible for phthalate toxicity. Phthalates bind peroxisome PPARs, and PPAR activation is responsible for phthalate toxicity in some tissues, such as the liver; however, PPARG is not required for phthalate-driven testosterone reduction, and phthalates do not induce a typical PPAR-mediated signaling response (Gazouli *et al.* 2002, Corton & Lapinskas 2004, Hannas *et al.* 2012). Phthalates antagonize cholesterol biosynthesis (Thompson *et al.* 2004), and the cholesterol-reducing drug, simvastatin, exacerbates phthalate effects on testosterone (Beverly *et al.* 2014). Dexamethasone also enhances phthalate toxicity, suggesting glucocorticoid receptor signaling as a possible mechanism (Drake *et al.* 2009). Phthalate exposure causes elevated plasma estradiol and gene expression patterns similar to estrogen receptor activation (Klinefelter *et al.* 2012, Veeramachaneni & Klinefelter 2014), and the soy estrogen, genistein, alters phthalate toxicity, possibly through effects on testicular macrophages, a little-explored area of fetal testis toxicity (Jones *et al.* 2015, Walker *et al.* 2020).

Given the number of nuclear receptor signaling pathways that are potentially altered by phthalates, we have hypothesized that there is a role of PPAR binding in fetal testicular phthalate toxicity, which may mediate adverse effects through crosstalk with other nuclear receptor signaling pathways. In fact, it has been demonstrated that MEHP has a cell type-specific effect on RAR signaling in mouse Sertoli cells, caused by PPAR–RAR competition for heterodimerization with retinoid X receptors (RXRs) (Dufour *et al.* 2003). Control of RAR signaling is critical for the development and function of the testis. We have shown that phthalates interact with exogenous retinoic acid in the fetal testis, modulating the effects of retinoic acid on cord development (Spade *et al.* 2019b). This hypothesis is consistent with the effects of phthalates on the regulation of genes and proteins involved in the specification of the supporting cell lineage to granulosa or Sertoli-like phenotypes (Wang *et al.* 2015, Spade *et al.* 2019a). Clearly, phthalates alter the function of multiple nuclear receptor signaling pathways that are critical for normal testis development. However, the search for a definitive set of molecular initiating events of phthalate toxicity continues.

Mechanistic clues in other classes of toxicants

Although phthalate toxicity presents a complicated case that occurs through multiple mechanisms and is not

fully described by classification as endocrine disruption, the effects of endocrine-disrupting chemicals (EDCs) – mostly estrogens and antiandrogens – on the fetal testis have been the subject of a great deal of research, and the effects of these compounds may prove relevant as comparisons for phthalates. Several excellent reviews describe the impacts of antiandrogens that disrupt fetal testis development and function by reducing testosterone (e.g. ketoconazole, prochloraz, statins, phthalates), antagonizing the androgen receptor (e.g. procymidone, DDT), or through both mechanisms (e.g. linuron, prochloraz) (Gray *et al.* 2001, Wilson *et al.* 2008, Scott *et al.* 2009). It is well established that there is a sensitive window for antiandrogenic effects on the masculinization of the male reproductive tract, termed the 'masculinization programming window' (MPW), and that antiandrogens have their most significant effects on male reproductive system development during the MPW, as described in the recent review from Sharpe (2020). While the exact mechanism of estrogenic toxicity in the fetal testis is not as well characterized as the antiandrogens, environmental estrogens also exert toxic effects on fetal testis development, which has been previously reviewed (Scott *et al.* 2009) and which may be attributable to the balance between androgen and estrogen levels. Diethylstilbestrol is a classic estrogenic fetal testis toxicant (Fielden *et al.* 2002). For a detailed description of these mechanisms, we refer the reader to the review papers cited here. Presently, we will discuss two endocrine-disrupting exposures that have received significant attention within the last decade: bisphenol A and analgesic medications.

An environmental estrogen bisphenol A and replacements

Bisphenol A (BPA), which was first commercially used in 1957, is one of the most highly produced chemicals worldwide and is used in numerous consumer products, such as plastic food and beverage containers, can linings, dental sealants, computers, and thermal paper. In almost all humans, BPA can be detected in the blood, urine, saliva, and other bodily fluids (Ikezuki *et al.* 2002, Sasaki *et al.* 2005). BPA is a controversial environmental exposure, due to its known estrogenic activity, on one hand, and its rapid metabolic clearance, on the other. Toxicokinetic studies of BPA in rodents have supported the argument that BPA is rapidly glucuronidated, and free BPA levels following oral exposure are very low (Doerge *et al.* 2010). Accordingly, the United States Food and Drug Administration (FDA) has maintained that BPA is safe as currently used. However, BPA can be transported across the placenta (Balakrishnan *et al.* 2010), and estrogenic compounds have demonstrated toxicity in the fetal testis. BPA has been identified as a modulator for both estrogen receptor alpha (ESR1/

ER α) and beta (ESR2/ER β) (Delfosse *et al.* 2012). At high concentrations *in vitro*, BPA can also bind to many other nuclear receptors, including the aryl hydrocarbon receptor (AHR), PPARs, AR, both thyroid hormone receptors (THRs), the pregnane X receptor (NR1I2/PXR), and estrogen-related receptor gamma (ESRRG/ERRg) (Moriyama *et al.* 2002, Matsushima *et al.* 2008, Molina-Molina *et al.* 2013). Collectively, by interacting with a variety of nuclear receptors, BPA can exert a profound impact on hormonal homeostasis.

Reports on the impact of BPA exposure on male reproductive development have varied widely. A recent review found a lack of consensus regarding the effect of BPA on fetal testis development, which may be attributed to differences in exposure protocol, animal models, BPA concentration range, and dosing time frame (Williams *et al.* 2014). In one recent publication, high-dose BPA exposure (5 or 50 mg/kg) in mice resulted in decreased Sertoli cell number, altered expression of Sertoli cell-related genes, disruption of spermatogenesis, impaired epididymal sperm counts, altered morphology, and reduced motility at postnatal week 6 (Tainaka *et al.* 2012). Another study of high-dose BPA in the fetal rat (0, 4, 40, and 400 mg/kg body weight) reported a dose-dependent decrease in serum testosterone level in male fetuses, only at 40 and 400 mg/kg compared to control. On GD 21, fetal Leydig cell number, but not Sertoli cell number, was reduced in the 400 mg/kg group, and expression of Leydig cell and Sertoli cell markers was reduced (Lv *et al.* 2019). Although this indicates that doses of 40 mg/kg/day and above could potentially disrupt the development of the male reproductive tract, these doses are greater than likely human exposure. Additionally, two other *in vivo* mechanistic studies have reported potential deleterious effects of BPA on fetal Leydig cell function, independent of any change in the number of the Leydig cells (N'Tumba-Byn *et al.* 2012, Ben Maamar *et al.* 2015).

It should be noted that BPA is being replaced with other bisphenol compounds in some manufacturing applications, as a result of consumer concern about BPA toxicity. One example is bisphenol C (BPC), which in one study strongly inhibited testosterone production in an *ex vivo* assay, but at 100 mg/kg/day *in utero* (GD 14-18) only weakly inhibited fetal testis testosterone production and testis gene expression *ex vivo* on GD 18. Further, *in utero* BPC exposure at 100 and 200 mg/kg/day did not significantly affect the reproductive tract of male offspring (Gray *et al.* 2019). In another result that is contrast to these reports of fetal testis toxicity of BPA, Dere *et al.* reported a whole-life BPA toxicity experiment in the rat model, which was conducted as part of the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) study. To address the concerns of the non-monotonic dose-responsive nature of the BPA, the CLARITY-BPA consortium study investigated a wide BPA dose range

from 2.5 to 25,000 µg/kg/day in rats from GD 6 until parturition, followed by treatment from PND 1 to 90, and the testes and sperm were collected for evaluation. Due to the design, outcomes of this study cannot be attributed solely to fetal exposure, and fetal endpoints were not directly measured. However, this study did not find any significant alterations in the histopathologic, morphometric, and molecular endpoints evaluated in adult rats, suggesting that under the conditions of this consortium study, the rat testis is not sensitive to BPA-induced toxicities (Dere *et al.* 2018). In summary, the current data from rodent studies provide mixed evidence that gestational BPA exposure can negatively affect male reproductive system development. The relevant BPA exposure level in humans is relatively low (Vandenberg *et al.* 2007), but there are reports of non-monotonic BPA dose-response for many endpoints (Heindel *et al.* 2020). Therefore, caution should be taken while extrapolating animal data to human effect.

An antiandrogenic class of drugs analgesic medications

Unsurprisingly, studies on fetal testicular toxicity of analgesics and NSAIDs predate other categories of fetal testicular toxicants, with papers published as early as 1967, but the compounds investigated in these papers are quite diverse, and the rate of publication on this topic has also increased since 2015. Epidemiological evidence supports an association between *in utero* exposure to over-the-counter analgesics, including some non-steroidal anti-inflammatory drugs (NSAIDs), and the development of male reproductive disorders. A recent review concluded that there is increasing evidence that analgesics impair testis development and male reproductive function (Hurtado-Gonzalez & Mitchell 2017). Data from experimental models largely support claims of adverse effects. In one study, gestational exposure to indomethacin, a cyclooxygenase inhibitor and NSAID, led to decreased rat testis weight and testicular prostaglandin E₂ (PGE₂) level but had no effect on intratesticular testosterone or AGD (Dean *et al.* 2013). Another study by the same group reported that exposure to indomethacin (0.8 mg/kg/day) from GD 15.5 to 18.5 or acetaminophen (350 mg/kg) from GD 13.5 to 21.5 significantly reduced fetal testicular germ cell number and accelerated differentiation of germ cells (Dean *et al.* 2016). A human fetal testis xenograft experiment found a similar effect of exposure to acetaminophen and ibuprofen on fetal germ cells, including a decrease in fetal germ cell number and altered expression of germ cell differentiation markers (Hurtado-Gonzalez & Mitchell 2017). Antiandrogenic effects have been reported, as well. A recent study reported that the analgesic dipyrone (metamizole) and its two main metabolites (4-methylaminoantipyrine (MAA) and 4-aminoantipyrine (AA)) inhibit testosterone

biosynthesis *in vitro* at high concentration but not at therapeutically relevant doses *in vivo* (Passoni *et al.* 2018). These experimental results are consistent with epidemiological evidence that use of analgesics, including acetaminophen, ibuprofen, and aspirin, during pregnancy is associated with reduced AGD and increased risk of undescended testis (Jensen *et al.* 2010, Kristensen *et al.* 2011, Fisher *et al.* 2016, Kilcoyne & Mitchell 2019).

The molecular mechanisms of analgesic-induced disruption of fetal testis development are partially understood. Prostaglandins (PGs) are important endogenous mediators in paracrine and autocrine signaling processes, involved in various reproductive functions. Cyclooxygenase 1 (COX1) and COX2 are critical enzymes involved in the synthesis of PGs from arachidonic acid. Because acetaminophen and the NSAIDs exert their effect by inhibiting the PG synthesis pathway and can cross the placenta, altered PG levels in the fetal testis may disturb fetal steroidogenesis, making pregnant women and children vulnerable (Passoni *et al.* 2018). Although most animal studies show a marginal effect of gestational analgesic exposure on testis development, analgesics are taken intentionally and at higher exposure levels than typical environmental exposures, which means that these effects may pose significant risks during pregnancy. NSAIDs also present a possibly relevant comparison for phthalates, as some papers have hypothesized that disrupted cholesterol metabolism or arachidonic acid signaling contribute to phthalate toxicity (Beverly *et al.* 2014, Clewell *et al.* 2020).

PPAR signaling disruption perfluorinated alkyl substances (PFAS)

Perfluoroalkyl substances (PFAS) are synthetic chemicals widely used as industrial lubricants and surfactants, as well as in products like clothing, household utensils, and food wrapping. Like polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzodioxins (PCDDs), and organochlorine pesticides, PFAS are classified as persistent organic pollutants (POPs), toxic environmental chemicals that are generally resistant to environmental degradation, widespread in soil, water and air. Because PFAS are extremely environmentally persistent and widespread in environments around the world, wild animals and humans are ubiquitously exposed to PFAS. PFAS exert general and developmental toxicity. Several publications in 2003 reported that gestational perfluorooctane sulfonate (PFOS) exposure in rodent models resulted in extensive pup mortality, growth restriction, and developmental delays (Lau *et al.* 2003, Thibodeaux *et al.* 2003). Similar to phthalates, a possible mechanistic component of PFAS toxicity is binding to PPARs (Szilagyi *et al.* 2020).

Conversely, data on the impact of low-dose PFAS exposures on the fetal testis are scarce. Several recent studies have evaluated the effects of *in utero* PFOS exposure in mouse and rat fetal testis, as well as later-life effects, focusing mostly on Leydig cell function. Low-dose PFOS, ranging from 0.3–20 mg/kg in various studies, has led to altered neonatal (PND 1), pubertal (PND 21), young adult (PND 35–63), and adult (PND 90) testis outcomes. Reported effects have included reduced fetal testosterone biosynthesis, perturbations of lipid mediators in neonatal testis, reduced testosterone production, reduced Leydig cell number, and lower epididymal sperm count in young adults. PFOS decreased mRNA and protein expression of Leydig cell and Sertoli cell markers in adult testis, altered the Wnt signaling pathway, down-regulated GSK-3 β phosphorylation, and up-regulated phosphorylation of β -catenin and cyclin D1, which could inhibit steroidogenesis directly and lead to the impairment of Leydig cell mitosis, development, and function (Zhao *et al.* 2014, Lai *et al.* 2017, Zhang *et al.* 2020). One study reported a non-monotonic effect of PFOS on Leydig cell function at PND 70, with testosterone levels increased by 1 mg/kg PFOS but decreased testosterone and Leydig cell loss in the 2.5 and 5 mg/kg groups (Song *et al.* 2018). In one well-designed *ex vivo* culture study, PFOA reduced testosterone, progesterone, cAMP, and the expression of the critical steroidogenic gene, *Star*, in both fetal and adult rat testes (Eggert *et al.* 2019), suggesting that PFAS may exert antiandrogenic effects through a phthalate-like mechanism in the rat testis. This is consistent with a report in which gestational exposure of mice to 5 mg/kg/day PFOA led to reduced testosterone in PND 21 male offspring. The authors also reported histopathological indicators of testicular injury; however, without quantification, it was unclear whether the reported effects reflected slight differences in the developmental stage or fixation artifacts (Bao *et al.* 2021).

As PFOS and perfluorooctanoic acid (PFOA) are phased out of the market, many more PFAS are introduced as substitutes, including hexafluoropropylene oxide dimer acid [(HFPO-DA), GenX]. In one experiment, exposure of pregnant rats to 1 to 500 mg HFPO-DA/kg/day during male reproductive development (GD14–18) did not result in changes in *in vitro* receptor activity, testosterone production, or expression of key genes involved in male reproductive development in the fetal testis but reduced male reproductive tissue weights (Conley *et al.* 2019). Exposure to another PFAS, perfluorononanoic acid (PFNA) during gestation caused alterations in Leydig and Sertoli cell function that were detectable in neonatal testis as decreased expression of SF-1, Leydig cell markers (STAR, CYP17, HSD3B, and HSD17B), and Sertoli cell markers (WT1 and AMH) (Singh & Singh 2019). Given wide exposure to PFAS and evidence that PFAS threaten male reproductive system development, future studies will be needed to clarify

the PFAS mode of action on the male reproductive axis, including more studies of emerging PFAS chemicals, using physiologically relevant exposure routes and concentration levels.

Remaining questions and future perspectives

Resolving questions about phthalate toxicity

Comparison to other fetal testis toxicants provides some relevant insights about phthalate toxicity. The idea that phthalates act through an estrogenic mechanism has been largely, but not completely, abandoned. Phthalate toxicity bears little resemblance to bisphenol toxicity, as described above. However, the rapid metabolism of BPA limits its toxicity in most animal studies. On the other hand, disruption of arachidonic acid signaling by analgesics and activation of PPARs by PFAS result in some effects that are phthalate-like. PFAS cause germ cell death and reduce testosterone through a similar mechanism to the phthalates, by reducing the expression of steroidogenic enzymes. NSAIDs, meanwhile, alter germ cell differentiation and survival, similar to phthalates. Many of the effects of phthalates remain unique, however, including the induction of MNGs in the fetal testis and the testicular dysgenesis phenotype, which limits the utility of comparing phthalate toxicity to any other class of fetal testicular toxicants.

Two of the major knowledge gaps in phthalate toxicity can be illustrated using the adverse outcome pathway (AOP) concept (Fig. 2). First, as mentioned above, hypothesized AOPs to describe phthalates' antiandrogenic mode of action and the consequences of suppressing testosterone production during fetal testis development explicitly denote that there is no consensus molecular initiating event (Howdeshell *et al.* 2015, Conley *et al.* 2018, Arzuaga *et al.* 2019, Clewell *et al.* 2020, Gray *et al.* 2020). Methods for addressing this gap could include screening for protein-small molecule interactions, and in particular, revisiting questions about PPAR-driven mechanisms of phthalate toxicity. Phthalates are peroxisome proliferators, and like many other peroxisome proliferators, they are linked to cancers of the liver in rodents (Oshida *et al.* 2015, Corton *et al.* 2018). There is evidence that activation of PPAR α signaling is not a component of the phthalate toxicity mechanism in the fetal testis (Ward *et al.* 1998, Hannas *et al.* 2012). However, the possibility has not been ruled out that phthalates bind to PPARs other than PPAR α or cause effects downstream of PPARs other than direct activation of gene expression, such as antagonism of other PPAR-ligands interactions, non-canonical signaling, or inflammatory actions (Varga *et al.* 2011). In fact, many phthalates are predicted *in silico* to bind to all three human PPAR isoforms. Further, interactions between PPARs and other nuclear receptors, such as RARs, could explain a portion of the phthalate toxicity mechanism (Dufour *et al.* 2003, Spade *et al.* 2019b).

Figure 2 Hypothesized adverse outcome pathway (AOP) for phthalate toxicity in the fetal testis. Based solely on experimental evidence, there are a number of gaps in the present understanding of phthalate toxicity. Hypothetical steps in the AOP and hypothesized relationships between key events are represented with dashed lines. The biggest knowledge gap is the lack of a confirmed molecular initiating event(s). It is clear that ortho-phthalates are metabolized to monoesters, which cause toxicity in the fetal testis. Proposed molecular initiating events are numerous but have included PPAR binding, decreased arachidonic acid (AA) release, and prolonged expression of the nuclear receptor COUP-TFII in Leydig cells. There is no evidence that phthalates inhibit the androgen receptor or directly activate the PPARA-mediated gene expression. The relationship between Leydig-reduced synthesis of Leydig cell-derived hormones, INSL3 and testosterone, and adverse outcomes, such as cryptorchid testis, is very clear. Cryptorchid testis is associated with testicular cancer epidemiologically, but there is no accepted rodent model of germ cell cancer in which to demonstrate this link experimentally. Sertoli cell effects of phthalates are less frequently studied, but the relationship between altered Sertoli cell proliferation and cytoskeletal processes with changes in cord morphogenesis (tubulogenesis) and adverse germ cell outcomes is well supported. Animals treated with phthalates during gestation have reduced spermatogenesis in later life. This could be caused by functional deficits in Sertoli cells or simply by reduced Sertoli cell number, but there is reason to hypothesize that these changes contribute to reduced fertility. Finally, it is likely that there are connections between the Leydig- and Sertoli cell-mediated effects of phthalates. For example, androgen insufficiency could contribute to germ cell loss. The long-term impact of multinucleated germ cells and germ cell apoptosis is unclear, given the high rate of germ cell loss in normal testes in the early postnatal development window.

Beyond activation or inhibition of PPARs or crosstalk between PPARs and other nuclear receptors, at least two additional MIEs have been proposed in phthalate AOPs, both primarily related to testosterone. First, there is experimental evidence that phthalates prolong the expression of the nuclear receptor COUP-TFII in rat, but not in mouse, Leydig cells, leading to suppression of Leydig cell SF-1-regulated gene expression and reduced production of testosterone (van den Driesche *et al.* 2012b). This could explain the mechanism of phthalates' antiandrogenic effects, as well as the species differences. Secondly, it has been hypothesized that phthalates disrupt Leydig cell arachidonic acid release by inhibiting the enzyme cPLA2, leading to decreased expression of steroidogenesis-associated genes (Clewett *et al.* 2020).

The second gap is the incomplete understanding of the mechanisms that underlie the species differences between rats, mice, and humans. There is a paucity of mouse data, relative to the vast number of phthalate studies that have been conducted in the rat model. Further comparisons between mice and rats could

help to resolve this knowledge gap. Notably, far fewer experiments have addressed the effects of phthalates on Sertoli cells than Leydig cells or even germ cells (Table 1). This is likely due in part to the relative difficulty of directly measuring features like Sertoli cell number, differentiation, cytoskeletal structure, or germ cell–Sertoli cell contacts, compared to more readily quantified outcomes like testosterone or even MNGs. MNGs are the one feature that has been clearly identified in the fetal testes of all tested species following phthalate exposure. Additionally, no class of toxicants other than phthalates has been reported to induce MNGs in the fetal testis, which means that identifying the molecular mechanisms of MNG induction could provide key clues for understanding phthalate toxicity. Notably, we have demonstrated that in the rat fetal testis, medium chain-length phthalates that are antiandrogenic also disrupt seminiferous cord development and cause induction of MNGs (Spade *et al.* 2018). Given the consistency of MNG induction, this effect or other measures of impaired seminiferous cord development, such as cord diameter,

may be the most human-like metrics for phthalate dose-response analysis. Further dose-response assessment of phthalate-induced histopathology will help to determine whether the fetal rat testis has a similar sensitivity to the antiandrogenic and cord-related effects of phthalates. However, whether a single MIE or two MIEs explain the effects of phthalates on Leydig cells and Sertoli cells, respectively, there is a qualitative difference between the Leydig cell response to phthalates in rats, relative to humans and mice. Additional studies of phthalate toxicity in the mouse model may help to resolve the cause of this difference.

Later-life effects of fetal testicular toxicant exposures

There is extensive evidence that environmental exposures and pharmaceuticals can alter multiple processes that are required for normal fetal testis development. This is most relevant to human health if these changes result in diminished fertility or increased risk of other adverse reproductive health outcomes in later life. Because latent effects are more difficult to demonstrate definitively, some questions about later-life effects of fetal testis toxicity remain. Some of the most straightforward evidence for later-life adverse outcomes of fetal testis toxicity is associated with antiandrogen exposure. Antiandrogens inhibit masculinization of the male reproductive tract, causing congenital defects, such as undescended testis and hypospadias (Hotchkiss *et al.* 2004, Noriega *et al.* 2005, Conley *et al.* 2018, Gray *et al.* 2020). These effects are caused by gestational phthalate exposure in the rat (Fisher *et al.* 2003, Wilson *et al.* 2004, Foster 2006), and despite the lack of clear experimental evidence that phthalates are antiandrogenic in the human fetal testis, some epidemiological studies have found a link between gestational phthalate exposure and hypospadias in humans. The strength of this link is debated (Lotttrup *et al.* 2006, Jensen *et al.* 2015), and other studies have concluded that there was no significant association between phthalate exposure and cryptorchidism or hypospadias in human cohorts (Anand-Ivell *et al.* 2018). Hypospadias and cryptorchid testis are common human male reproductive defects that require surgical correction. Moreover, it is presumed that any environmental exposure causing an increased risk of cryptorchidism has later-life effects, given the relationship between undescended testes and reduced fertility and seminoma risk (Yavetz *et al.* 1992, Pettersson *et al.* 2007).

The impact of phthalates on testicular structure, as observed by histopathology (i.e. 'testicular dysgenesis'), is persistent. Phthalate-induced testicular histologic lesions, germ cell loss, and tubular atrophy persist throughout adulthood in the rat (Barlow & Foster 2003, Barlow *et al.* 2004, Mahood *et al.* 2007, Gray *et al.* 2009, Repouskou *et al.* 2019). Phthalate-induced long-term decreases in Sertoli cell number may mediate a

decrease in fertility (Dostal *et al.* 1988). Rats treated with phthalates *in utero* also exhibit decreased sperm count and quality later in life, leading to early reproductive aging and senescence (Barakat *et al.* 2017, Axelstad *et al.* 2018, Dostalova *et al.* 2020). MNGs are a unique toxicologic marker of fetal testicular phthalate exposure. It was previously suggested that MNGs or developmentally delayed fetal germ cells were potential carcinoma *in situ* (CIS) cell precursors (Ferrara *et al.* 2006, Sonne *et al.* 2008). However, MNGs are degenerative and do not persist postnatally in WT mice (Saffarini *et al.* 2012). While these germ cell changes may not directly diminish later-life testis function, they are relevant markers of phthalate-driven Sertoli cell toxicity, which can persist into adulthood. In addition to effects that are mediated by structural changes caused by altered Sertoli cell development, fetal phthalate exposure disrupts later-life development of adult Leydig cells (Kilcoyne *et al.* 2014, Chen *et al.* 2017). Similarly, other fetal testis toxicants, many of which have not been studied as thoroughly as phthalates, are likely to cause later-life effects on testis function. Several publications address the later-life effects of DES and BPA and adult Leydig cell development and fertility (Fielden *et al.* 2002, Ivell *et al.* 2013). There is also a rich literature on the transgenerational effects of some *in utero* exposures on male reproductive biology (Anway *et al.* 2005, Manikkam *et al.* 2012, 2013, Stenz *et al.* 2019, Barakat *et al.* 2020), which implies a persistent effect of *in utero* exposures on the development and function of the testis, resulting in altered spermatozoa.

Mixtures

To this point, we have only reviewed studies performed on a single-chemical basis, as is the standard of practice for chemical risk assessment and most toxicology studies. Moreover, we have focused heavily on phthalates, for which tolerable exposure levels below which toxicity is not observed *in vivo* are relatively high. Low doses of phthalates found to have antiandrogenic or seminiferous cord effects in animal studies are often in the range of 10–100 mg/kg/day for a single phthalate (Boekelheide *et al.* 2009, Heger *et al.* 2012, Ungewitter *et al.* 2017). Although phthalates are ubiquitous in built environments and many consumer products, normal human exposure levels to single phthalates are much lower than 100 mg/kg/day. Most humans are not exposed to phthalate doses that exceed the EPA reference dose of 20 µg/kg/day, although exposures can exceed the reference dose in sensitive populations. Exposure levels are highest in medical settings, especially in premature neonates, who undergo treatments such as total parenteral nutrition that can raise the risk of exposure to phthalates used in medical plastics (Kavlock *et al.* 2006). Exposure estimates in neonates in some neonatal intensive care settings have ranged from

5 to 22.6 mg/kg/day (Loff *et al.* 2000, Calafat *et al.* 2004, Kavlock *et al.* 2006, Koch *et al.* 2006).

Additionally, humans, including pregnant women, are exposed to multiple chemicals simultaneously. Drug–drug and drug–environmental chemical interactions during pregnancy might impose a serious risk on both mothers and children. According to the FDA, 50% of pregnant women self-report taking at least one medicine during pregnancy. In addition to analgesics, which we have reviewed, these include drug therapies for the management of the pregnancy complications, such as diabetes, morning sickness, or high blood pressure. As drugs are taken deliberately and usually at a relatively high dose compared to involuntary exposure to environmental chemicals, the assessment of their potential adverse impact on both mothers and their children is critical. For example, betamethasone, a glucocorticoid, is used in women at risk of preterm delivery. Recent studies found that *in utero* betamethasone exposure can disrupt fetal male reproductive development (Borges *et al.* 2016, Pedrana *et al.* 2016, de Barros *et al.* 2018). Similar investigations have been published recently on caffeine, the anti-diabetic drug, metformin, the antiviral drugs, acyclovir and ganciclovir, the anti-epileptic drug, carbamazepine, the antiandrogen and hair loss drug, finasteride, the cholesterol-reducing drug, simvastatin, and others (Bowman *et al.* 2003, Tartarin *et al.* 2012, Andretta *et al.* 2014, Nihi *et al.* 2014, Ogunwole *et al.* 2015, Veroniki *et al.* 2017, Meyer *et al.* 2018, Beverly *et al.* 2019). Collectively, research regarding the potential risk of medications used in pregnancy on the developing male reproductive system remains inadequate, particularly given the higher exposure levels for drugs relative to environmental chemicals, as indicated in the review by Kilcoyne and Mitchell (2019). Therefore, additional studies on many therapeutic drugs used during pregnancy are needed.

In light of this challenge, studies of toxicant mixtures using *in utero* rodent exposure models have been reported with increasing frequency, as described in a recent review (Howdeshell *et al.* 2017). Many of these studies have been designed to test the hypothesis that mixture response for antiandrogenic chemicals with similar modes of action is predicted better by dose-addition models than response-addition models or, in other words, that exposure at sub-toxic doses to two or more compounds that act on the same targets can produce a toxic result. In an early example of this effort, rats exposed to vinclozolin and procymidone, which are dicarboximide fungicides and androgen receptor antagonists, showed significantly higher rates of hypospadias and vaginal pouch than either compound alone (Gray *et al.* 2001). More recently, large studies of chemical combinations with similar mechanisms of action have been reported, with a focus on phthalates. In these experiments, dose-addition modeling has accurately predicted the reduction of

fetal testosterone production and androgen-sensitive postnatal male reproductive tract development in up to five phthalate mixture studies (Howdeshell *et al.* 2008, 2015). Dose-addition modeling also predicts outcomes for mixtures with multiple mechanisms that target the same pathway. For example, co-exposure to DBP, a fetal testosterone production inhibitor, and procymidone, an androgen receptor antagonist, at doses that were individually sub-toxic, caused an increased incidence of hypospadias and vaginal pouch (Hotchkiss *et al.* 2010). Recent studies have addressed much more complex antiandrogenic combinations: androgen receptor antagonists (procymidone, vinclozolin, and pyrifluquinazon), phthalates as testosterone synthesis inhibitors (BBP, DBP, DEHP, DiBP, DiHeP and DPeP), pesticides with dual mechanisms of androgen receptor antagonism and testosterone synthesis inhibition (prochloraz and linuron), and a cholesterol synthesis inhibitor (simvastatin) (Rider *et al.* 2010, Conley *et al.* 2018), with similar findings. These results have been supported by a study comparing antiandrogenic effects of endocrine-disrupting pharmaceuticals and environmental chemicals in cultured human fetal testis tissue (Gaudriault *et al.* 2017). On the other hand, dose addition is not the best model to predict mixture toxicity for chemicals working on different pathways but affecting the same target tissue. With DBP (androgen pathway) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD, AHR pathway), response addition more accurately models some of the adverse endpoints in the co-exposure group (Rider *et al.* 2010). To serve the goal of accurately assessing the hazard posed by environmental chemicals and pharmaceuticals, many more mixture studies may need to be performed, including mixtures of compounds from different classes and with dissimilar modes of action, at environmentally relevant dose levels. Mixture studies involving phthalates and classes of compounds that may share some aspects of their toxicity mechanisms, such as analgesics or PFAS, could also help to clarify the knowledge gaps in the phthalate AOP.

Emerging technologies and mechanisms

Several future directions are likely to grow from the last decade of fetal testis toxicity research, aided by advances in research technologies. The advent of single-cell RNA-sequencing (scRNA-seq) has enhanced understanding of testis biology. The testis is an ideal organ to apply such technology, because of its many and varied cellular differentiation states. However, to date, only two experiments have used this technology to describe fetal testis development (Stevant *et al.* 2018, Law *et al.* 2019). These experiments have confirmed existing knowledge but have also added knowledge, for example, about the timing of specification of supporting cell and steroidogenic cell precursors from SF-1-positive progenitors. This technology will be an important tool for toxicologists seeking to

answer questions about the influence of compounds such as phthalates on cell type specification and differentiation, which are easily lost in the average values produced by bulk gene expression analysis.

The last decade has also produced advances in the understanding of fetal testis development and maintenance of the differentiated testis phenotype. Recent work has further clarified the interplay between 'testis' and 'ovary' signaling processes in fetal supporting cells (Nicol & Yao 2015, Nicol *et al.* 2018), as well as the under-appreciated role of resident macrophages in testicular development (DeFalco *et al.* 2014). We have been interested in the interaction between phthalates and retinoic acid, which can disrupt these cell type specification processes (Spade *et al.* 2019a,b). Several studies have also begun to address the question of phthalate impacts on testicular macrophage function, following both fetal exposure and prepubertal exposure (Murphy *et al.* 2014, Walker *et al.* 2020). It is likely that the keys to understanding phthalate toxicity mechanisms more fully lie in cellular developmental processes that have not as yet been the focus of toxicology studies.

Conclusion

The fetal testis is a remarkable organ. Testis development happens in a short time span beginning later than most organs, relies on multiple signaling processes, and involves migration and differentiation of multiple cell types, ultimately giving rise to structures that support spermatogenesis and the endocrine functions that program the masculinization of the male fetus. This process is highly susceptible to disruption by environmental toxicants. Phthalates are a key class of fetal testis toxicants, and although they have been studied intensely over the past 20 years, the molecular initiating events in phthalate toxicity remain to be identified. Filling these knowledge gaps will be a significant challenge, but it is likely to contribute to the knowledge of basic testis biology and a better understanding of how *in utero* toxicant exposures impact long-term and even transgenerational health outcomes.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0592>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was funded by the National Institute of Environmental Health Sciences [R00 ES025231].

Author contribution statement

H L and D S contributed equally to the concept, research, and writing of this manuscript. D S designed the figures.

Acknowledgement

The authors thank Vinald Francis for scientific illustrations.

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Received 2 November 2020

First decision 4 January 2021

Revised manuscript received 2 July 2021

Accepted 27 July 2021