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Proposed Key Characteristics of Female Reproductive Toxicants as an Approach for Organizing and Evaluating Mechanistic Data in Hazard Assessment

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Background: Identification of female reproductive toxicants is currently based largely on integrated epidemiological and *in vivo* toxicology data and, to a lesser degree, on mechanistic data. A uniform approach to systematically search, organize, integrate, and evaluate mechanistic evidence of female reproductive toxicity from various data types is lacking.

Objective: We sought to apply a key characteristics approach similar to that pioneered for carcinogen hazard identification to female reproductive toxicant hazard identification.

Methods: A working group of international experts was convened to discuss mechanisms associated with chemical-induced female reproductive toxicity and identified 10 key characteristics of chemicals that cause female reproductive toxicity: 1) alters hormone receptor signaling; alters reproductive hormone production, secretion, or metabolism; 2) chemical or metabolite is genotoxic; 3) induces epigenetic alterations; 4) causes mitochondrial dysfunction; 5) induces oxidative stress; 6) alters immune function; 7) alters cell signal transduction; 8) alters direct cell–cell interactions; 9) alters survival, proliferation, cell death, or metabolic pathways; and 10) alters microtubules and associated structures. As proof of principle, cyclophosphamide and diethylstilbestrol (DES), for which both human and animal studies have demonstrated female reproductive toxicity, display at least 5 and 3 key characteristics, respectively. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), for which the epidemiological evidence is mixed, exhibits 5 key characteristics.

Discussion: Future efforts should focus on evaluating the proposed key characteristics against additional known and suspected female reproductive toxicants. Chemicals that exhibit one or more of the key characteristics could be prioritized for additional evaluation and testing. A key characteristics approach has the potential to integrate with pathway-based toxicity testing to improve prediction of female reproductive toxicity in chemicals and potentially prevent some toxicants from entering common use. https://doi.org/10.1289/EHP4971

Introduction

Successful reproduction requires multiple systems to function properly. Malfunction can result in difficulty or inability to conceive, failure to carry a pregnancy successfully to term, or difficulty nourishing an infant. Organs that must function properly for these tasks include the hypothalamus, pituitary gland, ovary, fallopian tubes, uterus, placenta, and mammary gland. Interference with molecular, cellular, or tissue functions within any of these organs can, in principle, result in female reproductive dysfunction. Disorders of the female reproductive system are relatively common. For example, in the United States, nearly 7% of married women are infertile and 12% of women 15–44 y of age have impaired fecundity (CDC 2018). Disorders of the female reproductive system can adversely affect a woman's health and quality of life beyond their impacts on reproduction. For example, endometriosis and uterine myomas (fibroids), and uterine adenomyosis can cause pain and excessive menstrual bleeding (Fortin et al. 2018; Rolla 2019), whereas early menopause increases risk of osteoporosis, cardiovascular disease, and dementia (Dubey et al. 2005; Shuster et al. 2008; Silva et al. 2001; Svejme et al. 2012).

The process of chemical risk assessment involves determining whether exposure to the chemical at any dose can cause adverse health effects (hazard identification), assessing the range of human exposures to the chemical (exposure assessment), and integrating the hazard and exposure data in dose-response assessment and risk characterization (DiBartolomeis 2007). Currently female reproductive hazard identification is based on an integration of epidemiological, *in vivo* toxicology, and mechanistic data, with most emphasis placed on epidemiological and toxicological data. There are tens of thousands of chemicals in commerce, but very few have had *in vivo* reproductive toxicity testing and even fewer have epidemiological studies with female reproductive end points. Each of these types of data has strengths and limitations for hazard identification. Epidemiological data collected on human populations are usually observational, meaning individuals are not randomly allocated to exposed and unexposed groups so as to prevent the influence of factors other than the chemical exposure of interest on the end points. Validated methods for measuring chemicals or their metabolites in biological samples do not exist for the vast majority of chemicals, and even when they do exist may lack sensitivity or

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may not be collected during a relevant time window. Moreover, humans live in a world with multiple simultaneous chemical exposures. Rarely, although growing in frequency, do studies consider exposure to mixtures, in part because measurement is expensive and the study of mixtures poses statistical and sample size challenges (Braun et al. 2016).

Animal studies also have significant limitations. Table 1 shows apical end points that are routinely measured in *in vivo* animal studies for female reproductive toxicity performed according to guidelines of the U.S. EPA (1998a, 1998b) or Organization for Economic Cooperation and Development (OECD) (ECETOC 2002). Studies using only these end points may fail to identify some female reproductive toxicants. For example, they do not include evaluation of the placenta, mating behavior, or serum reproductive hormone concentrations. Species and dosage differences can also limit the ability to extrapolate from animals to humans, resulting in false positives and negatives. For example, rats and mice are by far the most commonly used animals in regulatory testing, but these species do not develop endometriosis (Story and Kennedy 2004). Catarrhine primates (humans, apes, some monkeys) and a few other rodent and bat species are the only species known to menstruate (Alvergne and Högqvist Tabor 2018), and nonprimate species are not known to develop endometriosis (Story and Kennedy 2004). Finally, both animal and epidemiological studies are time consuming and expensive and have not been performed on many chemicals.

The ability to predict female reproductive toxicity from high-throughput mechanistic studies performed *in vitro* or *in vivo* would be extremely useful. Such studies can be performed on thousands of chemicals at relatively low cost and may not be subject to the same methodological challenges seen in epidemiological and traditional animal toxicology studies. At a minimum, using *in vitro* assays to predict potential female reproductive toxicity would be very useful in certain contexts, such as for conducting alternatives analysis of multiple chemicals that could be used in a particular application or for prioritizing chemicals for more in-depth assessment.

Here we argue that predicting the potential for human hazard from *in vitro* assays would be aided by an understanding of the key characteristics of chemicals that induce female reproductive toxicity. Previously, Smith et al. (2016) identified 10 key characteristics of human carcinogens. This framework has been utilized by the International Agency for Research on Cancer (IARC) in their Monographs Program as described by Guyton et al. (2018). The key characteristics of carcinogens create a uniform approach for searching, organizing, and evaluating mechanistic evidence to support carcinogenic hazard identification. A 2017 National Academy of Sciences report recommended that the key characteristics approach be expanded to other end points, including reproductive effects, endocrine disruption, and cardiovascular disease (National Academies of Science, Engineering, and Medicine 2017).

Objective

We have attempted to apply the key characteristics approach to female reproductive toxicants based on our current knowledge of the mechanisms by which chemicals cause female reproductive toxicity.

Methods

A working group of international experts was convened at the University of California, Berkeley from 7 to 8 March 2018 to review the key characteristics approach and determine whether this approach can also be applied to endocrine disruptors and male and female reproductive toxicants. Initial draft lists of key characteristics of endocrine disruptors and male and female reproductive toxicants and examples thereof were formulated at the meeting. In addition, three groups were convened to continue working together to further refine these key characteristics.

This paper presents the conclusions of the working group on key characteristics of female reproductive toxicants. The group consisted of experts with extensive knowledge of the literature on mechanisms of chemical-induced female reproductive toxicity as well as the epidemiological and *in vivo* toxicology literature on chemical exposures associated with adverse female reproductive outcomes. Members of the group had expertise in mechanistic toxicology, obstetrics and gynecology, occupational and environmental medicine, epidemiology, risk assessment, and regulation of hazardous chemicals. To provide a common understanding of the general approach, the group reviewed the key characteristics of carcinogens to illustrate the types of mechanistic effects of interest. The group then developed a *de novo* list of key characteristics of female reproductive toxicants independent of the key characteristics of carcinogens.

We focused on mechanistic end points, not the apical end points listed in Table 1, because apical end points are already used in hazard identification and the key characteristics approach is intended to emphasize early predictive cellular and tissue-level changes that could be identified using *in vitro* assays and other rapid screens. During the two-day meeting, the group deliberated to generate a comprehensive list of known early cellular and tissue-level changes associated with reproductive toxicity in females. In subsequent phases, conducted via conference call, the list was narrowed by removing apical end points and hypothetical mechanisms lacking sufficient supporting evidence. The list was also consolidated to merge concepts with similar biology. In addition to identifying mechanisms of chemicals with female reproductive toxicity based on its collective expertise, the group reviewed conclusions of expert bodies that identify female reproductive toxicants (NTP; OEHHA 2018) and conducted literature searches (PubMed, Google Scholar). The intent of the searches was not to conduct a systematic review of female reproductive toxicants. Rather the intent was to identify a set of chemicals for which there was a body of published research demonstrating female reproductive toxicity and associated mechanisms. Chemicals to illustrate each of the key characteristics were selected from among the chemicals identified by consensus of the group based on its collective expertise. This was an iterative process in which the initial list of key characteristics and example chemicals was refined based on discussions of the entire group and the work of subgroups focused on developing and refining specific key characteristics and example chemicals.

Based on this process, 10 key characteristics of chemicals, which cause female reproductive toxicity, were identified, as well

Table 1. Female-specific apical end points of reproductive toxicity measured in guideline studies of the U.S. EPA and/or OECD.

Reproductive process or end point	Assay
Estrous cycling	Vaginal cytology
Reproductive organ size	Weights of ovaries, uterus (with oviducts and cervix), pituitary
Reproductive organ structure	Macroscopic and histopathological examination of ovaries, uterus, oviducts, cervix, vagina, pituitary, mammary gland. Enumeration of ovarian primordial follicles
Development	Puberty (vaginal opening, first vaginal estrus in rodents), anogenital distance, structure of external genitalia
Pregnancy	Pregnancy rate, number of implantation sites, preimplantation mortality, birth rate, number and sex of live and dead pups at birth, fetal/neonatal body weights

Note: EPA, Environmental Protection Agency; OECD, Organization for Economic Cooperation and Development.

as examples of chemicals known to have one or more of these characteristics. Although many of these key characteristics may appear to be general toxicity mechanisms, they are only relevant here where they act on the cells and tissues of the female reproductive system, including the hypothalamus, pituitary, ovary, uterus, oviduct, vagina, mammary gland, and placenta. The key characteristics, example toxicants, and associated mechanisms/pathways are summarized in Figure 1 and Table 2.

To illustrate the utility of the key characteristics for identifying chemicals that may be expected to elicit female reproductive toxicity, we selected three example chemicals based on two criteria: *a*) the existence of multiple epidemiological and animal toxicology studies of female reproductive end points of the toxicant, and *b*) the desire to choose toxicants with different mechanisms of action for illustrative purposes.

Description of the Key Characteristics of Female Reproductive Toxicants

Key Characteristic 1: Alters Hormone Receptor Signaling; Alters Reproductive Hormone Production, Secretion, or Metabolism

The hypothalamic-pituitary-ovarian (HPO) axis maintains hormonal balance within the female reproductive system. Gonadotropin releasing hormone (GnRH), a peptide hormone produced in the hypothalamus, stimulates the anterior pituitary via specific plasma membrane receptors to produce and release the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Padmanabhan et al. 2018). Gonadotropins, acting via plasma membrane receptors in turn promote follicle development, ovulation, maintenance of the corpus luteum and steroid hormone production by the ovary (Padmanabhan et al. 2018). The ovary in response to the gonadotropin stimulation produces two major steroid hormones—progesterone (P) and estradiol (E2)—as well as peptide hormones such as inhibin and activin. The ovarian hormones, in turn, regulate LH and FSH synthesis and secretion through positive and negative feedback to the pituitary and hypothalamus (Padmanabhan et al. 2018). Furthermore, the ovarian steroids regulate the function of target organs, such as the uterus and mammary gland, classically via intracellular receptors. Prolactin (PRL) from the anterior pituitary promotes mammary gland growth and milk production (Seachrist et al. 2018). PRL also plays a role in controlling ovarian function in laboratory rodents (Ben-Jonathan et al. 2008). Oxytocin, a hypothalamic protein hormone is released at the neurohypophysis (posterior pituitary), and stimulates milk secretion and, during parturition, uterus contraction (Seachrist et al. 2018). Toxicants may interfere at one or many of the steps described above, contributing to developmental and reproductive toxicological adverse effects.

Toxicants may interfere with the HPO axis. For example, exposure of female rats to the herbicide atrazine decreased LH and PRL secretion by the pituitary (Cooper et al. 2007). Evidence supports that these effects are mediated via a hypothalamic site of action. Exposure to atrazine increased preovulatory GnRH content in the hypothalamic median eminence, consistent with decreased GnRH release as the cause of decreased preovulatory LH secretion, and increased hypothalamic dopamine, the hypothalamic inhibiting factor for PRL secretion (Cooper et al. 2000, 2007; Stoker et al. 1999). There is evidence that TCDD alters the E2 positive feedback to the hypothalamus that causes the preovulatory gonadotropin surge by decreasing hypothalamic sensitivity to E2 in rats (Gao et al. 2001). Decreased levels of GnRH, LH (absence of LH surge), and FSH were observed in perfluorooctane sulfonate (PFOS)–exposed mice. Consistent with decreased GnRH levels, the number of kisspeptin neurons and the level of Kiss1 mRNA in hypothalamic nuclei that regulate GnRH release were reduced (Feng et al. 2015). Chemicals may directly disrupt steroid hormone production and metabolism or they may interact with hormone receptors or serum binding proteins. TCDD decreased E2 secretion without

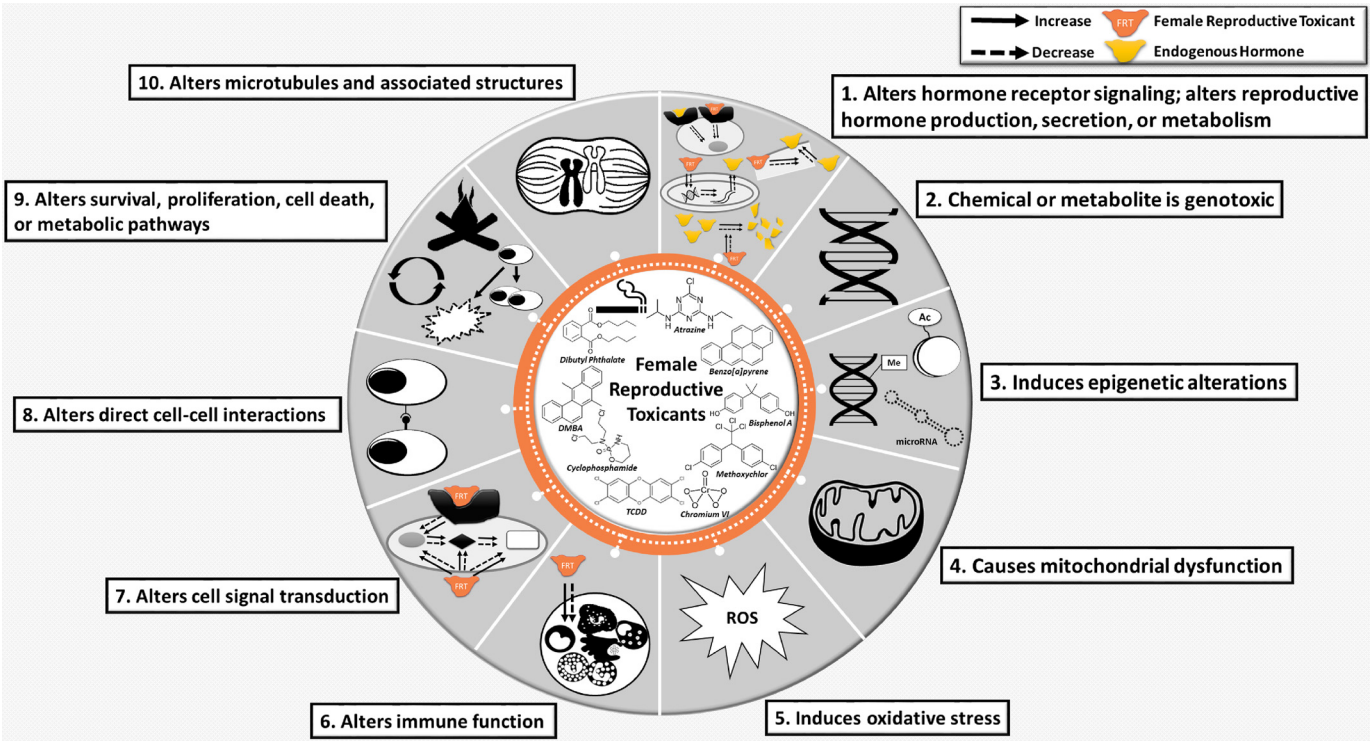

Figure 1. The ten key characteristics of female reproductive toxicants. Example toxicants and associated mechanisms are provided in Table 2. Image: © Linda Rieswijk.

Table 2. Key characteristics of female reproductive toxicants.

Characteristic	Example toxicants	Known mechanistic pathway associated with female adverse reproductive outcomes
1. Alters hormone receptor signaling; alters reproductive hormone production, secretion, or metabolism	Methoxychlor	ERα agonism and ERβ antagonism by metabolite HPTE (Gaido et al. 2000)
	Atrazine	Inhibition of LH and PRL secretion mediated by alteration of hypothalamic GnRH and dopamine signaling (Cooper et al. 2007)
2. Chemical or metabolite is genotoxic	Benzo[*a*]pyrene	Metabolism to DNA-damaging diol epoxide, radical cation, and quinone metabolites (Xue and Warshawsky 2005). DNA adduct formation resulting in mutations in ovaries and oviducts after prenatal exposure (Einaudi et al. 2014; Lim et al. 2015; Luderer et al. 2019)
	Cyclophosphamide	Phosphoramide mustard metabolite causes dsDNA breaks in oocytes of primordial follicles in cultured neonatal ovaries (Petrillo et al. 2011)
3. Induces epigenetic alterations	Bisphenol A	Altered DNA methylation of maternal imprinted genes in oocytes, embryos, placenta (Chao et al. 2012; Susiarjo et al. 2013)
	Diethylstilbestrol	Altered DNA methylation and histone acetylation and methylation in developing uterus (Bromer et al. 2009; Jefferson et al. 2013)
4. Causes mitochondrial dysfunction	Methoxychlor	Inhibition of OXPHOS, increased ROS causing follicle apoptosis (Gupta et al. 2006b)
	Rotenone	Inhibition of complex I of electron transport chain with decreased ATP production and increased ROS, resulting in failure of oocyte meiosis (Shen et al. 2018)
5. Induces oxidative stress	Chromium VI	Increased ovarian and placental hydrogen peroxide, lipid peroxidation, decreased antioxidant gene expression with *in utero* or lactational exposure (Banu et al. 2017a, 2017b; Stanley et al. 2013, 2014)
	Methoxychlor	Increased ovarian hydrogen peroxide, oxidative protein and DNA damage; atresia of cultured antral follicles (Borgeest et al. 2002; Gupta et al. 2006a, 2006b)
6. Alters immune function	NSAIDs	Inhibition of follicular prostaglandin synthesis resulting in inhibition of ovulation (Espey et al. 1982; Gaytán et al. 2006; Tsafriri et al. 1973)
	TCDD	Inhibition of natural killer cells and increased neutrophil activity at sites of ectopic endometrium in mouse model of endometriosis (Bruner-Tran et al. 2018; Cummings et al. 1999)
7. Alters cell signal transduction	TCDD	Disruption of EGFR, MAPK, PTK, c-Src signaling in monkey endocervical cells (Enan et al. 1998)
	Cyclophosphamide	Alters AKT signaling in oocytes of small follicles (Kalich-Philosoph et al. 2013)
8. Alters direct cell–cell interactions	Benzo[*a*]pyrene	Decreased sperm–egg binding and fusion (Sobinoff et al. 2012)
	DMBA	Decreased ovarian gap junction mRNA and protein levels (Ganesan and Keating 2014; Ganesan et al. 2015)
9. Alters survival, proliferation, cell death, or metabolic pathways	Dibutyl phthalate	Cell cycle arrest in antral follicles (Craig et al. 2013; Rasmussen et al. 2017)
	Benzo[*a*]pyrene, DMBA	Apoptosis of germ cells in *ex vivo* fetal ovary (Lim et al. 2016; Lim and Luderer 2018; Matikainen et al. 2001, 2002)
	DHEA	Inhibition of oocyte pentose phosphate pathway (Jimenez et al. 2013)
10. Alters microtubules and associated structures	Colchicine, colcemid, carbendazim	Meiotic spindle disruption, aneuploidy (reviewed by Luderer et al. 2018)
	Cigarette smoke	Oviductal cilia dysfunction (Talbot et al. 1999)

Note: AKT: protein kinase B; c-Src: proto-oncogene tyrosine-protein kinase Src; DHEA: dehydroepiandrosterone; DMBA: 7,12-dimethyl-benz[a]anthracene; dsDNA, double-stranded DNA; EGFR: epidermal growth factor receptor; ER: estrogen receptor; GnRH: gonadotropin-releasing hormone; HPTE: 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane; LH: luteinizing hormone; MAPK: mitogen-activated protein kinase; NSAIDs: nonsteroidal anti-inflammatory drugs; OXPHOS: oxidative phosphorylation; PTK: protein tyrosine kinase; PRL: prolactin; ROS: reactive oxygen species; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

altering aromatase activity in cultured human luteinized granulosa cells, most likely by decreasing the enzyme that produces androgen substrate for aromatization (Morán et al. 2000, 2003) (Sanderson 2006). In mice, PFOS exposure suppressed the biosynthesis of P and E2 by down-regulating the steroidogenic acute regulatory (StAR) protein (Feng et al. 2015). Some methoxychlor metabolites present estrogen receptor alpha (ERα) agonist activity and ERβ and androgen receptor antagonist activity (Gaido et al. 2000). Steroid mimics such as bisphenol A (BPA) can alter the ratio of steroid hormone bound to the serum binding protein sex hormone-binding globulin (SHBG) and free, unbound hormone if they bind or modify the amount of SHBG (Hodgert Jury et al. 2000).

Key Characteristic 2: Chemical or Metabolite Is Genotoxic

Genotoxicity is generally defined as alteration in genetic material caused by exposure to an external agent and comprises DNA damage and chromosomal changes. DNA damage caused by genotoxic chemicals within a cell can be repaired, induce cell death, or result in mutations that can be transmitted to daughter cells. Mutations in protooncogenes or tumor suppressor genes can initiate carcinogenesis (Armitage 1985; Barcellos-Hoff et al. 2013). Genetic damage to germ cells from pre- or postnatal exposures can adversely affect female reproduction, resulting in oocyte destruction, or if the oocyte survives, the damage may be transmitted to offspring (NRC 1989). In comparison with DNA/chromosome damage to sperm, there are fewer studies on oocyte DNA or chromosome damage, probably due to the much smaller number of these cells that can be recovered during a normal cycle or even with exogenous hormonal stimulation. However, DNA damage has been measured in the ovaries of animals exposed pre- or postnatally to mutagenic chemicals. We provide specific examples below.

Phase I metabolism of xenobiotic chemicals can convert relatively nonreactive parent compounds to electrophilic, reactive metabolites, which can potentially interact with macromolecules in tissues of the female reproductive system, resulting in reproductive toxicity. Important Phase I enzymes include the cytochrome P450 enzymes. Many of these reactive metabolites are also mutagenic because they can react with DNA, forming DNA adducts. Examples include polycyclic aromatic hydrocarbons (PAHs) (Xue and Warshawsky 2005) and 4-vinylcyclohexene (NTP 1986, 1989). DNA adducts formed as a result of exposure

to PAHs have been measured in the ovaries of rodents and in human placenta using ^{32}P-postlabeling and immunostaining with antibodies against the DNA adducts. PAH DNA adduct formation is thought to play a role in the ovarian toxicity and developmental toxicity of these compounds (Shum et al. 1979; Takizawa et al. 1984). Treatment with the PAH benzo[a]pyrene (BaP) causes ovarian DNA strand breaks and DNA adducts in mice (Einaudi et al. 2014; Lim et al. 2015) and rats (Ramesh et al. 2010). BaP-related albumin adducts have been demonstrated in human cord blood and DNA adducts in the human placenta (Autrup and Vestergaard 1996; Hansen et al. 1993).

Ovarian and oviductal mutations have been measured in female offspring of transgenic MutaMouse™ females exposed during pregnancy to oral doses of the PAH BaP that also result in ovarian follicle depletion (Luderer et al. 2019). Genotoxic anticancer drugs have also been shown to cause ovarian DNA damage. Culture of neonatal ovaries with the phosphoramide mustard metabolite of cyclophosphamide caused double-stranded DNA (dsDNA) breaks in oocytes of primordial and primary follicles measured using immunostaining for phosphorylated histone H2AX (Petrillo et al. 2011). *In vivo* treatment of mice with cisplatin (Gonfloni et al. 2009) or doxorubicin (Soleimani et al. 2011) caused dsDNA breaks in oocytes of primordial follicles. Culture of human ovarian cortical strips with doxorubicin caused dsDNA breaks in oocytes of primordial follicles (Soleimani et al. 2011).

Key Characteristic 3: Induces Epigenetic Alterations

The term epigenetic refers to stable changes in gene expression that are not a result of alterations in DNA sequence and that can be heritable through cell divisions (Jirtle and Skinner 2007). Epigenetic processes include DNA methylation, posttranslational histone modifications and chromatin remodeling, and expression of noncoding RNAs such as microRNAs (miRNAs). These processes coordinately guide normal development and enable dynamic responses to environmental stimuli. Developmental exposure to some female reproductive toxicants can cause persistent epigenetic changes, leading to the altered expression of genes and proteins important for development and function (for review see Bommarito et al. 2017; Cruz et al. 2014; Eichenlaub-Ritter and Pacchierotti 2015; Gore et al. 2015; Ho et al. 2017).

Prenatal exposure of mice to BPA altered methylation of maternal imprinted genes in oocytes (Chao et al. 2012) and in embryos and placentas (Susiarjo et al. 2013), resulting in abnormal oocyte development, folliculogenesis, and placental development. Expression of miRNA (miR)-146a was significantly increased and correlated with BPA accumulation in the placenta of pregnant women living in a polluted area (De Felice et al. 2015). Prenatal exposure of mice to BPA or DES increased protein levels and functional activity of the histone methyltransferase EZH2 in the adult mammary gland (Doherty et al. 2010). Neonatal exposure of mice to DES resulted in persistent hypomethylation of sites in several uterine gene promoters (Li et al. 1997; Tang et al. 2008), and prenatal exposure resulted in persistent hypermethylation of uterine homeobox A10 gene (*Hoxa10*) (Bromer et al. 2009). Neonatal DES exposure in mice also led to reduced uterine levels of EZH2 and histone deacetylases, persistently altered the site-specific occupancy of modified histones in oncogenic SIX homeobox 1 (*Six1*), and permanently altered expression of *Six1* in adult mice (Jefferson et al. 2013). These epigenetic changes caused by DES may play a role in the abnormal uterine morphology and function caused by DES exposure during female reproductive system development (Newbold 2004).

miRNAs targeting the transforming growth factor beta (TGF-β) pathway were reported to be dysregulated in preeclamptic placentas from women with elevated placental cadmium levels and in a human trophoblast cell model treated with cadmium *in vitro* (Brooks et al. 2016). *In vitro* cadmium exposure decreased placental trophoblast migration via increased signaling of the TGF-β pathway mediated by miRNAs (Brooks and Fry 2017). In an epigenome-wide association study of placental DNA from two U.S. birth cohorts, 17 differentially methylated CpG sites and increased expression of inflammatory signaling and cell growth genes were associated with elevated placental cadmium levels (Everson et al. 2018).

Key Characteristic 4: Causes Mitochondrial Dysfunction

Mitochondrial functions include oxidative phosphorylation, fatty acid beta oxidation, Ca^{2+} buffering, and apoptosis regulation (Westermann 2010). In ovarian granulosa cells and theca cells, mitochondria are also essential for steroid hormone synthesis (Uzumcu and Zachow 2007). Mitochondria are generally thought to be maternally inherited via oocytes. Insufficient energetics in oocytes/embryos leads to impaired spindle formation or chromosome misalignment, provoking aneuploidy, which can result in preimplantation embryonic growth restriction, malformations, spontaneous abortions, and stillbirths (Tilly and Sinclair 2013). Mechanisms by which mitochondrial function can be disrupted include mitochondrial DNA (mtDNA) mutations, decreased mtDNA copy numbers, dissipation of mitochondrial membrane potential, oxidative damage caused by highly reactive secondary reactive oxygen species (ROS) generated as by-products of oxidative phosphorylation, and insufficient elimination of damaged mitochondria (Meyer et al. 2013). Interplay between these mechanisms appears to be complex. For example, human fibroblasts with mitochondrial dysfunction or treated with electron transport chain inhibitors showed a senescent phenotype independent of ROS or DNA damage (Wiley et al. 2016). Mitochondria are dynamic organelles that frequently change their morphology by fusion and fission regulated by key high-molecular mass GTPases, such as dynamin-related protein 1 (Drp1). Oocyte-specific Drp1-deficient mice show subfertility, despite maintenance of the oocyte ATP content and mitochondrial membrane potential (Udagawa et al. 2014).

Culture of premeiotic embryonic mouse ovaries with rotenone, an inhibitor of Complex I of the electron transport chain, decreased ATP production, and prevented premeiotic DNA replication and the entry of germ cells into meiosis (Shen et al. 2018). Also acting like Complex I inhibitors, intraperitoneal methoxychlor administration inhibited mitochondrial respiration and increased ROS production, leading to atresia of mouse ovarian follicles (Gupta et al. 2006b). Mouse oocytes cultured with methoxychlor showed oxidative stress and impaired spindle formation (Liu et al. 2016). Methoxychlor and its metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) steroidogenic in cultured rat granulosa cells, including expression of the gene encoding the cholesterol side chain cleavage enzyme, which resides on the inner mitochondrial membrane (Zachow and Uzumcu 2006).

Key Characteristic 5: Induces Oxidative Stress

ROS and reactive nitrogen species (RNS)—such as the free radicals superoxide anion radical, hydroxyl radical, and nitric oxide and nonfree radicals such as hydrogen peroxide and peroxynitrite—are formed as a result of normal cellular metabolism and function as signaling molecules within the female reproductive system. For example, ROS are required for ovulation (Miyazaki et al. 1991; Shkolnik et al. 2011). ROS and RNS can also be formed during xenobiotic metabolism. Cells possess multiple enzymatic and nonenzymatic antioxidants that allow them to regulate levels of ROS and RNS. Oxidative stress occurs when the balance between ROS/RNS generation and antioxidant defenses is disrupted. Altered redox balance

can affect protein function and signaling pathways, and ROS and RNS can react with and damage nucleic acids, proteins, and lipids (Sies et al. 2017).

Oxidative stress has been shown to play a role in the apoptotic death of ovarian follicles caused by a number of toxicants (reviewed by Luderer 2014) including the heavy metal chromium (Cr) and the organochlorine insecticide methoxychlor. Reduction of CrVI to CrIII results in ROS generation. Administration of CrVI as potassium dichromate to lactating rats depleted ovarian follicles, increased plasma and ovarian hydrogen peroxide and lipid hydroperoxide levels, and decreased plasma and ovarian antioxidant enzyme activities and ascorbic acid levels in the female offspring (Stanley et al. 2013, 2014). Supplementation with ascorbic acid or the free radical scavenger edavarone was protective (Stanley et al. 2013, 2014). Increased apoptosis, cell cycle arrest, and decreased levels of antioxidants were also observed in granulosa and theca cells cultured with CrVI, and ascorbic acid supplementation was protective (Banu et al. 2011; Stanley et al. 2011, 2013). *In vivo* treatment of mice with the insecticide methoxychlor increased atresia of follicles at the antral stage of development and increased ovarian hydrogen peroxide levels and oxidative protein and DNA damage (Borgeest et al. 2002, 2004; Gupta et al. 2006a, 2006b). Culture of mouse antral follicles with methoxychlor induced atresia and inhibited growth, and supplementation with the antioxidant *N*-acetylcysteine was protective (Gupta et al. 2006a), supporting a role for oxidative stress in mediating these effects.

CrVI exposure also induced oxidative stress and initiated apoptosis in the placenta. Administration of potassium dichromate to pregnant rats decreased fetal weights; decreased placental trophoblast cell invasion; increased placental lipid peroxidation, protein oxidation, and hydrogen peroxide levels; and decreased placental mRNA and protein expression of antioxidant enzymes while inducing apoptosis (Banu et al. 2017a, 2017b). Consistent with these findings in rats, concentrations of chromium in human placentas were positively associated with markers of oxidative protein damage and apoptosis and negatively associated with expression of antioxidant genes (Banu et al. 2018).

Key Characteristic 6: Alters Immune Function

Another category of exposures that impact female reproduction are those that alter immune function. Alteration of immune function encompasses hypofunction or suppressed immunity and hyperfunction, including autoimmunity and inflammation. Reproduction in the female requires precise tuning of the immune system. Ovulation has been likened to a focal inflammatory reaction (Brännström et al. 1993; Duffy et al. 2019; Oakley et al. 2010). Implantation requires active participation from the maternal immune system (Robertson 2010). During pregnancy, the maternal immune system must "tolerate" the foreign paternal antigens present in the fetus while maintaining vigilance toward infection (Robertson 2010).

The preovulatory LH surge stimulates massive increases in macrophage and neutrophil recruitment to the preovulatory follicle, and these immune cells play an important role in ovulation, synthesizing prostaglandins and other molecules that are required for normal ovulation (Brännström et al. 1993; Duffy et al. 2019; Oakley et al. 2010). Therefore, it is not surprising that nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin synthesis, including diclofenac, fenoprofen, niflumate, tolmetin, phenylbutazone, and naproxen can inhibit ovulation (Espey et al. 1982; Gaytán et al. 2006; Tsafriri et al. 1973).

Endometriosis, a condition in which implants of endometrial tissue are found outside of the uterus, can impact reproduction by causing tubal infertility as well as pain. Endometriosis is known to occur only in humans and other primates that menstruate (Story and Kennedy 2004). The etiology is likely multifactorial, but it appears to involve immune dysregulation that creates a permissive environment in which endometrial tissue that reaches the peritoneal cavity due to retrograde menstruation can grow (Bruner-Tran et al. 2018). Exposure to TCDD and dioxin-like polychlorinated biphenyls, which, like TCDD, are ligands for the aryl hydrocarbon receptor (AhR), has been reported to increase incidence and severity of endometriosis in monkeys (Rier et al. 1993, 2001). In cocultures of human endometrial stromal and epithelial cells, TCDD decreased progesterone receptor-B expression and increased TGF-β expression, both of which likely play a role in increasing matrix metalloproteinase expression (Bruner-Tran et al. 2008). Matrix metalloproteinases are known to stimulate growth of endometrial implants (Bruner-Tran et al. 2008). TCDD also promotes the growth of endometrial implants and inhibits natural killer cell–mediated implant regression in rat and mouse models of endometriosis (Bruner-Tran et al. 2018; Cummings et al. 1999). These effects may be mediated by down-regulation of endometrial progesterone signaling and altered behavior of neutrophils at sites of ectopic endometrium, which promote inflammation and vascularization of implants (Bruner-Tran et al. 2008).

Acute or chronic inflammation in the human female reproductive tract has been identified as a cause of preterm birth (Goldenberg et al. 2008). *In utero* exposure of mice to TCDD caused preterm birth, and this effect was enhanced by inflammatory stimuli that did not cause preterm birth in control mice (Bruner-Tran and Osteen 2011).

Key Characteristic 7: Alters Cell Signal Transduction

Intracellular signaling cascades transduce signals external to cells, allowing them to respond to changes in the environment. Examples include activation of protein kinase A/cyclic AMP, protein kinase B (AKT), and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling by LH and FSH binding to their receptors (Conti et al. 2012) and membrane-localized estrogen receptors signaling via the ERK signaling pathway (Levin 2015). Exogenous chemicals may exert female reproductive toxicity by directly altering these signaling cascades without affecting reproductive hormone–receptor interactions.

A single *in vivo* dose of the potent AhR agonist TCDD disrupted mitotic signal transduction pathways in endocervical cells of cynomolgus monkeys, including decreased epidermal growth factor (EGFR) binding and casein kinase II (CKII) activity, and increased protein tyrosine kinase (PTK), cSrc tyrosine-protein kinase, and ERK2 activity; the exposed monkeys also developed squamous metaplasia in the endocervix (Enan et al. 1998). Consistent with the *in vivo* dosing results, culture of primary monkey endocervical cells with TCDD increased AhR-associated cSrc kinase activity and PTK activity and decreased CKII activity (Enan et al. 1998).

Treatment of mice *in vivo* with the antineoplastic agent cyclophosphamide depleted primordial follicles (Nguyen et al. 2018). Primordial follicle depletion induced by cyclophosphamide required the pro-apoptotic BH3-only BCL-2 family protein BCL2 binding component 3 (BBC3, formerly known as PUMA) (Nguyen et al. 2018). *In vivo* cyclophosphamide treatment resulted in phosphorylation of AKT and its downstream targets rp26 and the transcription factor forkhead box O3 (FOXO3) in oocytes of small follicles in mice (Kalich-Philosoph et al. 2013). Phosphorylation of FOXO3 in oocytes may have a dual role in primordial follicle depletion by cyclophosphamide, activating some dormant primordial follicles and inducing apoptosis of other primordial follicle oocytes by up-regulating transcription of BBC3 (Kalich-Philosoph et al. 2013; Kerr et al. 2012).

Key Characteristic 8: Alters Direct Cell–Cell Interactions

Direct cell–cell interactions play critical roles during ovarian follicle development and during the union of sperm and egg in the process of fertilization. Gap junctions between the outer somatic theca cells of maturing ovarian follicles, which possess a blood supply, and the inner somatic granulosa cells allow for the transport of nutrients, metabolites, and signaling molecules (Clark et al. 2018). Similarly, granulosa cells communicate via gap junctions with the developing oocyte (Clark et al. 2018). Gap junctional communication between granulosa cells and the oocyte is required for development of oocyte meiotic competence (Clark et al. 2018). Sperm–egg fusion collectively refers to the complete process of gamete interactions that result in sperm incorporation into the egg (Evans 2012). This process includes cell binding (i.e., attachment) that progresses to firm adhesion followed by interaction of and close apposition of the membranes. This process culminates in membrane fusion of the gamete plasma membranes and results in mixing of the lipid bilayers, formation of fusion pores and establishment of cytoplasmic continuity of the gametes. Membrane order and organization are critical for gamete membrane functionality. The initial step of membrane recognition (attachment) and contact is achieved through protein–protein- or protein–carbohydrate-mediated binding of the membranes, followed by membrane adhesion by fusion proteins (Stein et al. 2004).

Short-term BaP exposure of female mice during the neonatal period when the ovary contains almost exclusively immature primordial follicles led to impaired ability of oocytes ovulated 6 weeks after the exposure to fuse with sperm (Sobinoff et al. 2012). These results are consistent with what is seen among *in vitro* fertilization patients who smoke (Sobinoff et al. 2012).

The PAH 7,12-dimethyl-benz[*a*]anthracene (DMBA)-decreased mRNA and protein expression of the gap junction proteins connexin 43 and connexin 37 in cultured neonatal mouse ovaries coincident with follicle apoptosis (Ganesan and Keating 2014) and in ovaries of mice treated *in vivo* (Ganesan et al. 2015). These results suggest that disruption of cell–cell communication within ovarian follicles may play a role in the induction of follicle death by DMBA.

Key Characteristic 9: Alters Survival, Proliferation, Cell Death, or Metabolic Pathways

Alteration of survival, proliferation, cell death, or metabolic pathways in female reproductive tissues can result in female reproductive toxicity. Programmed cell death by apoptosis is induced in female reproductive tissues by several known female reproductive toxicants, as is cell cycle arrest resulting in decreased proliferation and growth. Disruption of metabolism of endogenous and exogenous small molecules can also cause female reproductive toxicity. Dysregulation of autophagy, a process by which intracellular organelles are broken down and recycled, has also been associated with female reproductive toxicity. Examples of each of these are described below.

Cell cycle arrest is involved in the ovarian follicle toxicity of the phthalate plasticizer dibutyl phthalate (DBP). DBP decreases growth of cultured mouse antral follicles, the last stage of ovarian follicle development before the preovulatory stage (Craig et al. 2013; Rasmussen et al. 2017). DBP causes arrest of follicle cells in the G1 phase of the cell cycle by up-regulating expression of cyclin-dependent kinase (CDK) inhibitors and down-regulating cyclins, resulting in decreased follicle growth (Craig et al. 2013; Rasmussen et al. 2017).

Induction of apoptotic cell death has been implicated in the destruction of ovarian follicles and germ cells in the developing ovaries by PAHs. The PAH BaP induces caspase-dependent apoptotic cell death of germ cells in cultured embryonic mouse ovaries (Lim et al. 2016; Lim and Luderer 2018). The PAH DMBA induces apoptosis in cultured fetal and neonatal mouse ovaries (Matikainen et al. 2001, 2002). Collectively, these studies demonstrated that germ cell death induced by PAHs in developing mouse ovaries is dependent on the pro-apoptotic BCL2-family protein BAX and on caspase activation.

Preconception and early gestational exposure to a mixture of BaP and DMBA decreased apoptosis in the placenta by up-regulating anti-apoptotic protein expression and down-regulating pro-apoptotic protein expression in an AhR-dependent manner and by increasing apoptosis in the yolk sac and the allantoic mesenchyme in an AhR-independent manner (Detmar et al. 2008). These changes were associated with alterations of the placental vasculature and intrauterine growth restriction.

Alterations of oocyte metabolism due to excessive exposure to some nutrients or dietary supplements can decrease oocyte quality. Elevated levels of nonesterified fatty acids during *in vitro* maturation of bovine oocytes resulted in aberrant early embryonic development (Sutton-McDowall et al. 2016; Van Hoeck et al. 2011). Dehydroepiandrosterone (DHEA) inhibition of the pentose phosphate pathway altered lipid metabolism in mouse oocytes (Jimenez et al. 2013).

Exposure to cigarette smoke depleted ovarian follicles in adult mice (Tuttle et al. 2009). Increased autophagy appears to be involved in the mechanism of follicle depletion because ovaries of exposed mice displayed increased expression of genes involved in autophagy and increased numbers of autophagosomes in the granulosa cells of follicles (Gannon et al. 2012; Furlong et al. 2015).

Key Characteristic 10: Alters Microtubules and Associated Structures

Microtubules have at least two distinct critical activities in female reproduction that are susceptible to disruption that leads to impaired fertility and other adverse outcomes. Microtubule spindle formation in oocytes undergoing meiosis is critical for proper chromosomal segregation. Errors in spindle formation have been shown to increase the risk of aneuploidy, polyploidy, and failures of oocyte maturation and embryogenesis (Vogt et al. 2008). Microtubules and related structures in cilia are also necessary for the propulsion of gametes and embryos along the oviduct (Lyons et al. 2006). Failure of ciliary propulsion is a factor in infertility and ectopic pregnancy (Ezzati et al. 2014).

Several drugs—including colchicine, vinca alkaloids, and taxanes—are known to target microtubules (Florian and Mitchison 2016). All of these agents bind to tubulin, although their subsequent effects differ significantly. Cigarette smoke and diesel exhaust have both been shown to alter meiotic spindles in mice, resulting in errors of spindle shape and chromosome congression (Jennings et al. 2011; Udagawa et al. 2018). Multiple studies in bovine (Campen et al. 2018; Ferris et al. 2015) and mouse (Can et al. 2005) oocytes reported that BPA and bisphenol S (BPS) are associated with chromosomal misalignment as well as delayed and altered spindle formation. Agents that produce ROS—including peroxynitrite, cyclophosphamide, acrolein, and D-galactose—have shown effects in several *in vitro* assays that identify derangement of the meiotic chromosomal scaffold (Jeelani et al. 2017; Khan et al. 2016; Thakur et al. 2017).

Ciliary transport of the oocyte–cumulus complex in the hamster oviduct was significantly slowed by cigarette smoke exposure (Knoll and Talbot 1998), suggesting an etiology for the observed association between smoking and reduced fertility (Lyons et al. 2006). Assays have been developed to evaluate effects on ciliary beat frequency, oocyte pick-up rate, and smooth muscle contraction in the oviduct. Cigarette smoke inhibited both ciliary beat frequency and oviductal smooth muscle contraction and increased adhesion of the oocyte complex to the cilia, resulting in reduced

oocyte pick-up and movement through the oviduct in hamsters (Gieseke and Talbot 2005; Talbot et al. 1999).

Application of the Key Characteristics Approach to Three Known Female Reproductive Toxicants

To illustrate the potential utility of the proposed key characteristics of female reproductive toxicants, we provide three examples of toxicants for which abundant *in vivo* toxicological and/or epidemiological data demonstrate female reproductive toxicity. For cyclophosphamide and DES, both human and animal studies demonstrate female reproductive toxicity. In contrast, for TCDD, the epidemiological database is mixed.

Cyclophosphamide is listed on the Proposition 65 list of known female reproductive toxicants (OEHHA 2018). Numerous studies show that women or girls treated with cyclophosphamide develop temporary or permanent amenorrhea and often have earlier onset of menopause due to depletion of the primordial follicle pool (Howell and Shalet 1998; Kumar et al. 1972; Morgan et al. 2012). Studies in mice and rats clearly show that treatment with cyclophosphamide or its metabolite phosphoramide mustard (PM) destroys primordial and small primary follicles (Plowchalk and Mattison 1991, 1992; Shiromizu et al. 1984). Cyclophosphamide also induces apoptosis in granulosa cells of secondary and antral follicles in rats, resulting in destruction of those follicles (Davis and Heindel 1998; Jarrell et al. 1987; Lopez and Luderer 2004). Mechanistic studies show that cyclophosphamide displays at least 5 of the 10 proposed key characteristics (KCs) of female reproductive toxicants. Cyclophosphamide is metabolically activated to PM, which possesses anticancer activity and is also thought to be the ultimate ovotoxicant (Plowchalk and Mattison 1991). Culture of neonatal rat and mouse ovaries with PM induced dsDNA breaks in oocytes of primordial follicles (Petrillo et al. 2011) (KC2). Cyclophosphamide metabolism results in ROS generation, and culture of a human granulosa cell line with 4-hydroperoxycyclophosphamide, a precursor of cyclophosphamide, caused oxidative DNA damage (Tsai-Turton et al. 2007) (KC5). Cyclophosphamide treatment in mice resulted in ovarian phosphorylation of AKT and its downstream target FOXO3 (Kalich-Philosoph et al. 2013) (KC7). Cyclophosphamide or its metabolites induced apoptosis in a cultured granulosa cell line and in granulosa cells of ovarian follicles with *in vivo* dosing (Devine et al. 2012; Lopez and Luderer 2004; Tsai-Turton et al. 2007) (KC9). Mice homozygous, but not heterozygous, for the genetic deletion of the pro-apoptotic BH3-only BCL-2 family member BBC3 (previously called PUMA) were completely resistant to primordial follicle destruction, consistent with activation of the mitochondrial apoptotic pathway in primordial follicles by cyclophosphamide (Nguyen et al. 2018) (KC9). Cyclophosphamide and its metabolite acrolein interfered with meiosis by causing microtubule disruption (Jeelani et al. 2017) (KC10).

DES is a synthetic estrogen formerly prescribed to pregnant women based on a hypothesis that some miscarriage was caused by estrogen deficiency. Although this was disproven in the 1950s, clinical use continued until the 1970s (CDC 2012). In 1971, a clinical report of eight cases of a rare cancer in young women (adenocarcinoma of the vagina) noted that their mothers had each been treated with DES when they were pregnant (Herbst et al. 1971). These clinical cases led to further epidemiological, pharmacologic, and basic studies that demonstrated malformations of the oviduct, uterus, cervix, vagina, and mammary glands as well as tumors or proliferative lesions in these organs in developmentally exposed women and mice (Newbold 2012). Mechanistic studies associate DES with at least three of the key characteristics. ERα is required for the induction of uterine abnormalities and tumors by DES in animals (Newbold et al. 2006) (KC1). DES altered expression of the transcription factor *Hoxa10* in cultured human endometrial cells and in the uteri of developmentally exposed mice (Bromer et al. 2009) (KC7). Developmental DES exposure altered epigenetic markers in the uterus, including differential methylation of *Hoxa10* (Bromer et al. 2009) and histone acetylation and methylation (Jefferson et al. 2013) (KC2). The ability to impact epigenetics likely explains the multigenerational effects seen in humans.

In vivo toxicology studies demonstrate that TCDD is a female reproductive toxicant when administered during prenatal development. A single oral dose of TCDD to pregnant rats, hamsters, or mice adversely affects development and function of the reproductive system of the prenatally exposed female offspring. Effects include abnormalities of the external genitalia and vagina, delayed onset of puberty, high incidence of constant estrus, uterine adenomyosis, and decreased fertility of the F1 female offspring (Bruner-Tran and Osteen 2011; Bruner-Tran et al. 2016; Flaws et al. 1997; Gray and Ostby 1995; Wolf et al. 1999). The prevalence of these effects varies with the species and the gestational stage at which exposure occurs. Some of these effects are transgenerationally transmitted to females of the F3 and F4 generations (Bruner-Tran and Osteen 2011; Bruner-Tran et al. 2016). Although mice and rats do not develop endometriosis, the reproductive phenotype of decreased fertility and adenomyosis observed in rodents after prenatal exposure is similar to the reproductive phenotype of women with endometriosis, and TCDD promotes growth of human endometrial tissue in a mouse model of endometriosis (Bruner-Tran et al. 2010). In the Seveso Women's Health Study, serum TCDD was associated with increased time to pregnancy and infertility (Eskenazi et al. 2010), longer menstrual cycle length (Warner et al. 2007), and earlier age at natural menopause (Eskenazi et al. 2005). In contrast to the data in animals, epidemiological studies in humans have not consistently observed associations between prevalence of endometriosis and biomarkers of exposure to TCDD (Eskenazi et al. 2002; Foster 2008).

Mechanistic studies show that TCDD displays at least 5 of the 10 proposed key characteristics of female reproductive toxicants. The effects of TCDD on these mechanistic end points related to female reproduction, as well as its myriad other toxicological effects are known or believed to be a consequence of AhR activation (White and Birnbaum 2009). TCDD alters estrogen receptor signaling (Madak-Erdogan and Katzenellenbogen 2012) (KC1). Developmental TCDD exposure decreased uterine progesterone receptor protein and mRNA expression and uterine responses to progesterone in F1 as well as F4 female offspring (Bruner-Tran and Osteen 2011) (KC1). Hypermethylation of the progesterone receptor promoter occurred in uteri of F1 and F3 female offspring of mothers exposed to TCDD during pregnancy (Bruner-Tran et al. 2017) (KC3). TCDD exposure altered endometrial immune function to promote the growth of ectopic endometrial implants in mice (Bruner-Tran et al. 2008, 2010, 2018) (KC6). TCDD disrupted mitotic signal transduction pathways in endocervical cells of the rhesus monkey, including EGFR, PTK, cSrc tyrosine-protein kinase, casein kinase II, and Erk2 (Enan et al. 1998). TCDD also disrupted EGFR, PTK, and protein kinase A activities in cultured luteinized human granulosa cells (Enan et al. 1996) (KC7). TCDD exposure decreased protein levels of the cell cycle inhibitor p21 and cyclin-dependent kinase (Cdk) Cdc2/Cdk1 and increased protein levels of Cdk4 in endocervical cells of rhesus monkeys (Enan et al. 1998) (KC9). Taken together, the *in vivo* toxicological and mechanistic evidence support the conclusion that TCDD is a female reproductive toxicant.

Discussion

Human health hazard assessment of chemicals for female reproductive toxicity has been hampered by the lack of *in vivo* toxicology or epidemiological studies for the vast majority of chemicals in commerce and a lack of a uniform approach to systematically

search, organize, integrate, and evaluate mechanistic evidence. The proposed key characteristics of female reproductive toxicants can provide an approach to systematically searching and organizing the abundant mechanistic literature to support evaluation of chemicals for female reproductive toxicity.

Although they were developed independently, the key characteristics for female reproductive function proposed herein overlap significantly with those previously described for carcinogens (Smith et al. 2016). Several of the key characteristics are essentially identical (e.g., KC3, "induces epigenetic alterations," and KC5, "induces oxidative stress"). Our KC2, "chemical or metabolite is genotoxic" encompasses the carcinogen key characteristics "is metabolically activated" and "is genotoxic." Our KC6, "alters immune function" encompasses key characteristics "induces chronic inflammation" and "is immunosuppressive" for carcinogens. Our KC1, "alters hormone receptor signaling; alters reproductive hormone production, secretion, or metabolism" and KC7, "alters cell signal transduction" relate to key characteristic "modulates receptor-mediated effects" for the carcinogens. It is important to recognize, however, that the key characteristics must occur in cells relevant to the female reproductive system to be considered potential evidence that a chemical causes female reproductive toxicity. For example, hormone receptor signaling would be specific to female reproductive hormone signaling within cells of the female reproductive system, and genotoxicity should manifest in cells or tissues of the female reproductive system. This specificity to organ system is different from that described in the key characteristics of carcinogens.

Certain key characteristics of carcinogens (Smith et al. 2016) are considered less relevant to female reproductive toxicity (e.g., "alters DNA repair or causes genomic instability"; "causes immortalization"). Although these effects may damage the female reproductive system, or lead to cancers in reproductive organs, these were not thought to significantly affect fertility and reproductive function. In contrast, we identify several characteristics that are important to female reproduction and not among the key characteristics of carcinogens, including disruption of microtubules, cell–cell interactions, and mitochondrial dysfunction.

Future efforts should focus on evaluating the proposed 10 key characteristics against other known female reproductive toxicants. For example, the California Proposition 65 list contains 67 chemicals listed as known female reproductive toxicants (OEHHA 2018), including one of the three examples discussed above (cyclophosphamide). An in-depth examination of those chemicals for the key characteristics would be illustrative, although it would undoubtedly identify many data gaps. As with the key characteristics approach for carcinogens, the implementation of the key characteristics approach for female reproductive toxicants could be realized by several approaches. Search terms relevant to the key characteristics could be developed for systematic interrogation of the literature using the Health Assessment Workspace Collaborative (HAWC 2014). A comprehensive assessment of assays and cell models for relevant key characteristics end points would be a useful resource in designing experiments and novel assays to address data gaps.

It is possible that the overlap between our key characteristics and those previously developed for carcinogens can be explained by cognitive biases, such as anchoring (Blumenthal-Barby and Krieger 2015). In our opinion, however, it is more likely due to underlying biological mechanisms that can cause more than one apical end point, depending on timing, dose, and other factors. If a common set of key characteristics underlies multiple different end points, then identifying chemicals that exhibit these characteristics becomes critical for predicting hazard.

Identification of chemicals that exhibit one or more of the key characteristics described here could be helpful in many decision contexts. Such chemicals might be prioritized for additional evaluation and testing to ensure that their potential reproductive toxicity is fully understood and addressed. Companies developing chemicals for various functional uses may choose to screen for these characteristics and avoid, where possible, selecting chemicals that screen positive. Chemical classes could be identified, defined, and regulated when multiple structurally similar chemicals display the same or similar key characteristics. They could be used in the hazard identification portion of risk assessments to supplement incomplete or inconsistent epidemiological and animal toxicology databases. Key characteristics could also be used by risk assessors when epidemiology or animal toxicology studies are not available to prioritize chemicals that may pose a hazard to female reproduction. Using the key characteristics as a replacement for epidemiological or animal toxicology studies seems unlikely in the near future, but as application and uses of the key characteristics are refined, this could be a future goal. Ultimately, the use of key characteristics avoids the narrow requirement to understand the precise adverse outcome pathway (AOP) or critical mode of action (MOA) for each chemical (Guyton et al. 2018), and it will also help reduce the reliance on animal testing. Instead, the key characteristics will capture a broad array of traits that are associated with chemical hazards and should allow chemicals that exhibit these characteristics to be flagged and prioritized for further action. Such an approach has the potential to prevent female reproductive toxicity and thereby possibly reduce the high rates of infertility, spontaneous abortion, premature birth, low birth weight, and other adverse outcomes that affect women and families worldwide.

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