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Proposed Key Characteristics of Male Reproductive Toxicants as an Approach for Organizing and Evaluating Mechanistic Evidence in Human Health Hazard Assessments

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Background: Assessing chemicals for their potential to cause male reproductive toxicity involves the evaluation of evidence obtained from experimental, epidemiological, and mechanistic studies. Although mechanistic evidence plays an important role in hazard identification and evidence integration, the process of identifying, screening and analyzing mechanistic studies and outcomes is a challenging exercise due to the diversity of research models and methods and the variety of known and proposed pathways for chemical-induced toxicity. Ten key characteristics of carcinogens provide a valuable tool for organizing and assessing chemical-specific data by potential mechanisms for cancer-causing agents. However, such an approach has not yet been developed for noncancer adverse outcomes.

Objectives: The objective in this study was to identify a set of key characteristics that are frequently exhibited by exogenous agents that cause male reproductive toxicity and that could be applied for identifying, organizing, and summarizing mechanistic evidence related to this outcome.

Discussion: The identification of eight key characteristics of male reproductive toxicants was based on a survey of known male reproductive toxicants and established mechanisms and pathways of toxicity. The eight key characteristics can provide a basis for the systematic, transparent, and objective organization of mechanistic evidence relevant to chemical-induced effects on the male reproductive system. https://doi.org/10.1289/EHP5045

Introduction

Exogenous Chemicals and Altered Male Reproductive Health

There has been a decline in sperm counts (Levine et al. 2017) and a simultaneous rise in the incidence of testicular cancer in most regions of the world (Skakkebaek et al. 2016). It has been proposed that environmental chemicals are contributors to a large proportion of these adverse effects on male reproduction and associated conditions such as testicular dysgenesis syndrome (Bergman et al. 2013; Skakkebaek et al. 2016). Further, several exogenous chemical exposures are associated with decreased human male fertility, including occupational exposures to lead, cadmium, welding fumes, certain pesticides (e.g., 1,2-dibromo-3-chloropropane), alcohol consumption, cigarette smoking, drug use (e.g., cocaine), and certain pharmaceuticals (Hotchkiss et al. 2008; du Plessis et al. 2015; Semet et al. 2017). The identification of chemicals that cause male reproductive effects, and the mechanisms underlying these effects, is critical to developing approaches to mitigate the risks of environmental, occupational, medical, and lifestyle exposures and to understand the etiology of population-level trends in dysfunction.

Apical End Points of Male Reproductive Toxicity

Proper development and structural organization of the male reproductive tract can be clinically assessed by examining testicular descent, anogenital distance, preputial separation, areola and nipple retention, external genitalia (e.g., hypospadias, cleft phallus) and reproductive organ size and morphology. Such evaluations can be performed visually and by ultrasound in humans and by necropsy in animals (e.g., small testes; presence/absence of vas deferens, seminal vesicles, epididymis, prostate) (Chapin and Creasy 2012; Foster and Gray 2013; Nikolaidis 2017). Analyses of gross pathology (e.g., size, organ weights, malformations) and histopathology can uncover developmental defects (e.g., abnormal/delayed sexual development, Sertoli cell–only tubules, spermatogenesis arrest) as well as insults at different life stages that interfere with male fertility (e.g., atrophy, edema, inflammation, seminiferous tubule vacuolation) (Creasy et al. 2012; Nikolaidis 2017). Detailed sperm evaluation at multiple levels is essential, including testicular spermatogenesis and spermiation, ejaculated semen analysis (count, concentration, motility, morphology), assessment of sperm functions (e.g., Catsper channels, capacitation, acrosome reaction, hyperactivation, DNA integrity), and fertilizing ability (e.g., sperm penetration assay) that individually contribute novel information and insights regarding the effects of

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potential reproductive toxicants (U.S. EPA 1996a; Creasy and Chapin 2018). Hormone measurements [e.g., luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, estradiol, inhibin] can inform interference with the hypothalamic–pituitary–testicular axis, which systemically controls reproduction and aids in identifying primary versus secondary hypogonadism (Chapin and Creasy 2012). Finally, assessments of sexual behavior and performance (e.g., libido, erection, mounting behaviors, intromission, ejaculation) are key end points that affect fecundity. These activities may be adversely influenced by alterations in endocrine and neural systems and structural aberrations (Nikolaidis 2017).

Role of Mechanistic Evidence in Hazard Evaluation

Hazard identification as part of a human health risk assessment consists of an analysis of the available evidence on chemical-induced adverse health effects that are focused on cancer and noncancer outcomes including male reproductive toxicity. For example, the State of California maintains a list of carcinogens and reproductive toxicants and indicates those that are specific to male and/or female effects (OEHHA 2019). Evaluation of epidemiological and toxicological studies for direct evidence of effects after chemical exposures plays a critical role in the hazard identification process. Mechanistic evidence is also an important component. Analysis of events that are precursors to apical end points seen in animals and humans supports evidence of a hazard, identifies potential susceptible populations and life stages, and informs the human relevance of effects observed in animals (NRC 2007, 2017). Evaluation of *in vivo* and *in vitro* mechanistic evidence can also identify data gaps in the current understanding of how a chemical may cause adverse effects in experimental models and humans. Finally, there continues to be a shift away from whole animal testing for apical end points toward high-throughput *in vitro* testing and toxicogenomic profiling due to cost, timelines, ethical considerations, regulatory constraints on animal testing, and the large volume of chemicals needing evaluation (NRC 2007, 2017). Although some of these animal model alternatives may be useful for male reproductive toxicants, it is important to note that cell culture of germ cells through maturation in organoids and even tissue explants has had far less success than for some other tissues, which will limit their use for testicular analyses in the near future (Nakamura et al. 2019). Nonetheless, the authors concur that increasingly in the future, large, diverse, and complex mechanistic data sets will likely comprise much of the data available for evaluating the hazards of chemicals used in commerce and their breakdown products.

The identification of male reproductive toxicants that pose a hazard to human health typically requires the integration of evidence from epidemiological studies of different designs, populations, and exposures; animal evidence from studies of various apical end points and experimental designs; and consideration of the varied mechanistic data that support (or diminish support for) the chemical posing a reproductive hazard (Rooney et al. 2014; OHAT 2019). Various organizations provide evidence evaluation frameworks that can be used to categorize and analyze toxicological end points indicative of male reproductive toxicity (see Table S1), but currently there is no generally accepted approach for systematically and transparently identifying and organizing mechanistic data for male reproductive hazard identification. Consequently, there is a lack of uniformity in the mechanistic topics addressed across assessments and an absence of a standard procedure to efficiently identify, organize, and summarize the voluminous data from mechanistic studies. The proposed key characteristics of male reproductive toxicants were developed as a tool that can improve the systematic and transparent screening and evaluation of mechanistic data related to chemical-induced male reproductive toxicity.

Identifying the Key Characteristics of Male Reproductive Toxicants

Recently, the 10 key characteristics of human carcinogens were introduced to provide a uniform and objective approach for identifying and organizing mechanistic evidence to support cancer hazard identification (Smith et al. 2016; Guyton et al. 2018). The key characteristics were identified by an international working group of experts organized by the International Agency for Research on Cancer (IARC) (Smith et al. 2016). The 10 key characteristics of human carcinogens comprise the properties of known human carcinogens, including their ability, for example, to be genotoxic or immunosuppressive or to modulate receptor-mediated effects. Established human carcinogens commonly exhibit one or more of these characteristics, and therefore such data can provide independent evidence of carcinogenicity when human epidemiological evidence is lacking. Such data can also help in interpreting the relevance and importance of findings of cancer in animals and in humans. In its 2017 report "Using 21st Century Science to Improve Risk-Related Evaluations," the National Academy of Sciences, Engineering and Medicine (NASEM) opined that the key characteristics approach "avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence" (NRC 2017). Indeed, the key characteristics of carcinogens have been successfully used by the IARC Monographs Programme to evaluate the mechanistic data compiled for the more than 30 agents evaluated in Meetings 112–121 of the IARC Monographs (Guyton et al. 2018). The 2017 NRC report also recommended that the key characteristics approach be expanded to other end points, including reproductive effects, endocrine disruption, and cardiovascular disease (NRC 2017).

Here, we have attempted to develop a set of key characteristics of male reproductive toxicants based on our current knowledge of the mechanisms by which chemicals cause reproductive toxicity. A working group consisting of regulatory experts, toxicologists, and epidemiologists with a background in reproductive biology and mechanisms associated with chemical-induced adverse health effects was convened at the University of California, Berkeley from 7 to 8 March 2018 to address this topic. The objective was to review the key characteristics approach and to determine whether this methodology can also be applied to the evaluation of male reproductive toxicants. The workgroup participants consulted comprehensive lists of chemicals known to target the male reproductive system (OEHHA 2019) and also addressed current male reproductive outcomes considered in evaluations of experimental and epidemiological evidence of toxicity induced by agents such as radiation and viral and bacterial pathogens. Peer-reviewed publications and U.S. government health assessments [e.g., U.S. EPA Integrated Risk Information System (https://cfpub.epa.gov/ncea/iris/search/index.cfm), U.S. Agency for Toxic Substances and Disease Registry (ATSDR)] provided reviews and examples of chemical-induced effects in the male reproductive system, including the "Toxicological Profile of Cadmium" (ATSDR 2012), the "Toxicological Profile of DDT" (ATSDR 2002), and the "Toxicological Review of Benzo[a]pyrene" (ATSDR 2002, 2012; U.S. EPA 2017). Based on the initial analysis, several key characteristics of male reproductive toxicants were identified (Figure 1 and Table 1), as well as examples of chemicals known to affect those specific characteristics (Table 2).

Each key characteristic is described in the context of mechanisms or pathways by which exposure to male reproductive toxicants (including environmental toxicants, pharmaceuticals, and drugs of abuse) can lead to adverse health effects. This is not intended to be an exhaustive discussion of all potentially available evidence from experimental studies. Instead, this narrative relies on previous literature reviews and mechanistic analyses of toxicant-

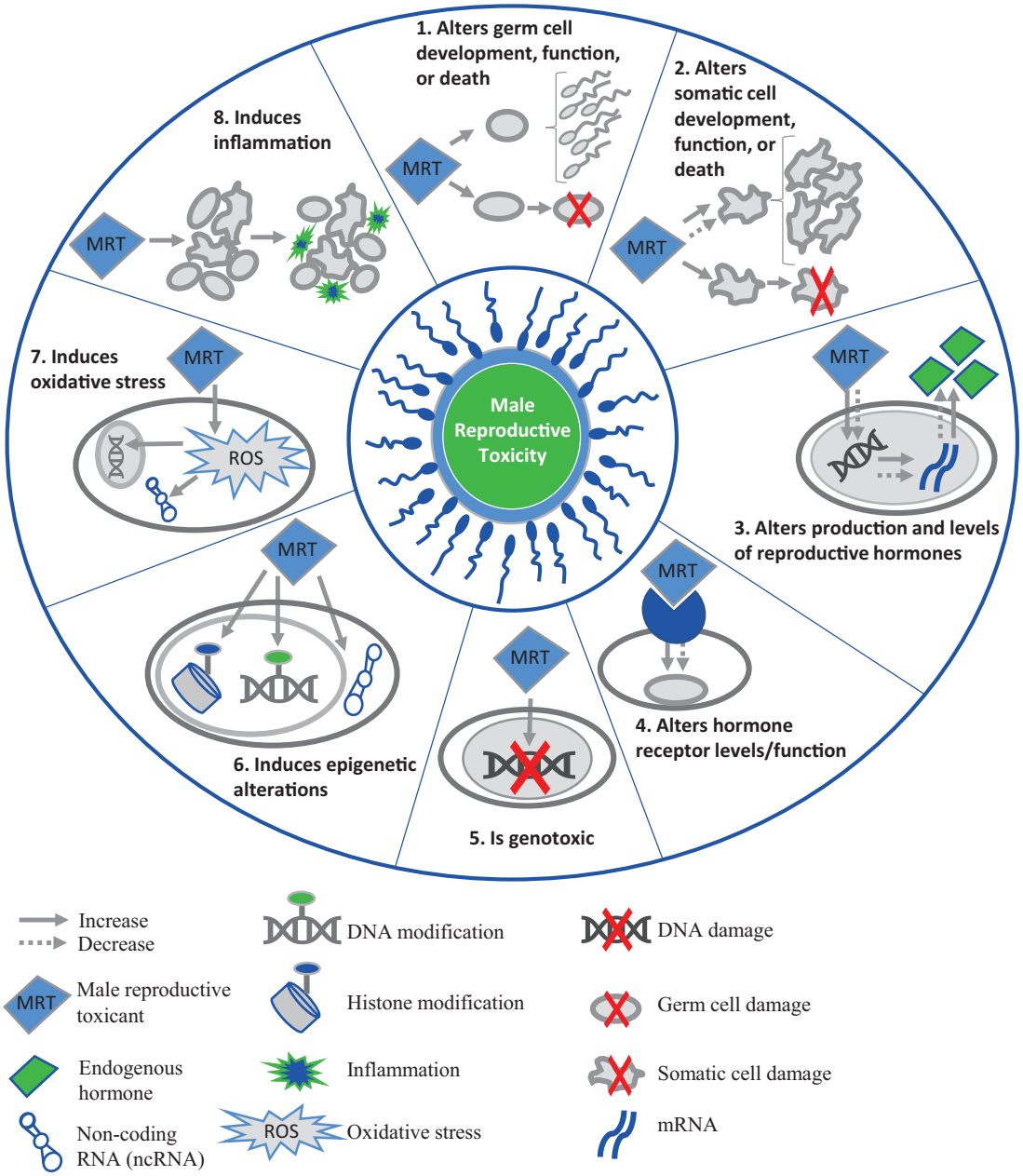

Figure 1. Key characteristics of male reproductive toxicants. Exposure to male reproductive toxicants (MRT) resulting in (1) altered spermatogenesis, normal functions (e.g., acrosome reaction), or increased cell death; (2) disruptions in somatic cell development (e.g., increased or decreased proliferation), functions (e.g., alterations in blood–testis barrier), or death; (3) changes in hormone production/levels; (4) modifies hormone receptor functions/cellular levels; (5) increases DNA damage; (6) epigenetic alterations of cellular macromolecules (DNA, RNA, and/or proteins); (7) reactive oxygen species (ROS)-induced cellular damage; and (8) increases inflammation (e.g., elevated production/levels of pro-inflammatory cytokines and edema). In combination with the male-specific end points of reproductive toxicity described in Table S1, the key characteristics of male reproductive toxicants can be applied for the evaluation of toxicological and mechanistic evidence for hazard identification.

induced adverse male reproductive health effects. Examples of chemicals known to affect the male reproductive system via mechanisms that fall under the key characteristics described herein are presented in Table 2.

The Eight Key Characteristics of Male Reproductive Toxicants

1. Alters Germ Cell Development, Function, or Death

The multistep process of sperm formation begins early in embryonic and fetal life. During the first trimester, migrating primordial germ cells embed in the developing gonadal primordium that contains somatic supportive cell precursors. At the time of colonization, the primordial germ cells and somatic supportive cell precursors are still bipotential. During the second and third trimesters, the somatic cell precursors are induced by the sex determining region Y (SRY) to differentiate into Sertoli cells in the gonadal anlage, whereas the pluripotent gonocyte lineage commits to differentiate into spermatogonia (Del Valle et al. 2017). During the first months after birth, a developmental stage known as minipuberty, characterized by an increased testosterone level, occurs due to a brief activation of the pituitary–gonadal axis (Grinspon and Rey 2010; Rey 2014). Throughout the duration of

Table 1. Key characteristics of male reproductive toxicants.

Key characteristic	Examples of relevant evidence
1. Alters germ cell development, function, or death	Increased germ cell apoptosis; alterations in sperm acrosome reaction and motility
2. Alters somatic cell development, functions, or death	Increased Sertoli cell apoptosis; alterations in Sertoli cell functions, cytoskeleton, and interactions with germ cells; alterations in Leydig cell development
3. Alters production and levels of reproductive hormones	Decreased Leydig cell steroidogenic functions; increased hepatic metabolism and excretion of sex hormones
4. Alters hormone receptor levels/functions	Androgen receptor antagonism, estrogen receptor activation, decreased LH receptor expression
5. Is genotoxic	DNA damage, chromosome fragmentation, altered sperm cell chromosome numbers
6. Induces epigenetic alterations	Altered sperm ncRNAs, germ cell DNA methylation patterns, and histone retention sites
7 Induces oxidative stress	Reduced tissue antioxidant levels
8. Induces inflammation	Increased testicular expression of pro-inflammatory markers and prostaglandin levels

Note: LH, luteinizing hormone; ncRNA, noncoding RNA.

this developmental stage, some germ cells may still show fetal characteristics. However, germ cells are generally quiescent in childhood and classified as spermatogonia until meiosis starts at the beginning of puberty with the production of sperm (Müller and Skakkebæk 1983; Masliukaite et al. 2016). Spermarche (first production of sperm) occurs around 13 y of age (currently the data are limited to studies of Caucasian boys) (Nielsen et al. 1986; Del Valle et al. 2017; Creasy and Chapin 2018). Although the underlying mechanism behind the onset of puberty is not known, the initial maturation steps involve hypothalamic and pituitary centers leading to activation of the pituitary–gonadal axis, resulting in increased LH and testosterone levels, and the subsequent maturation of external genitalia (Sørensen et al. 2010).

Germ cells are vulnerable to external stressors during all developmental stages. Human genetic models (Lottrup et al. 2013) and experimental animal findings show that the differentiation process of pluripotent fetal germ cells into mature spermatogonia committed to spermatogenesis is particularly sensitive to toxicant-induced alterations in signaling from surrounding cells such as Sertoli and Leydig cells (Sharpe and Skakkebaek 2008). It is assumed that seminomas (germ cell tumors) and nonseminomas in young adults originate in fetal pluripotent germ cells that failed to differentiate correctly during the perinatal period. The fetal hypothesis also links germ cell cancer to other symptoms of the testicular dysgenesis syndrome, including cryptorchidism, hypospadias, and decreased spermatogenesis (Skakkebaek et al. 2016). Furthermore, animal studies consistently report alterations in germ cell development and germ cell loss after gestational exposure to phthalate esters (Martino-Andrade and Chahoud 2010; Albert and Jégou 2014).

The adult testis has been generally considered to be less sensitive than the immature gonad to toxicant-induced alterations. However, modern chemotherapy has—as proof of principle—shown that human spermatogenesis can be disrupted by exposures to chemicals (Brennemann et al. 1997). Further, recent studies have shown that endocrine-disrupting chemicals may mimic progesterone effects on CatSper, a calcium channel in the human sperm cell that regulates sperm motility and acrosomal exocytosis (Schiffer et al. 2014). Although the clinical significance of these findings remains to be established, they demand consideration in light of the high frequency of abnormal and immotile sperm among men in the western world (Levine et al. 2017). The workshop consensus is that there is a continued need for a comprehensive approach where combined male and female factors are considered together to elucidate a possible role of male reproductive toxicants during the fertilization process.

Environmental chemicals and other substances that directly or indirectly target proliferation, differentiation, or death processes in germ cells (at any stage of production) include heavy metals (e.g., cadmium), various phthalate esters, and drugs of abuse such as cocaine (Table 2). Exposure to these agents has been shown to lead to male reproductive toxicity.

2. *Alters Somatic Cell Development, Functions, or Death*

Exposures to toxic compounds has been shown to adversely affect the male reproductive system by targeting somatic cells in several organ systems that are essential for healthy reproduction outcomes. Under normal conditions, somatic cells provide structural support and nourishment and regulate endocrine functions that are necessary for normal spermatogenesis and fertility (Woldemeskel 2017; Creasy and Chapin 2018). Chemicals and substances that interfere with the development, integrity, or function of these somatic cells can have deleterious effects on male fertility (Boekelheide et al. 2005; Scott et al. 2009; Sansone et al. 2018). Within the testes is a network of seminiferous tubules where spermatogenesis occurs. Sertoli cells line the walls of these tubules and provide essential nourishment and support for developing gametes through paracrine factors and cell–cell communication. Peritubular myoid cells lie at the base of the tubules and provide contractile elements for sperm transport and contribute to the blood–testes barrier formed by Sertoli cell tight junctions. Between the tubules are the Leydig cells, which synthesize androgens and are essential for sperm production and growth of the male reproductive tract (van den Driesche et al. 2012). Chemicals such as hydrocarbons, cadmium, and some phthalate esters have been shown to induce Sertoli cell apoptosis, alterations in Sertoli cell cytoskeleton and Sertoli-germ cell interactions, and decreased steroidogenesis in Leydig cells (Table 2). In addition, resident macrophages that interact with Leydig cells as well as endothelial cells are present in the testicular interstitial space and can become targets for toxicant-induced damage (Hales 2002; Zheng et al. 2010; Harris et al. 2016). Accessory reproductive organs such as the epididymis and vas deferens are the excurrent ducts where sperm mature and are stored and properly transported during emission/ejaculation (Evans and Ganjam 2017). The prostate gland and seminal vesicles produce seminal plasma for sperm transport and capacitation. Normal accessory reproductive organ functions can be altered by exposure to reproductive toxicants such as vinclozolin and flutamide (Dent et al. 2015). The hypothalamus and pituitary play a critical role in male reproductive development and functions by regulating Sertoli and Leydig cell functions via LH and FSH secretion (Foster and Gray 2013), and exposure to environmental contaminants such as cadmium may impact normal gonadotropin secretion, resulting in altered testicular functions (Lafuente 2013). In addition, alterations in somatic cells of other organ systems can impact the male reproductive system. For example, dichlorodiphenyltrichloroethane (DDT) and nonylphenol may alter hepatic metabolism of endogenous hormones, which in turn alters available androgen and estrogen levels (Laurenzana et al. 2002; Kretschmer and Baldwin 2005; Medina-Díaz et al. 2007; Hotchkiss et al. 2008).

Table 2. Key characteristics of male reproductive toxicants and examples from chemicals known to affect the male reproductive system.

Key characteristic	Example toxicants	Known mechanism/pathway associated with adverse male reproductive outcomes	References
1. Alters germ cell development, function, or death	B[a]P	Increased spermatogenic cell apoptosis; altered sperm motility and acrosome reaction	Ramesh et al. 2017; U.S. EPA 2017
	Cadmium	Increased spermatogenic cell apoptosis, reduced sperm count, altered sperm motility	ATSDR 2012; Jenardhanan et al. 2016
	Phthalates	Germ cell degeneration and reduced cell number	Howdeshell et al. 2008; Martino-Andrade and Chahoud 2010; Habert et al. 2014
	4-Methylbenzylidenecamphor	Altered sperm motility via disrupted Ca^{2+} channel function	Schiffer et al. 2014
	Cocaine, sirolimus, sulfasalazine, cannabinoids, DES	Decreased sperm count and motility, altered sperm morphology	Li et al. 2003; Semet et al. 2017
2. Alters somatic cell development, functions, or death	B[a]P	Increased Sertoli cell apoptosis	Ramesh et al. 2017
	Phthalates	Altered Sertoli-germ cell interactions; decreased testosterone production in Leydig cells	Boekelheide et al. 2005; Scott et al. 2009
	Cadmium	Disruption of the blood–testis barrier via alterations in Sertoli cell actin filaments, and assembly of tight junctions	Siu et al. 2009; Gao et al. 2015; Li et al. 2016; de Angelis et al. 2017
	PCBs	Decreased Sertoli cell metabolic functions and viability	Jenardhanan et al. 2016
	Alcohol, phthalates	Increased Fas-mediated Sertoli and germ cell apoptosis	Lucas et al. 2009; Pourmasumi et al. 2017; Sansone et al. 2018
3. Alters production and levels of reproductive hormones	DDT	Increased hepatic expression of CYP3A4 and metabolism of sex hormones	Laurenzana et al. 2002; Medina-Díaz et al. 2007
	Linuron	Decreased fetal androgen production/levels	Hotchkiss et al. 2008; Wilson et al. 2008; Scott et al. 2009; Dent et al. 2015
	Phthalates, sirolimus	Decreased expression of steroidogenic enzymes and reduced androgen production	Hotchkiss et al. 2008; Bergman et al. 2013; Semet et al. 2017
	Ketoconazole, prochloraz	Inhibition of the steroidogenic enzyme CYP17A1 activity	Scott et al. 2009; Dent et al. 2015
	PCBs, B[a]P	Decreased serum levels of reproductive hormones; decreased androgen production in Leydig cells	Meeker and Hauser 2010; Jenardhanan et al. 2016; Ramesh et al. 2017; U.S. EPA 2017
	Opiates	Reduced androgen levels and secretion of gonadotropin-releasing hormone; increased aromatase expression	Bawor et al. 2015; Drobnis and Nangia 2017; Semet et al. 2017
	Cadmium	Alterations in LH associated with changes in prolactin secretion; decreased Leydig cell steroidogenic enzyme activity, cAMP levels, and expression of the LH receptor	Siu et al. 2009; Lafuente 2013
4. Alters hormone receptor levels/functions	Prochloraz, linuron, procymidone, vinclozolin, flutamide, cyproterone acetate, DDT	AR antagonism	ATSDR 2002; Hotchkiss et al. 2008; Wilson et al. 2008; Scott et al. 2009; Dent et al. 2015; Semet et al. 2017
	DES	Activation of estrogen receptor	Henley and Korach 2006
	B[a]P	Activation of AHR resulting in increased expression of xenobiotic metabolic enzymes and formation of reactive metabolites and ROS	Ramesh et al. 2017
	Cadmium	Reduced levels of the LH Receptor	Gunnarsson et al. 2007; Wan et al. 2011
5. Is genotoxic	Acrylamide	Increased germ cell formation of gylcidamide-DNA adducts	Estill and Krawetz 2016
	Cadmium, PCBs	Chromatin fragmentation, and ROS-dependent DNA damage in germ cells	Meeker and Hauser 2010; Tavares et al. 2016; de Angelis et al. 2017
	B[a]P, cisplatin, carboplatin	Increased DNA adducts and DNA fragmentation in spermatozoa and testicular tissue	Vakalopoulos et al. 2015; Tavares et al. 2016; Ramesh et al. 2017; U.S. EPA 2017
	Alcohol	Altered sperm chromosome number (aneuploidy), and increased DNA fragmentation	Kapp 2010; Pourmasumi et al. 2017
	Chlorambucil, cyclophosphamide, procarbazine, melphalan	DNA alkylation, altered DNA structure and function	Vakalopoulos et al. 2015
	Ethane-methane sulfonate	Increased/irreversible spermatogonia DNA damage resulting in necrosis	Woldemeskel 2017
6. Induces epigenetic alterations	TCDD, methoxychlor, alcohol	Altered germ cell DNA methylation patterns	Anway et al. 2005; Paoloni-Giacobino 2014; Skinner 2016; Chastain and Sarkar 2017; Pilsner et al. 2017; Ding et al. 2018
	Vinclozolin	Altered sperm ncRNAs, DNA methylation, histone retention sites	Brieño-Enríquez et al. 2015, 2016; Ben Maamar et al. 2018
	Diethylhexyl phthalate	Altered sperm ncRNAs associated with testicular dysgenesis syndrome in mice	Stenz et al. 2017
7. Induces oxidative stress	Cadmium, B[a]P	Reduction in antioxidant enzyme activity, and antioxidant levels	Kapp 2010; Rezk and Sikka 2011; Lafuente 2013; de Angelis et al. 2017; Ramesh et al. 2017
	TCDD	Decreased tissue antioxidant levels	Lavranos et al. 2012
	Lindane, methoxychlor	Reduction in antioxidant enzyme activity	Jenardhanan et al. 2016
8. Induces inflammation	Cadmium, TCDD, silver nanoparticles	Increased testicular expression/levels of pro-inflammatory markers. Increased testicular edema	Siu et al. 2009; Sengupta 2013; de Angelis et al. 2017; Pilsner et al. 2017
	TCDD	Increased testicular prostaglandin levels	Bruner-Tran et al. 2014

Note: AHR, aryl hydrocarbon receptor; AR, androgen receptor; B[a]P, benzo[a]pyrene; Ca^{2+}, calcium ion; cAMP, cyclic adenosine monophosphate; CYP3A4, cytochrome P450 family 3 subfamily A member 4; CYP17A1, cytochrome P450 family 17 subfamily A member 1; DDT, dichlorodiphenyltrichloroethane; DES, diethylstilbestrol; LH, luteinizing hormone; ncRNA, noncoding RNA; PCBs, polychlorinated biphenyls; ROS, reactive oxygen species; TCDD, tetrachlorodibenzodioxin.

3. Alters Production and Levels of Reproductive Hormones

Gonadotropins, sex steroids, and thyroid hormones produced by the hypothalamic–pituitary–gonadal (HPG) axis, the hypothalamic–pituitary–adrenal axis, and the hypothalamic–pituitary–thyroid axis play an important role in the normal development and function of the male reproductive system (Dent et al. 2015). Chemical-induced alterations in reproductive hormones during developmental and sexually mature stages have been shown to result in adverse effects including malformations and infertility (Sharpe and Skakkebaek 2008; Scott et al. 2009; Mocarelli et al. 2011; Semet et al. 2017). Environmental chemicals such as DDT, linuron, polychlorinated biphenyls (PCBs), and phthalate esters have been shown to interfere with the normal development and function of the male reproductive system via alterations in hormone production, hormone levels, and the balance between male and female hormones (Table 2). Reproductive hormone production and levels have also been shown to be altered after exposure to pharmaceuticals (Semet et al. 2017) as well as drugs of abuse (Bawor et al. 2015; Drobnis and Nangia 2017; Semet et al. 2017). These compounds can alter reproductive steroid hormone levels by directly inhibiting steroidogenic enzyme activity, reducing their cellular expression, or accelerating their metabolism and excretion (Hotchkiss et al. 2008). For example, marijuana and its metabolites adversely affect the HPG axis—specifically, a reduction in LH levels—which in turn reduces testosterone levels and compromises spermatogenesis (du Plessis et al. 2015). Toxicant effects on steroid hormones are not restricted to reproductive organs; for example, hepatic expression and activity of CYP3A4, an enzyme known to metabolize androgens (Niwa et al. 2015), can be altered by DDT exposure (Medina-Díaz et al. 2007). Gonadotropic hormones (gonadotropic releasing hormone, FSH, LH, and inhibin), prolactin, and thyroid hormones also play an important role regulating development and normal functions of the male reproductive system (Wan et al. 2013; Reis et al. 2015), and levels of these hormones can be altered by several toxicants (Table 2).

4. Alters Hormone Receptor Levels/Functions

Chemicals that interact with steroid and protein hormone receptors may alter their normal functions. This can occur through various mechanisms, including binding to and activating target cellular receptors, occupying a receptor's active site and blocking normal activation by endogenous hormones, modulating recruitment of co-activators (or co-repressors) to the transcriptional complex or interfering with normal crosstalk between membrane and nuclear hormone receptors (Wilson et al. 2008; Rezk and Sikka 2011; Yeung et al. 2011). Studies in androgen receptor knockout mice revealed ablation of the masculinization of reproductive organs and the emergence of female phenotypical appearance (Matsumoto et al. 2008). Similarly, chemicals that alter androgen receptor functions during critical periods of sex differentiation and maturation or in the adult can result in male reproductive toxicity by adversely affecting male sexual development, the male phenotype, and maintenance of reproductive functions. Some chemicals known to cause toxicity via this pathway include dicarboximide fungicides (e.g., vinclozolin and procymidone), linuron, and flutamide (Table 2). Estrogen receptors (ERα, ERβ, G protein-coupled ER) have also been shown to play important roles in male reproductive function, and toxicants that agonize or antagonize these receptors, such as diethylstilbestrol (Gill et al. 1979), have been shown to have adverse effects on this system. As described above gonadotropic hormones regulate development and normal male reproductive system functions, and agents that interfere in their receptor signaling can lead to adverse effects (Wan et al. 2013). Several environmental chemicals (e.g., cadmium, lead) have been shown to interfere with expression of the LH receptor without affecting LH mRNA levels (Wan et al. 2013).

5. Is Genotoxic

Human evidence suggests that DNA damage in sperm is associated with lower fertility rates, poor embryo quality, and pregnancy loss (Zini and Sigman 2009; Rezk and Sikka 2011; Ioannou et al. 2016). Toxicant exposure can damage sperm DNA through direct mechanisms, for example, through DNA strand breaks or DNA binding, or indirectly, for example, through the induction of oxidative stress (Delbès et al. 2010). There are also a variety of intrinsic factors that contribute to sperm DNA damage, including protamine deficiency (which reduces sperm chromatin compaction) and high endogenous reactive oxygen species (ROS) levels (Zini and Sigman 2009; González-Marín et al. 2012). Mature sperm have no capacity for DNA repair, so epididymal or ejaculated sperm exposed to these stressors may become damaged with no possibility of repair prior to fertilization (González-Marín et al. 2012). Examples of chemicals known to promote DNA/chromosome damage in male germ cells include hydrocarbons {e.g., benzo[a]pyrene [B(a)P]}, acrylamide, chemotherapy drugs, alcohol, PCBs, and cadmium (Table 2).

Toxicant exposure can also cause aneuploidy in sperm, which is the gain or loss of whole chromosomes or segments of chromosomes as a result of nondisjunction that occurs when meiosis is disrupted during gametogenesis. Sperm aneuploidy is associated with infertility, pregnancy loss, and congenital abnormalities (Mandrioli et al. 2016; Ioannou et al. 2016). Sperm aneuploidy may result from exposure to chemicals classified as genotoxicants (Mandrioli et al. 2016) but might also result from indirect effects of endocrine-disrupting chemicals (Perry et al. 2016).

6. Induces Epigenetic Alterations

The term epigenetic alterations refers to stable changes in gene expression that are not caused by alterations in the DNA sequence and can be heritable across cell divisions (Tammen et al. 2013). Examples of epigenetic phenomena include changes in DNA methylation (Jirtle and Skinner 2007; Skinner 2016; Donkin and Barrès 2018), histone modification (Skinner 2016; Donkin and Barrès 2018), chromatin packaging, and noncoding RNA (ncRNA) (Donkin and Barrès 2018), all of which can affect the activity and availability of DNA for expression. Studies have shown that some endocrine-disrupting chemicals and other male reproductive toxicants alter DNA and histone methylation patterns, suggesting that epigenetic modifications are part of the mechanism of action for these chemicals (Crews and McLachlan 2006; Wu et al. 2015; Estill and Krawetz 2016). Epigenetic changes in germ cells may also be inherited by offspring with potential for transgenerational inheritance (Youngson and Whitelaw 2008). An example is the transgenerational inheritance of compromised male fertility following gestational exposure to the fungicide vinclozolin (Anway et al. 2005).

There is growing interest in the role played by the sperm epigenome in male fertility, embryonic development, and offspring health. During sperm DNA maturation, histones are largely replaced by specialized protamines, creating a tightly compacted packaging that protects sperm DNA from damage (Carrell et al. 2012). Nonetheless, the retained histones play fundamental roles in offspring development and transgenerational inheritance (Siklenka et al. 2015) and together with DNA methylation and ncRNA are sites for environmental reprograming (Siklenka et al. 2015; Belleau et al. 2018; Ben Maamar et al. 2018). The sperm epigenome is also involved in genomic imprinting, whereby methylation of certain

genes in parental gametes determines the allelic expression of those genes in offspring Kitamura et al. (2015). Consequently, alterations in sperm DNA methylation patterns are suggested to have negative implications for male fertility (Aston et al. 2015; Urdinguio et al. 2015; Jenkins et al. 2016), embryonic development (Aston et al. 2015), and offspring disease susceptibility (Jenkins et al. 2014). Chemicals known to alter male germ cell DNA methylation patterns, ncRNAs, and histone methylation or retention include vinclozolin, alcohol, methoxychlor, and diethylhexyl phthalate (Table 2).

7. Induces Oxidative Stress

Oxidative stress is caused by an increased production of ROS that overwhelms cellular and tissue antioxidant capacity (Chen et al. 2013; Sabeti et al. 2016). Although ROS are necessary for normal sperm functions such as capacitation and oocyte fertilization, excessive production of oxygen radicals is associated with sperm abnormalities such as tail defects and acrosome abnormalities, increased DNA damage, and decreased sperm mobility and viability (Lavranos et al. 2012; Chen et al. 2013; Sabeti et al. 2016). Furthermore, male germ cells are susceptible to damage caused by oxygen radicals because sperm cells do not have DNA repair mechanisms (Agarwal et al. 2014; Sabeti et al. 2016), possess low levels of antioxidant enzymes (Sabeti et al. 2016), and have high levels of plasma membrane polyunsaturated fatty acids that are targets for lipid peroxidation (Chen et al. 2013; Agarwal et al. 2014). Oxygen radicals in the male reproductive tract are commonly produced by immune system cells (including leukocytes and macrophages) (Lavranos et al. 2012; Agarwal et al. 2014). ROS in sperm cells are produced in the mitochondria and the plasma membrane (Sabeti et al. 2016; Agarwal et al. 2014). Chemicals known to cause adverse effects in the male reproductive system (e.g., reduced sperm counts and motility, increased abnormal sperm morphology) via increased production of ROS and downstream cellular damage include pesticides, tetrachlorodibenzodioxin (TCDD), and heavy metals such as cadmium (Table 2).

8. Induces Inflammation

Acute or chronic inflammation of the male reproductive tract caused by infection, hormonal perturbations, or exposure to environmental contaminants are known causes of male factor infertility (Lavranos et al. 2012; Fijak et al. 2018). The defined clinical manifestations include urethritis, prostatitis, seminal vesiculitis, epididymitis, and orchitis. Although bacteria and other common uropathogens represent the most frequent cause of inflammatory conditions (Fijak et al. 2018), noninfectious causes of inflammation, including environmental contaminants such as cadmium, should also be considered (de Angelis et al. 2017). Inflammatory responses caused by pro-inflammatory agents are amplified by activated lymphocytes and macrophages through the release of a variety of cytokines, chemokines, and growth factors present in human semen. These factors include interleukins, tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, soluble receptors and antagonists, granulocyte and macrophage colony-stimulating factors, interferons, chemokines, macrophage inflammatory proteins, transforming growth factor, monocyte chemotactic and activating factor, hepatocyte growth factor, and prostaglandins (Fraczek and Kurpisz 2007, 2015; Frungieri et al. 2015). Increased levels and/or production of these pro-inflammatory cytokines is associated with decreases in sperm number, motility, and concentration; increased ROS production; and reduced testosterone production by Leydig cells (Fraczek and Kurpisz 2007). Inflammation is also a source of oxidative stress (Bachir and Jarvi 2014; Ko et al. 2014) leading to an imbalance in the oxidant/antioxidant system, and it may cause scarring of the delicate ductal system leading to subsequent anatomic obstruction (Bachir and Jarvi 2014). Environmental contaminants such as cadmium and TCDD have been shown to induce inflammation in the male reproductive system (Table 2).

Discussion and Conclusions

The identification and analysis of mechanistic evidence for the evaluation of chemical-induced male reproductive toxicity is an increasingly essential aspect of hazard evaluation and human health risk assessment, especially when epidemiological and apical animal toxicity data are inadequate or sparse. Approaches that facilitate the systematic evaluation of toxicological and mechanistic evidence can improve the transparency and strength of evidence analyses performed as part of a risk assessment. Here, we have developed a set of eight key characteristics of male reproductive toxicants (Figure 1) that can provide a structure for systematically identifying and organizing the relevant literature on mechanistic information in support of an evaluation of a chemical for male reproductive toxicity.

The workshop participants concluded that chemicals that are male reproductive toxicants would be expected to have strong evidence for having one or more of the key characteristics, although it should be noted that simply the presence of one or even several of these characteristics does not conclusively identify a chemical as a male reproductive toxicant. Indeed, several key characteristics of male reproductive toxicants overlap with the key characteristics of carcinogens. The key characteristics of male reproductive toxicants, as well as the key characteristics of carcinogens, are not checklists but, instead, provide a starting point for identification, organization, and analysis of mechanistic data that may inform whether a chemical can cause male reproductive effects or cancer, respectively. The key characteristics of male reproductive toxicants are intended to be used as part of an evaluation of chemical-induced reproductive toxicity that includes data from studies of experimental animal and epidemiological outcomes, if available, and requires expert judgment. Evaluation of the available mechanistic evidence for male reproductive effects of a chemical should therefore be focused on organs and systems that can impact male reproductive functions.

The key characteristics can be leveraged at multiple steps in the chemical evaluation process, including literature identification and problem formulation efforts. For example, the key characteristics can be used to develop a targeted literature search strategy to identify mechanistic data using appropriate combinations of Medical Subject Headings (MeSH) search tools and keyword terms for end points that can ultimately inform mechanisms relevant to male reproductive toxicity (see Table S2). The resulting identified papers can then be further screened and evaluated in more detail, including considerations of study design, dose–response effects, life stage, and reporting features. Accordingly, a full evaluation of the mechanistic database available for each chemical can be achieved, and strength of the evidence descriptors may be assigned to each key characteristic, for example, whether they are strong, limited, or inadequate (IARC 2019).

The authors suggest another potential application of the key characteristics approach is the development of literature inventories that can facilitate the review of the available evidence informing each of the key characteristics and the proposed network by creating a summary-level sortable list of the available evidence. This becomes particularly relevant when working with a large database of studies using diverse experimental models and designs. The inventory should include study design features as well as a description of the findings that inform each of the key characteristics. Once completed, the assessor can use the

inventory to navigate through the available evidence according to information categories captured in it (e.g., species, strain, dose, life stage, evidence for each of the key characteristics). An example of the types of information that can be captured in a literature inventory is presented in Excel Table S3. The studies captured in Excel Table S3 were identified from literature reviews by Siu et al. (2009), Lafuente (2013), and de Angelis et al. (2017).

The key characteristics of male reproductive toxicants may also be applied to guide prioritization of data-poor chemicals for further evaluation. In a hazard evaluation of a chemical for which animal and human data are sparse, the key characteristics could be used to organize and integrate relevant high-throughput, toxicogenomic and other mechanistic data and identify the potential for male reproductive toxicity. This would encompass inclusion and analyses of new high-throughput assays as well as new mapping of assays to the key characteristics. The analysis can carry less uncertainty when done in read across fashion wherein one or more structurally or mechanistically similar anchor chemicals have robust animal or human apical end point data sets showing adverse male reproductive outcomes (NRC 2017).

Information obtained from screened studies can be organized in a mechanistic network describing the potential pathways associated with chemical-induced male reproductive toxicity. An example using cadmium is presented in Figure 2 (Siu et al. 2009; Lafuente 2013; Wan et al. 2013; Gao et al. 2015; Jenardhanan et al. 2016; de Angelis et al. 2017; Flora and Agrawal 2017). Potential key characteristics involved in cadmium-induced male reproductive toxicity include *a*) alters germ cell development, functions, or death; *b*) alters somatic cell development, functions, or death; *c*) alters production and levels of reproductive hormones; *d*) alters hormone receptor levels/functions; *e*) is genotoxic; *f*) induces oxidative stress; and *g*) induces inflammation (Table 2).

Mode of action (MOA) analyses were developed in an attempt to link key events in a theoretical biological sequence so that a relatively simple hypothesis as to the mechanism involved in the toxic effect could be generated (U.S. EPA 1996b, 2005). Adverse outcome pathways (AOPs) are a more recent expansion of MOA concepts that include a molecular initiating event and an adverse outcome in an organism linked by all key events measured at various levels of organization (Carusi et al. 2018). Both MOAs and AOPs are linear, reductive models of complex physiology, but they may nonetheless be useful for understanding how chemicals exert their toxic effects (Escher et al. 2017). A challenge to the practical application of MOA and AOP approaches for chemical safety decision-making is limitations in the current understanding of disease processes that may be shown to be incorrect or incomplete (Guyton et al. 2009). This limitation was recognized by Sir Bradford Hill, who formalized the research of causality in humans while noting that, "what is biologically plausible depends upon the biological knowledge of the day" (Hill 1965). The authors believe the key characteristics are agnostic with respect to current or future knowledge of downstream adverse effects and of the precise mechanistic pathways leading to these outcomes. The value of this approach for reproductive toxicants is that, as for carcinogens, gaps in mechanistic data that delineate the complete pathway from exposure to adversity need not hamper the identification of reproductive toxicants. If strong evidence is present for one or more likely key characteristics, this can directly inform the hazard identification process. This emancipates the risk assessor from "connecting the dots" between a molecular initiating event and a specific adverse outcome that is the basis of the AOP approach to hazard identification. The key characteristics approach we describe here can be viewed as identifying molecular initiating events or early key events as described in the MOA and AOP frameworks based on our current knowledge of the molecular mechanisms of reproductive toxicant action and their role in health and disease. Using key characteristics to assemble mechanistic data about a putative reproductive toxicant does not require an exhaustive understanding of how the characteristics are causally linked to the adverse response or an *a priori* hypothesis about the MOA or AOP.

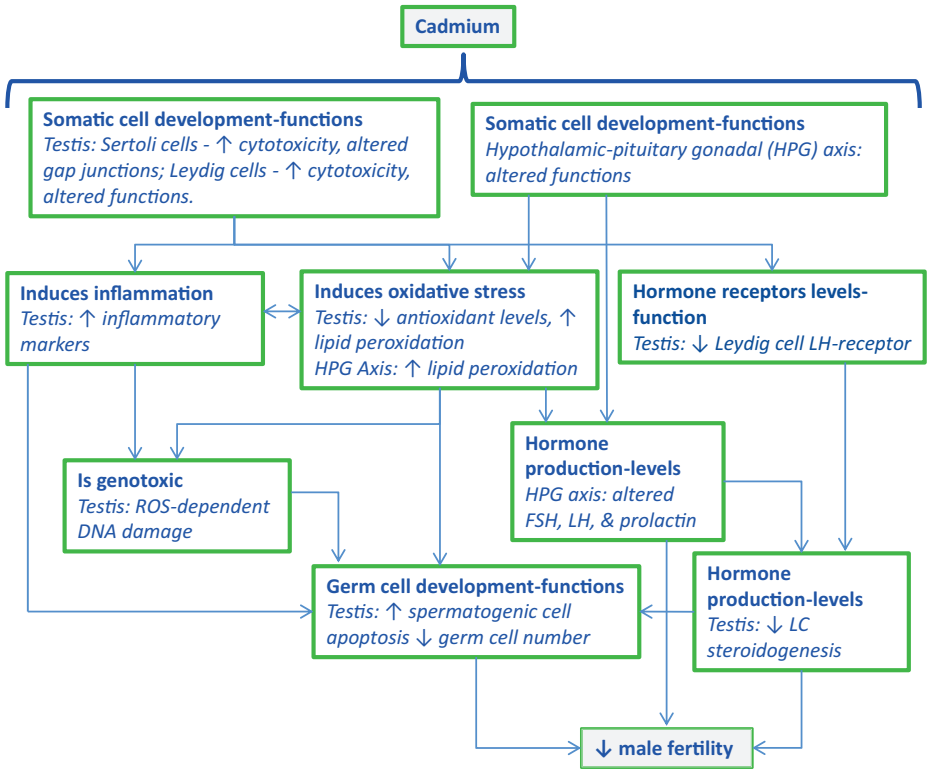

Figure 2. Application of the key characteristics to develop a network on cadmium-induced male reproductive effects.

The proposed key characteristics of male reproductive toxicants thus form an objective approach for organizing and evaluating the complex and ever-accumulating mechanistic evidence on a given chemical or group of chemicals. This evaluation of the mechanistic evidence is inclusive of data from both human observational studies and *in vivo* and *in vitro* experimental systems, encompassing molecular epidemiological studies using biomarkers and high-throughput *in vitro* tests. Once the available evidence for a chemical (or group of chemicals) suspected of targeting the male reproductive system is extracted and sorted into the different key characteristics, biological networks can be developed if required to facilitate a qualitative and quantitative evaluation (Figure 2 presents an example using cadmium). Furthermore, mechanistic evidence analyses that rely on the key characteristics of male reproductive toxicants can also help identify significant data gaps and areas that could benefit from additional research and provide guidance on the development of high-throughput assays to systematically evaluate each of the proposed key characteristics. Finally, the key characteristics of male reproductive toxicants should be updated as new mechanisms and pathways for chemical-induced male reproductive toxicity are discovered and described in the peer-reviewed literature and as more chemical-specific case studies and health assessments using the key characteristics approach are conducted.

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