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Male reprotoxicity and endocrine disruption

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Abstract

Mammalian reproductive tract development is a tightly regulated process that can be disrupted following exposure to drugs, toxicants, endocrine disrupting chemicals or other compounds via alterations to gene and protein expression or epigenetic regulation. Indeed, the impacts of developmental exposure to certain toxicants may not be fully realized until puberty or adulthood when the reproductive tract becomes sexually mature and altered functionality is manifested. Exposures that occur later in life, once development is complete, can also disrupt the intricate hormonal and paracrine interactions responsible for adult functions, such as spermatogenesis. In this chapter, the biology and toxicology of the male reproductive tract is explored, proceeding through the various life stages including *in utero* development, puberty, adulthood and senescence. Special attention is given to the discussion of endocrine disrupting chemicals, chemical mixtures, low dose effects, transgenerational effects, and potential exposure-related causes of male reproductive tract cancers.

Introduction

The concept of developmental origins of disease is certainly not new, yet only recently has the idea been supported by epidemiological research. In the mid-1990s, Dr. David Barker observed a correlation between low birth weight and an increased incidence of diseases later in life such as stroke, hypertension, coronary artery disease, and diabetes [1, 2]. Barker suggested that human fetuses permanently adapt their developing cellular physiology and metabolism to survive in conditions of inadequate maternal nutrition in preparation for a presumptively similar nutrient-poor postnatal environment. The result is an enhanced susceptibility to certain diseases in postnatal life due to an altered cellular metabolism which cannot adjust to conditions of adequate nutrition. One such example of this is the famine of the Dutch Hunger Winter from late 1944 to early 1945, during which thousands of people in the Netherlands experienced severely reduced caloric intake due to a German embargo on food imports and an early winter freeze that hindered canal transport. During this time, thousands of pregnant women were exposed to famine conditions. The children of this cohort have shown higher incidences of hyperlipidemia [3], altered DNA methylation patterns [4], early onset coronary artery disease [5], altered glucose tolerance and insulin secretion [6] and obesity [7] compared to unexposed controls. These findings lend credence to Barker's hypotheses and demonstrate that patterns set during critical programming windows of development can adversely influence health later in life.

While epidemiological studies have underscored the significance of the developmental origins of adult disease, human health risk assessment for environmental toxicant exposure is grounded primarily in animal studies. Many specific study designs are aimed at characterizing the potential for effects on reproduction and fertility, and are performed in

conjunction with developmental toxicity assessments. Although each regulatory agency has different testing requirements (reviewed in [8]), multigenerational tests represent the cornerstone of developmental and reproductive toxicity testing. This type of testing ensures detection of effects caused by pre- and postnatal exposures, effects with delayed onset, and effects that are transgenerational and could be missed with single generation testing [9]. A well-designed multigenerational test should examine three dose levels of the chemical in question that together produce a gradation of toxic effects; some reprotoxicity should be seen with the highest dose, while the lowest dose should exhibit minimal to no effects. This information can then be used to establish a “no observed adverse effect level” (NOAEL) that helps extrapolate data to determine safe exposure levels for humans.

Rats are the most commonly used species in multigenerational reproduction studies. Generally, healthy parental (F0) animals (both male and female) between 5 and 9 weeks of age are administered the chemical orally (through diet, drinking water, or gavage) on a daily basis for ten weeks prior to and during mating; exposure in the ten weeks prior to mating allows for the manifestation of effects on gametogenesis. Administration continues daily during gestation and through the weaning of the F1 offspring. The chemical continues to be administered to selected F1 generation male and female offspring into adulthood and through mating and production of an F2 generation until the F2 offspring are weaned. Using this study design, both F1 and F2 offspring are continuously exposed *in utero* from conception until birth and during the pre-weaning period; in this way, effects from exposures throughout development can be detected, including those that occur in the peripubertal and young adult phases [10].

There are several different reproductive endpoints that can be evaluated as part of a multigenerational study. While all animals are monitored for body weight and mating and fertility indices, there are endpoints specifically relevant to the male animal, including gross examination and morphology of the reproductive organs, observation of developmental effects, and measurement of sperm effects. Weight and histopathological analyses of the testes, epididymides, and accessory sex glands, including the prostate and seminal vesicles, are conducted; because these accessory sex glands are androgen-dependent, they may reflect changes in the animal's endocrine status or testicular function. Normal physical development may also be effected by exposure, including testicular descent, ano-genital distance, and structure of the external genitalia. Finally, evaluations of sperm number, morphology, and motility can suggest exposure-related damage; sperm number data are derived from counts of homogenization-resistant spermatid heads in the testis and epididymis [11]. Histopathological evaluation can be a sensitive indicator of damage; some signs of adverse effects include changes in seminiferous tubule diameter, spermatid head retention, vacuolization, sloughing, and germ cell apoptosis, among other endpoints.

While this study design is ideal for identifying potential adverse outcomes of a specific chemical or group of chemicals, it does not look at the mechanism of action for the chemicals in question. The National Research Council of the National Academies [12] has recently published a report that seeks to incorporate testing strategies that will move towards understanding the mode of action of chemicals. In “Toxicity in the 21st Century: A Vision and a Strategy,” they outline a systems biology approach driven by high-throughput testing strategies that utilize modern tools and toxicogenomic testing and that will provide important mechanistic data [13]. While finding *in vitro/ex vivo* cell models that mimic the complex interactions of reproductive and developmental cell types presents a potential challenge to the high-throughput testing strategies envisioned in the report, there are some promising models in development that are able to assess perturbations of the developing fetus and reproductive organs. As described by Chapin and Steadman [14], stem cells are able to differentiate into different cell types, and therefore could play an important role in

developing new assays to predict developmental and reproductive toxicity in vitro. Furthermore, stem cells can be evaluated for toxic responses in their undifferentiated state or during the process of differentiation. In addition to the development of in vitro models for reprotoxicity testing, future safety assessments will likely incorporate epigenetic evaluations as the relationship between epigenetic changes and health outcomes is better understood, particularly as more information becomes available on the potential transgenerational effects of toxicants [13].

We now look at the biology and toxicology of the male reproductive tract, starting with early development, followed by puberty, adulthood, and senescence.

Biology and toxicology of the developing male reproductive tract

The early fetal gonad

Prior to the onset of sexual differentiation, the human gonad is composed of proliferating coelomic and mesenchymal cells, primordial germ cells, and endothelial cells (gestational weeks 7-8) [15]. This early fetal gonad has the precursors of both male (Wolffian) and female (Mullerian) reproductive tracts and maintains the ability to differentiate into either sex [16]. Development of a male reproductive system requires two crucial events: formation of the testis, and differentiation and maintenance of the Wolffian duct through adulthood [17]. This process is induced by Mullerian Inhibiting Substance (MIS, also known as Anti-Mullerian Hormone, AMH), which is produced by the pre-Sertoli cells of the early testis. In the presence of MIS and testosterone, the Mullerian duct regresses and the Wolffian duct differentiates into the vas deferens and epididymis [18]; in their absence, the Wolffian duct regresses and the Mullerian duct develops into the oviduct, uterus, and upper vagina, resulting in a female phenotype [19].

Genetic regulation of male sexual differentiation

Sexual differentiation is primarily controlled by the SRY sex-determining gene on the short arm of the Y chromosome. When present, development of the male pathway is initiated through activation of molecular and cellular cascades that lead to testis formation and regression of the Mullerian duct [17, 20, 21]. The SRY gene likely acts as a genetic switch, encoding for transcription factors that allow cells of the early gonad to further develop; organizational changes of the cells are seen around 36 hours after the initial expression of SRY [22]. Sertoli cells are the first to differentiate within the gonad; there is strong evidence that it is the pre-Sertoli cell that expresses SRY [23], initiating differentiation of gonad to testis [24]. Changes in the early testis include seminiferous cord formation through the organization of pre-Sertoli cells around germ cells, increase in size of the XY gonads compared to the XX gonads, and development of a characteristic blood vessel on the periphery of the XY gonad [18]. Formation of the Sertoli cell lineage is strictly controlled by the expression of SRY-activated genes [23].

There are three major genes activated by SRY that are critical to male sexual differentiation – SOX9 (SRY-box containing gene 9), Fgf9 (fibroblast growth factor-9), and Sf1 (steroidogenic factor 1) [25, 26]. Transcription in the promoter region of SRY binding sites is activated when WT1 encodes a transcription factor that binds to the SRY protein [27]. First, null mutations in both WT1 and Sf1 can alter the development of the gonad and may be involved in initiating signals that control expansion of the genital ridge [28, 29]. Sf1 may also activate the transcription of hormones that masculinize both Sertoli and Leydig cells [21]. Second, because the transcription factor SOX9 can induce full male differentiation in the absence of SRY, it likely plays an important role in Sertoli cell formation [21]. At the time of sex determination, SOX9 moves into the nucleus and binds to the promoter site on the gene that codes for anti-Mullerian hormone, providing a pathway to male phenotype

development [21]. Finally, the *Fgf9* gene is responsible for the proliferation and differentiation of Sertoli cell precursors and aids in testis cord formation by initiating the migration of mesonephric cells into the gonad [21]. Following activation of these genetic regulators of sex differentiation, early cell types become apparent in the developing gonad.

Precursors of supportive reproductive cell types of both sexes, the Sertoli cell of the testis and the granulosa cell of the ovary, are believed to be present in the early gonad [18]. It is also likely that in the early gonad there is a single precursor of steroidogenic cells of both the male (Leydig cells) and female (theca cells) [30]. Unlike Sertoli cells, which require the expression of *SRY* for development, fetal Leydig cells do not express *SRY* or *SOX9*, indicating that they differentiate as a result of the paracrine action of Sertoli cells [17], which is driven by two main signaling molecules: desert hedgehog (DHH) and platelet-derived growth factors (PDGFs) [31]. Sertoli cells, without further involvement of *SRY*, direct the development of other cell types in the early testis, including peritubular myoid cells, endothelial cells and uncharacterized cells within the interstitial space [18, 23]. These additional cell types aid in seminiferous cord formation by surrounding Sertoli cells with peritubular myoid cells and help form the blood-testis-barrier (BTB) by creating a basal lamina that, in conjunction with Sertoli cells, encloses germ cells within the testis cords [18].

Hormonal regulation of male sexual differentiation

Hormonal control of male sexual differentiation begins with the formation of the Sertoli cells. Sertoli cells secrete anti-Müllerian hormone, inducing the regression of the Müllerian duct and allowing Leydig cells to initiate further differentiation of the Wolffian duct and accessory glands through secretion of androgens and insulin-like factor 3 (*Ins13*). Production of these hormones is partially regulated by the presence of human chorionic gonadotropin (hCG) and luteinizing hormone (LH) [17, 32]. The Wolffian duct is further stabilized through secretion of testosterone by the Leydig cell, allowing for differentiation of the vas deferens, epididymis, seminal vesicles, and ejaculatory duct from the Wolffian duct; once developed, these become dependent on androgens secreted by Leydig cells [17]. Differentiation of the urethra, prostate gland, penis and scrotum, and tissue between the anus and genital orifice is dependent on the conversion of testosterone to dihydrotestosterone (DHT) by 5- α reductase [16, 33]. *Ins13* helps induce testicular descent towards the end of sexual development [16, 17].

Testicular descent from the intra-abdominal site to an extracorporeal site (scrotum) allows the mature testis to maintain normal spermatogenesis later in life [32]. The cranial suspensory ridge (CSL) anchors the upper poles of the regressing mesonephrous and developing testis [34]. The genito-inguinal ligament (gubernaculum) connects the lower poles of the testis and the epididymis with the future inguinal canal [15]. Testicular descent occurs in two major phases: first, hormonal regulation of complex anatomical rearrangements begins around gestational week 25 in humans, stimulating transabdominal descent of the testes [15]. This phase is primarily regulated by *Ins13*, which initiates thickening of the gubernaculum by binding to its receptor, LGR8 (leucine-rich repeat-containing G protein coupled receptor 8) [34, 35]. Thickening of the gubernaculum anchors the testis in the inguinal region until the second phase of testicular descent, the inguinoscrotal phase, which begins approximately ten weeks after the completion of transabdominal descent [15]. While androgen expression is not necessary for transabdominal descent, it is required in the second phase to induce regression of the CSL [36]. When these two steps are complete, the testes are pulled into the scrotum and further anchored during gestational weeks 35-40, completing the process of testicular descent [15, 32].

Congenital abnormalities

Both genetic (SRY-Sox9) and hormonal (androgens) regulation is necessary in mammals for the development and organization of the male reproductive tract. If this delicate process is disturbed, either by genetic abnormalities or chemical exposure, reproductive tract disorders can result, including cryptorchidism, hypospadias, impaired spermatogenesis and testicular cancer [37]. The increased incidence of these abnormalities has led to a theory of testicular dysgenesis syndrome (TDS), which is discussed in further detail later in this chapter [37]. Cryptorchidism and hypospadias are fairly frequent congenital abnormalities that have been studied in detail. They may occur as isolated disorders, or may be associated with other congenital syndromes, such as those described below.

Cryptorchidism is the failure of one or both testes to descend properly into the scrotal sac. The most common congenital birth defect in male children, it occurs at a rate of 2-4% in full-term births; about 50% of cryptorchid testes spontaneously descend in the first few months after birth [38]. The etiology is largely unknown; however, it is likely that both genetic and environmental factors (acting as endocrine disruptors) contribute to cryptorchid outcomes [39]. Several genetic causes and polymorphisms associated with cryptorchidism have been identified in human patients, including mutations in *Ins13* and its receptor, *RXFP2*, as well as in the *AR* gene (which is associated with Androgen Insensitivity Syndrome) [38]. While there is a strong association of cryptorchidism with infertility and testicular cancer, the mechanisms underlying this association are unknown [38].

Hypospadias results from abnormal penile and urethral development, commonly arising from a defect in the midline fusion of the male urethra, leading to a misplaced urethral meatus. It is the most frequent genital malformation in male newborns [40]. As with cryptorchidism, the increased incidence of hypospadias has largely been attributed to environmental factors [37]. Despite this, there are several genes involved in penile development that may influence a hypospadias outcome, including *HOXA13*, *FGF10*, and those involved with androgen synthesis and action (LH receptor gene, 17-hydroxysteroid-reductase, and *AR*) [40].

In addition, several other congenital disorders have been studied with an eye to the etiology of disease states: congenital adrenal hyperplasia, androgen insensitivity syndrome, Klinefelter syndrome, and hypothalamic hypogonadism may also result from chromosomal or genetic abnormalities or may be induced by chemical exposure during fetal development.

Congenital adrenal hyperplasia (CAH) refers to a group of autosomal recessive disorders caused by a reduced or complete lack of activity of one of the steroidogenic enzymes involved in cortisol biosynthesis in the adrenal cortex [41]. The specific enzymatic defect results in different biochemical and clinical phenotypes whose severity depends on the location of normal enzymatic action in the adrenal steroidogenic pathway and the degree of enzyme deficiency. In about 90-95% of cases, CAH is caused by a deficiency in the 21-hydroxylase enzyme, resulting in decreased levels of aldosterone or cortisol. Lacking the negative feedback normally present, the pituitary secretes hormones that stimulate glucocorticoid and/or mineralocorticoid production. Increased glucocorticoid and mineralocorticoid precursors accumulate and are shunted into the androgen synthesis pathway, leading to hyperandrogenism. This excessive androgen production influences the development of external genitalia in both sexes [42]. Hyperandrogenic effects that present later in life include precocious pseudopuberty (in both sexes) and infertility resulting from excess androgen and estrogen production [41].

Androgen Insensitivity Syndrome (AIS) is characterized by a resistance to androgens and can manifest in a variety of ways depending on the degree of androgen receptor (*AR*)

disruption. Generally, males with AIS present with a female phenotype due to one or various AR mutations. In the extreme case of complete AIS, a lack of activity at the androgen receptor prevents testicular descent and further development of the external male genitalia; however, normal production of MIS during fetal development inhibits uterus and fallopian tube development, resulting in an XY male genotype with a female phenotype [43].

Hypothalamic hypogonadism, also known as Kallmann syndrome, is caused by improper migration of GnRH-secreting neurons to the hypothalamus during fetal development. Because GnRH stimulates FSH and LH hormone production in the pituitary, which stimulates the gonads, a GnRH deficiency leads to decreased or absent function of the testes. Symptoms can include the absence of secondary sexual characteristics, delayed puberty, underdeveloped testes, and/or micropallus. Several genetic mutations known to cause Kallmann syndrome are reviewed by Fechner et al. [44].

Klinefelter syndrome refers to a group of chromosomal disorders characterized by one or more additional X chromosomes added to a normal male karyotype. The classic form of Klinefelter is 47,XXY; this occurs at a rate of 1:500, and represents the most common chromosomal disorder among men [45, 46]. Because the X chromosome carries genes responsible for normal testis function, brain growth and development, and many others, X chromosome polysomy carries significant consequences [45, 47]. The most common signs of Klinefelter syndrome are spermatogenic and steroidogenic dysfunction, and as a result the disorder often remains undiagnosed until later in life. Other symptoms include small, firm testes with slightly decreased testicular volume, low serum testosterone resulting in erectile dysfunction and poor libido, and primary testicular failure resulting in infertility (azoospermia or severe oligospermia) [45].

Permeability of placental barrier

It is well established that *in utero* exposure to cigarette smoke, drugs, and alcohol can adversely affect fetal development. However, it was originally believed that the fetus was uniquely protected from exposure to chemicals due to the nature of the placental barrier. Charged with the tasks of delivering nutrients, removing wastes, modulating endocrine activity and regulating immune surveillance, the placental barrier is imperfect by design and fails to keep all harmful compounds in the maternal circulation from reaching the fetus. Moreover, the developing fetus does not have the protective factors of an adult – it has immature detoxification, metabolic, excretory, and immune functions that increase its susceptibility to toxic insult. The idea that the placenta formed a perfect barrier around the fetus was challenged in the 1950s and 1960s when chemical-induced birth defects demonstrated the fragility of the unborn fetus to insults by maternal exposures. In the 1950s, *in utero* thalidomide exposure led to drug-induced phocomelia (limb-shortening); by 1961 this sedative drug was withdrawn from the market. Similarly, consumption of methylmercury-contaminated shellfish by pregnant mothers has led to neurological dysfunction in their children, most prominently in Minimata Bay, Japan, where a population was exposed from 1932 to 1968. The symptoms that together are known as “Minimata disease” were first recognized in that area in 1956.

Examples of insults from environmental exposure are not limited to the human population. The 1962 publication of Rachel Carson's seminal work, “Silent Spring,” suggested the overuse of the pesticide dichlorodiphenyltrichloroethane (DDT) affected non-target species such as birds, impacting their ability to reproduce and resulting in a decline in wildlife populations. Legislation over the past 50 years has been aimed at minimizing environmental pollution and mandating stronger regulations for drug safety. The legacy of these pivotal events has been instrumental in shifting public attitudes towards an increased awareness of the environment, chemical exposures, and the vulnerability of the developing fetus.

Endocrine disrupting chemicals

The ability of chemicals to affect reproduction and development has garnered significant attention. The term “endocrine disrupting chemical” (EDC) was coined in 1993, when it was suggested that certain chemicals could mimic endogenous hormones, effectively disrupting hormone homeostasis. Further, it was suggested that exposure to these chemicals was responsible for the increased incidences of lowered fertility, intersexed animals, and disrupted sexual development observed in certain wildlife species [48]. Implicated compounds included a wide variety of chemical pesticides (DDT, methoxychlor, tributyl tin, and others), PCBs (polychlorinated biphenyls), dioxin, and many other types of anthropogenic chemicals. It is clear that toxicant-induced modulation of the endocrine system can have myriad downstream effects on reproduction and development. Side effects from exposure are not necessarily negative, however; from a pharmacological perspective, modulation of hormonal signaling could have potentially therapeutic effects. Receptor antagonists such as Tamoxifen and Flutamide are routinely used in the treatment of breast and prostate cancer, respectively. Other drugs, such as ethinyl estradiol (EE), a stable derivative of estradiol used in birth control pills, alter hormonal signaling through estrogen receptor binding and inhibit the release of gonadotropins, effectively preventing ovulation. Birth control pills are the most widely used form of contraception in the United States [49]. Not surprisingly, the widespread use of oral contraceptives has resulted in the detection of EE in treated waste water and has been linked to endocrine disruptive effects in fish, such as altered hormone levels, feminized or intersex species, and increased production of the estrogen-dependent protein vitellogenin [50, 51].

Epidemiological analysis of EDCs

Despite this evidence, the vast majority of chemicals in our environment have yet to be adequately assessed for their potential as endocrine disruptors. The 1996 passage of the US Food Quality Protection Act calls on the Environmental Protection Agency (EPA) to develop and implement an Endocrine Disruptor Screening Program (EDSP) to test chemicals for their ability to affect the endocrine systems of humans and other wildlife. Similarly, the 2007 legislation of the European Union's REACH (Registration, Evaluation, Authorization and Restriction of Chemical Substances) will eventually require manufacturers to identify hazards associated with their products based on the toxicity of the individual constituents. Although testing has not yet commenced, laying the framework for appropriate testing methodology is an important and necessary first step in determining the endocrine disrupting potential of compounds in our environment.

Primary research has been able to demonstrate the endocrine disrupting activities of some of these chemicals. However, it has been difficult to establish a definitive link between exposure and altered reproductive development and function. In animal experiments of EDC exposure, the incidence of endocrine disruption is often widely varied due to differences in dose, timing, metabolism and animal model. Additionally, the mechanisms driving these effects are not fully understood, further complicating the extrapolation of primary data to clinical relevance for human health and reproduction. A case of drug-induced endocrine disruption in 1971 underscores some of the challenges faced in conducting epidemiological analyses. In that year, physicians at Massachusetts General Hospital observed 8 cases of vaginal clear cell adenocarcinoma in young women between the ages of 14 and 22. Because it is a rare cancer normally seen in older women, these cases drew attention, and another link was found: 7 of the 8 girls were born to women prescribed the synthetic estrogen diethylstilbestrol (DES) during the first trimester of pregnancy [52]. DES was initially prescribed to pregnant women as an off-label use to help prevent miscarriage and support a healthy pregnancy. However, a full analysis later suggested an array of reproductive tract problems in both sons and daughters exposed to DES during the first trimester of

development, prompting the conditions of pregnancy to be considered a contraindication for use [53-55].

“DES daughters” are known to be at an increased risk of cancer, ectopic pregnancy, preterm delivery, infertility, and alterations to uterine and cervical structure [56]. Research has suggested that some of these effects may be mediated through multiple mechanisms, including the activation of proto-oncogenes, delayed expression of genes regulating reproductive structure, and altered gene methylation patterns [57-59]. “DES sons” have shown reproductive problems as well, including epididymal cysts, hypospadias, cryptorchidism and micropallus [56]. Experiments in male mice and rats demonstrate that this estrogen analog causes Leydig cell dysfunction via an estrogen receptor alpha-dependent mechanism, along with a reduction in insulin-like hormone 3 (InsI3), resulting in improper gubernacular development and testis maldescent [60-62]. DES also causes decreased testosterone production resulting from decreased synthesis of steroidogenic proteins cytochrome 17a-hydroxylase/17,20 lyase/17, 20 desmolase (Cyp17a1) and steroidogenic acute regulatory protein (Star) [63]. A major challenge in identifying putative mechanisms is the highly variable incidence of the assorted reproductive effects observed in DES sons and daughters. This is likely due to differences in exposure duration, timing, dosing, and genetic heterogeneity among a diverse human population. While current findings may not fully explain all manifestations of endocrine disruption following developmental DES exposure, the spectrum of reproductive defects observed demonstrates human fetal susceptibility to endocrine-active compounds.

Testicular dysgenesis syndrome

In the years since EDCs first took the spotlight, interest has expanded beyond simple estrogen mimicry. It is now known that compounds can alter endocrine activity in varying ways, covering the spectrum of pro- and anti-estrogenic, androgenic, and goitrogenic effects. At the same time, epidemiologic research has noted adverse trends in reproductive health and fertility of men in developed countries. In particular, epidemiologists have cited an increase in the incidence of reproductive congenital malformations and adult onset diseases over the past 70 years [64]. Congenital defects include varying degrees of cryptorchidism and hypospadias, as well as other associated anomalies of the epididymis, seminal vesicles, and vas deferens. Adult onset diseases of concern include alterations in sperm quantity, morphology, and motility, as well as testis germ cell cancer. Observation of these symptoms has resulted in the proposal of a “Testicular Dysgenesis Syndrome” (TDS), suggesting that these effects may share a common etiology in altered reproductive development [37]. According to this hypothesis, perturbations to the developmentally-critical *in utero* or perinatal environment, possibly due to EDC exposure, may result in subsequent dysgenesis of the male reproductive tract. Epidemiological support for this theory comes from examination of the contralateral testis of adult men with testis germ cell cancer, where focal areas of altered seminiferous tubule morphology, Leydig cell hyperplasia, and immature Sertoli cells have been observed, suggesting a contribution of altered germ cells to the development of cancer as a result of a disrupted developmental environment [65]. While the prevalence of the diseases and disorders that comprise TDS are highly variable between individuals, the manifestation of one may be considered a risk factor for developing another, as with unresolved cryptorchidism and subsequent testicular cancer.

While some rare genetic defects and point mutations can lead to altered reproductive structure and function, the overall prevalence of these genetic abnormalities cannot explain the increasing incidence or variable expressivity of the full array of TDS disorders. As a result, researchers have looked towards the use of transgenic animal models to examine the potential molecular mechanisms of the various TDS phenotypes observed in humans. Previous work in developmental biology has observed TDS-like effects with the loss or

disruption of genes regulating reproductive growth and development. Testes from Fgf9-null mouse pups at embryonic day 18.5 exhibit a wide range of altered testicular phenotypes with disorganization of seminiferous cords and disrupted peritubular myoid cell organization [66]. Dax-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1) deficient males exhibit infertility due to obstruction of the efferent ducts and rete testis as well as Leydig and Sertoli cell tumors [67]. Loss of the gene *Insl3* causes bilateral intraabdominal cryptorchidism with secondary infertility and testicular atrophy [68]. The aforementioned models have provided valuable information about the multifaceted roles of signaling molecules and transcription factors in male reproductive development and function. While each model produces one or two of the phenotypes described in human TDS cases, other undesirable pathological effects such as lung hypoplasia and ovarian-like testis organization limit their usefulness as a model for human TDS.

Phthalates

The phthalate ester class of chemicals has recently received considerable attention due to its widespread use and reported endocrine disruption activities. Phthalate esters are high production volume chemicals used as plasticizers in polyvinyl chloride (PVC) plastics to impart flexibility. They are also used as emulsifying agents, surfactants, and lubricants in numerous industrial, medical, and cosmetic products. As these compounds are not covalently bound to the PVC polymer, they can leach with age, use and ultraviolet light exposure, making them available for biological exposure [69]. Detectable levels of various phthalate metabolites have been observed in the urine of the general population by the United States Centers for Disease Control and Prevention [70]. Additionally, critically ill neonates receiving intensive medical treatment can be exposed to up to 10-20mg/kg of phthalate esters due to the use of PVC-based medical devices [71]. As such, phthalates have been suggested to be associated with the development of TDS due to the potential for significant exposure during development and the induction of reproductive tract defects in rats following gestational exposure (Figure 1) [72-74]. Importantly, the suppression of fetal steroidogenesis has resulted in an expression of “serious concern” for phthalate exposure of intensively medically treated infants by an Expert Panel for the Center of the Evaluation of Risks to Human Reproduction [75].

Early work on the endocrine disrupting activities of phthalates by the National Toxicology Program in 1991 demonstrated alterations to reproductive tract structure, seminiferous tubule degeneration, and lowered sperm counts in male pups exposed to di-(n-butyl) phthalate (DBP) during mid- to late-gestation [76]. Interestingly, these effects were not observed in the parental generation, suggesting that additional studies would be needed to more fully characterize the effects of developmental phthalate exposure. While estrogenic properties of phthalates had previously been reported, subsequent research pointed towards an anti-androgenic mechanism, as female rats gestationally exposed to DBP exhibited no change in reproductive organ weights, estrous cyclicity or vaginal opening, as was observed with weakly estrogenic compounds [77-79]. Experiments in rats showed that DBP and other similarly acting phthalates exhibit dose-dependent effects on the developing male reproductive tract, including cryptorchidism, hypospadias, hypospermatogenesis, increased seminiferous cord diameter, decreased anogenital distance, and the formation of multinucleated germ cells (MNGs) [79-82]. The anti-androgenic mode of action was confirmed when it was shown that DBP and its active monoester metabolite, monobutylphthalate (MBP), could lower both the expression of steroidogenic genes and intratesticular testosterone content without interacting with the androgen receptor [83-85]. It is now accepted that decreased *Insl3* and lowered testosterone production underlie the manifestation of cryptorchidism, and hypospadias and altered secondary reproductive

structures, respectively [86]. Although the pathogenesis of MNG formation is still unknown, it is believed that these rogue cells may arise from altered Sertoli-germ cell communication and altered cytokinesis.

The effects of in utero exposure to phthalates have been well characterized, demonstrating clear anti-androgenic effects on the rat male reproductive tract. While most work has focused on the rat as a susceptible species to the anti-androgenic effects of phthalates, published work in 2007 attempted to extend the rat model to mice in order to explore potential species differences in response. The findings of this study showed that *in utero* exposure of mice to equivalent doses of endocrine active phthalates exhibited similar toxicokinetics, and caused the same induction of MNG formation and alterations to seminiferous cords seen in the rat model. Interestingly, however, these fetal murine effects were observed in the absence of any measureable differences in steroidogenic gene expression or intratesticular testosterone content. This important species difference in response suggested that the effects of developmental phthalate exposure on seminiferous cords (increased diameter, MNG induction) are not mechanistically linked to lowered steroidogenesis and testosterone production.

The divergent response observed with developmental phthalate exposure in mice and rats highlights the inherent difficulty in relying on single species toxicity testing approaches. Differences in susceptibility and sensitivity hold particular importance for the extrapolation of risks to humans following toxicant exposure. As in utero experimentation is both costly and labor intensive, *in vitro* organ culture has been explored as a straightforward and inexpensive method for studying cellular effects of phthalates. Unfortunately, these models have been fraught with many limitations, including inconsistent responses in testosterone production following phthalate exposure, a treatment-related loss of germ cells, and failure of MNG induction [87-90]. For these reasons, in vitro approaches have thus far appeared inadequate for examining at least some phthalate-induced effects on the testis. However, the single species *in utero* exposure models used with phthalate research to date do not allow for determining whether the disparity in effect on Leydig cell steroidogenesis is an intrinsic response of the species, or an inherent property of the testis itself. This could be examined using a cross-species xenotransplant model, consisting of mouse and rat testis transplants into species either resistant (mouse) or sensitive (rat) to phthalate-induced effects on steroidogenesis. The interspecies approach would allow for direct assessment of the testicular response to phthalates on both a gene and histological level, while examining the role of host-specific factors in species responses.

Biology and toxicology of the pubertal male reproductive tract

A neonatal surge of testosterone during the first four months of life leads to testosterone levels that resemble those of a healthy adult male [16]. By six months of age, testosterone levels decline to near negligible levels and remain low until puberty, when the male acquires adult reproductive capabilities, develops secondary sex characteristics, and experiences a growth spurt [16].

The hypothalamus-pituitary-gonadal (HPG) axis is critical to sexual maturation, both at the fetal and pubertal stages. To initiate puberty, the hypothalamus secretes gonadotropin releasing hormone (GnRH), which causes an increase in nocturnal pulsatile gonadotropin (LH and FSH) secretion from the pituitary gland [91]. In adult males, pulsatile secretion occurs approximately every 90 minutes; the frequency with which this occurs is an important factor in normal gonadal response [92]. When the gonadotropin pulse frequency (and thus secretion of gonadal sex hormones) reaches a critical level, secondary sexual characteristics begin to form; this marks the beginning of phenotypic puberty [91]. While

FSH is not used until sperm maturation, LH is released during sleep alongside pulsatile GnRH, causing gonadal stimulation and inducing Leydig cell hyperplasia that leads to testosterone release [93]. During puberty, the testis cords hollow to form seminiferous tubules, and the germ cells migrate towards the periphery of the tubules where they begin to differentiate into sperm [21]. The HPG axis is critical for both gonadal development and steroid production; if it is disrupted, a hypogonadal state can result, leading to abnormalities such as the congenital disorders previously described.

It is during puberty that the blood-testis barrier (BTB) develops as an important barrier to both environmental toxicants and the immune system – this occurs alongside the proliferation of spermatogonia to primary spermatocytes [94]. Adjacent Sertoli cells in the seminiferous epithelium form tight junctions (TJ) using actin-based adherin junctions (AJ) characterized by actin filament bundles. The bundles join the Sertoli cell plasma membrane and the subsurface cistern of the endoplasmic reticulum (ER). This is identified as a typical structural feature of the basal ectoplasmic specialization [95]. There are three types of tight junction transmembrane proteins that are associated with the BTB: claudins, occludins (in rats), and junctional adhesion molecules [96-98]. The two AJ proteins associated with the BTB are the classic cadherins and nectin-2 [99, 100]. Both the TJ and AJ proteins become linked to actin filaments by adaptors, strengthening the BTB to ensure separation of spermatogonia and preleptotene/leptotene spermatocytes from the spermatocytes that are completing meiosis in the adluminal compartment [101]. In this way, the BTB serves as both an immunological and reproductive toxicant barrier by separating post-meiotic spermatocytes from systemic circulation. During spermatogenesis, preleptotene and leptotene spermatocytes pass through the dynamic BTB; when this process is disrupted, germ cells cannot develop or differentiate normally.

Because of the intricate hormonal events required to initiate puberty and the transition to sexual maturity, this developmental stage is anticipated to be sensitive to alteration by endocrine disrupting chemicals. Wook Joon et al. [102] observed severe reproductive histopathology after the administration of vinclozolin, a systemic dicarboximide fungicide with antiandrogenic activity, to male rats during the pubertal period. Rats dosed with 100 mg/kg/d displayed a decrease in the weight of accessory sex organs such as epididymal and seminal vesicle weights. Histopathology of rats dosed with 200 mg/kg/d included hypertrophy of Leydig cells, decreased prostate weight, and detached debris and sloughed germ cells in the caput epididymis, indicating a spermatogenic disorder in the testis. At the high dose of 300 mg/kg/d, an increase in serum testosterone was observed as well. Blystone et al. [103] saw a similar effect following administration of iprodione, another antiandrogenic fungicide, with decreases in prostate and seminal vesicle weights at 100 mg/kg/d. This study also consisted of a pubertal mixture study, examining the effect of an iprodione and vinclozolin co-exposure. Administration of both compounds together produced an additive effect on androgen-sensitive endpoints. Interestingly, however, iprodione appeared to have an inhibitory effect on the vinclozolin-induced increase in testosterone. Administration of vinclozolin and other EDCs to pubertal male rats appears to impair normal differentiation of reproductive organs during puberty, possibly by disturbing the precise endogenous endocrine landscape.

The end of puberty marks male sexual maturity, at which point reproductive organs and accessory sex glands have reached their adult state. The major components of the adult male reproductive system, including the testes, the epididymis and the prostate, are described below.

Biology and toxicology of the adult male reproductive tract

Structure and function of the adult male reproductive system

The testes are firm, oval shaped glands found in the mammalian scrotum. The adult testis is a complex organ whose two major functions, spermatogenesis and steroid hormone production, are highly dependent upon the coordinated regulation of interacting cell types, namely, Leydig cells, peritubular myoid cells, Sertoli cells, and germ cells (Figure 2). Structurally, the testes are covered by the tunica albuginea, a layer of connective tissue. The testis contains two major compartments – the seminiferous tubules and the interstitial spaces. Blood vessels, lymphatic vessels, and Leydig cells are found in the interstitial space; as the interstitial cells directly adjacent to the seminiferous tubules, Leydig cells are responsible for the production and release of testosterone. The seminiferous tubules, covered by peritubular myoid cells, are finely coiled tubes organized in loops throughout the organ that connect to the excurrent duct system and contain both Sertoli cells and germ cells. The Sertoli cells form the blood-testis-barrier and act as “nurse” cells that provide the nutrients and environment necessary for spermatogenesis. They also phagocytose both apoptotic germ cells during normal spermatogenesis and the residual spermatid cytoplasm during spermiogenesis [104]. Additionally, Sertoli cells secrete fluid that forms a tubular lumen and transports sperm to the epididymis. Typically, Sertoli cells do not proliferate once they are fully differentiated, so by the time spermatogenesis has begun, the Sertoli cell population is fixed in number [104]. Spermatogonial germ cells line the basement membrane of the seminiferous tubules and act as sperm progenitor cells; they maintain close contact with the Sertoli cells and move inward toward the lumen as they proliferate and differentiate. The seminiferous tubules converge at the rete testis; from here, the immature sperm formed in the testis are transported through the efferent ducts to the caput epididymis.

The testes are critical end-organ components of an endocrine feedback system responsible for testosterone synthesis. Testosterone is produced by a highly regulated pathway that begins with the secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus. GnRH stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) [104]. LH receptors on Leydig cells are sensitive to FSH-induced up-regulation, making the cells more responsive to LH. Leydig cells respond to LH stimulation by enhancing cholesterol desmolase activity, which converts cholesterol to pregnenolone, leading to testosterone synthesis and secretion [104]. Testosterone is necessary for normal spermatogenesis by activating pathways in Sertoli cells that promote differentiation of spermatogonia.

Spermatogenesis occurs in three phases: spermatogoniogenesis, meiosis, and spermiogenesis [105]. During spermatogoniogenesis, the spermatogonia in the basal compartment undergo multiple mitoses to build a large population of cells for subsequent meiosis and differentiation. In humans, there are three subtypes of spermatogonia: Type A(d) cells, Type A(p) cells and Type B cells [104]. Type A(d) cells have dark nuclei and replicate to ensure a constant supply of spermatogonia to fuel spermatogenesis. Type A(p) cells have pale nuclei and divide by mitosis to produce Type B cells. Type B cells divide to give rise to primary spermatocytes. From here, each primary spermatocyte moves into the adluminal compartment of the seminiferous tubule, where it enters the second phase of spermatogenesis by undergoing meiosis I to produce two secondary spermatocytes [104]. These maturational steps are sources of genetic variation, such as chromosomal crossover or random inclusion of either parental chromosome, increasing the genetic variability of the gamete. Secondary spermatocytes rapidly enter meiosis II and divide, yielding haploid spermatids. These spermatids differentiate into spermatozoa through a process called spermiogenesis, during which the spermatids begin to grow a tail and develop a thickened mid-piece where the mitochondria gather and form an axoneme [104]. In addition, the

haploid nucleus is streamlined and the Golgi apparatus surrounds the condensed nucleus, forming the acrosome. The nuclear cytoplasm is eliminated and chromatin compaction occurs, whereby the somatic and testis-specific histones are replaced with transition proteins and protamines [106]. The male germ cell differentiation program requires an array of finely tuned levels of gene regulation [106]. Chromatin compaction causes the cessation of transcription in elongating spermatids. However, high levels of messenger RNAs (mRNAs) are found in round spermatids before transcriptional arrest and are stored in a stable form in preparation for their translation during the later stages of spermiogenesis [106]. mRNA storage and subsequent translational activation are very important in regulating the synthesis of many sperm proteins. Maturation takes place under the influence of testosterone. The excess cytoplasm of the spermatids forms into residual bodies and is phagocytosed by surrounding Sertoli cells. The resulting spermatozoa, mature but lacking motility, are released from the Sertoli cells into the lumen of the seminiferous tubule in a process called spermiation [104]. The non-motile spermatozoa are transported to the epididymis by peristaltic contraction, where they gain motility and become capable of fertilization. Mature sperm consist of a haploid nucleus, a propulsion system, and an acrosomal sac of enzymes that enable the nucleus to enter the oocyte.

Sperm were originally regarded as a vessel for the transportation of the male genome to the oocyte, devoid of translational activity (due to their lack of cytoplasm and ribosomal RNAs); the oocyte, on the other hand, was believed to be responsible for producing all of the mRNA and proteins necessary for fertilization and embryogenesis. However, recent data suggest that sperm play a greater role in this process than originally believed; in addition to their genome, sperm transmit mRNA and provide the oocyte with vital organellar and male-specific proteomic components. At least 5,000 different mRNA transcripts are currently known to exist in sperm [107]. These characterized sperm transcripts are involved in cell signaling/communication, cell division, gene/protein expression, metabolism, cell structure and motility, and organism defense [108]. Many functions have been proposed for these sperm transcripts, including roles in sperm structure and stress response, *de novo* translational replacement of degraded proteins, oocyte fertilization, embryogenesis/morphogenesis, and epigenetic regulation and establishment/maintenance of the parental imprint [108, 109]. At present, it is understood that the quantity and types of mRNA transcripts may indicate the state of spermatogenesis [107].

Structure and function of the adult male reproductive system - Epididymis

The epididymis is a complex coiled tube that connects the testis to the vas deferens and functions to transport, nurture and mature the sperm. Its structure is designed to facilitate these processes both hormonally and physically. The epididymis is divided into three sections, the caput, the corpus, and the cauda, each of which plays a role in the development of the sperm as it travels through the organ. Secretory products produced by the epididymal epithelium lead to numerous functional changes of the sperm that include acquisition of motility and increased capacity to fertilize [110, 111].

Structure and function of the adult male reproductive system - Prostate

The prostate surrounds the urethra just below the bladder; this location allows for the control of urine flow during ejaculation. The prostate is a complex organ composed of both glandular and nonglandular tissues encased in a fibromuscular capsule. The two major regions of the prostate, the peripheral zone and the central zone, compose about 70% and 25% of the glandular mass, respectively [112], while a smaller transition zone consists of about 5-10% of the prostatic glandular tissue. The ducts from the different glandular regions drain into the urethra at different points. The prostate stores and secretes a milky white, slightly alkaline fluid (pH 7.29) that combines with spermatozoa and other secretions during

ejaculation. The prostatic fluid contributes about 25-30% of the volume of the ejaculate, and, along with secretions from the seminal vesicles, neutralizes the acidity of the vaginal tract to increase sperm survival and facilitate fertilization. Prostatic fluid also contains Prostate Specific Antigen (PSA), which further liquefies the semen after ejaculation, improving sperm motility [113].

Model adult testicular toxicants

2,5-Hexanedione (HD) is the active metabolite of the common industrial solvent, n-hexane (Figure 3A). Although the most significant exposures occur in occupational settings, humans are ubiquitously exposed to low levels of n-hexane as a chemical component of gasoline [114]. HD is both a neuronal and testicular toxicant with clear neurotoxic clinical manifestations and subtle indications of testicular injury. Animal studies indicate that HD targets Sertoli cell microtubule assembly by the induction of tubulin cross-linking both *in vitro* and *in vivo* [114]. HD exposure causes altered microtubule-dependent transport in Sertoli cells and disturbs the germ cell niche by impeding seminiferous tubule fluid secretion. Disruption of the Sertoli cell microenvironment stimulates germ cell apoptosis and ultimately results in testicular atrophy [114].

1,2-Dibromo-3-chloropropane (DBCP) is a nematocide that has been shown to reduce fertility and induce sterility in humans exposed in occupational settings (Figure 3B) [115]. DBCP was used from 1950 to 1979, at which time it was banned in the United States due to the adverse health effects that had been identified a few years earlier. In 1977, workers at the Dow chemical company complained of deleterious health effects in connection with the chemical, and indeed, published laboratory findings of these workers showed that they suffered from oligospermia, azospermia and elevated hormone levels [115, 116]. Animal experiments have also revealed the testicular toxicity of DBCP; exposed rats display altered seminiferous tubule morphology, malformations of the sperm, and reduced sperm counts. [117]. Studies also indicate that DNA is the subcellular target of this toxicant. DBCP can be converted to reactive metabolites both by cytochrome P450 and glutathione S-transferase-dependent (GST) pathways [118], and these resulting metabolites can induce single-strand breaks in DNA. The amount of GSTs in the spermatogenic cell types increases with spermatogenic cell development [118], which may indicate a higher potential to activate DBCP in germ cells at later stages of development. In addition, cells in S-phase are more susceptible to DBCP-induced apoptosis than cells in the growth phases [118]. In accordance with the model, proliferating and differentiating spermatogenic cells (e.g. round spermatids) are the cells most sensitive to DBCP-induced apoptosis. Because round spermatids have less compacted DNA than elongating/elongated spermatids, the DNA in these cells is a more accessible target.

Ethylene-1,2-dimethanesulfonate (EDS) is a toxicant that selectively and temporarily destroys the adult Leydig cell population through apoptosis via the Fas/FasL pathway (Figure 3C) [119]. An intraperitoneal (IP) injection of 75 mg/kg in adult rats decreases testicular and serum testosterone and increases the pituitary secretion of LH and FSH [120]. Overall, EDS induces a depletion of germ cells in the seminiferous epithelium. Leydig cells disappear from the interstitial space by 7 days post-IP injection, as evidenced by histological examination. Additionally, there is a decrease in elongating (step 9-13) spermatids in late stage seminiferous tubules (IX-XIII) [120], although the early stages remain intact. Single new Leydig cells with characteristics of progenitor type Leydig cells are observed at 2 weeks post-exposure. There is also a loss of all stages of elongated spermatids and germ cell sloughing into the lumen [120]. At 3 weeks post-exposure, there is an expansion of immature Leydig cells and signs of recovering spermatogenesis including the presence of elongated spermatids in late but not early stages [120]. At 5 weeks post-exposure, mature adult type Leydig cells are visible in the interstitial space. The late stages are almost

completely recovered (there is evidence of all germ cell types), although there are still no elongated spermatids found in early stages [120]. By 7 weeks post-exposure, the Leydig cell population has resumed a normal histological appearance. Spermatogenesis appears recovered in most of the tubules, although some of tubules still contain only Sertoli cells [120]. There is a close relationship between germ cell and Leydig cell changes; on the sub-cellular level, it is believed that the EDS-induced testosterone reduction causes apoptosis of germ cells (particularly of haploid germ cells) and causes the temporary arrest of spermatogenesis [120].

Carbendazim (CBZ) is the active metabolite of benomyl, a benzimidazole fungicide used to prevent and eliminate fungal plant diseases (Figure 3D) [121, 122]. Mammals are exposed to CBZ orally and it is readily absorbed and metabolized rapidly. Overall, CBZ has low acute toxicity but has many negative effects on the male reproductive system [121, 122]. CBZ is a Sertoli cell toxicant that targets those cells by inhibiting microtubule polymerization by binding to the beta-tubulin subunit of the tubulin heterodimer. This ultimately decreases the rate and stability of microtubule assembly [121]. Acute exposure of adult male Fisher 344 rats to CBZ results in increased testis weights and seminiferous tubule diameters as well as increased rates of germ cell sloughing, retained spermatid heads, and apoptotic germ cells one hour post-exposure in a dose dependent manner [121]. Subchronic exposure of adult male Wistar rats to CBZ resulted in many histopathological changes of the testis including atrophic seminiferous tubules, decreased germ cells, and increased sloughing in a dose dependent manner [122]. These rats had smaller testes and decreased epididymal sperm counts and motility and mating studies demonstrated a decreased fertility index, which was dose-dependent [122]. Luteinizing hormone was the only hormone significantly altered in the high dose group [122]. Flow cytometric analysis of the testicular tissue indicated an interference of the spermatogenic process, showing a dose dependent increase in primary spermatocytes and a decrease in the number of spermatogonia, spermatids, and DNA synthesizing cells [122]. Overall, CBZ disrupts proper Sertoli cell function and spermatogenesis and ultimately reduces fertility in male rats [121, 122].

Exogenous Hormones

Anabolic-androgenic Steroids (AASs) are synthetic analogues of testosterone. AASs are used to treat a variety of conditions including refractory anemia, hereditary angioedema, breast cancer, and starvation states [123]. At super-therapeutic doses (10x-100x), AASs are known to improve athletic ability and increase muscle mass. However, using these drugs for off-market purposes has negative effects on both human and animal physiology. In humans, AAS users have reported alterations in libido, changes in mood, reduced testis volume, acne, and gynecomastia [124]. In adult rats exposed to the AAS nandrolone decanoate for 2 weeks (followed by a 2 week recovery period), decreases were reported for mean testis volume, length of seminiferous tubules, sperm count, and motility [123]. Rats exposed to a cocktail of AASs (nandrolone decanoate, metenolone acetate, and dromostanolone) subcutaneously once a week for 6 weeks followed by a 4 week recovery period and an additional 6 weeks of treatment had increases in serum testosterone and serum dihydrotestosterone [125] and showed signs of hair loss, aggressive behavior and self-inflicted tail wounds. There was observable physical damage to the heart, testis and adrenal gland, and histology showed reduced numbers of Sertoli cells, Leydig cells and spermatozoa [125]. Exogenous administration of synthetic testosterone analogs resulted in negative feedback on the hypothalamic-pituitary axis and an inhibition of FSH and LH, which ultimately led to an inability to maintain spermatogenesis due to hypogonadotropic hypogonadism and testicular atrophy [123].

Protection from testicular injury

Ubiquitin marks aberrant proteins for 26S-proteasome degradation, and it is believed that a mutated form of ubiquitin (K48R) offers protection from testicular injury in both acute (experimental cryptorchidism) and chronic (aging) cases. This is true for mice with the K48R ubiquitin mutation, who are resistant to the effects of acute and chronic testicular injury [126]. Experimental cryptorchidism increases testicular heat stress, leading to a cascade of events that include altered protein degradation, germ cell loss and testicular atrophy; aging can also lead to germ cell loss and atrophy of the testes. When compared to wild type mice, ubiquitin K48R mutant mice have greater testis weights after both types of injury [126]. Testis cross sections of experimentally cryptorchid animals indicate that the average number of germ cells per seminiferous tubule is greater in ubiquitin K48R mutant mice than in their WT counterparts. The seminiferous tubules of aged mutant mice have larger diameters, with a greater number of germ cells, than WT mice, indicating that the K48R mutation does indeed serve to protect against the testicular injury of aging. It was also observed that the mutation protected blood vessels in the interstitial space from becoming enlarged with hyaline material accumulations [126].

Testicular atrophy can be the result of a variety of insults, including environmental toxicant exposure, chemotherapy, irradiation, and aging [127]. It is believed that treating males with gonadotropin releasing hormone (GnRH) analogues after testicular insult can prevent or reverse atrophy [127], because GnRH is a peptide hormone that induces the release of LH and FSH from the anterior pituitary. GnRH analogues (agonists and antagonists) are used to treat many clinical conditions, including prostate cancer, because they offer reversible medical castration with effects similar to that of orchiectomy [128]. GnRH agonists directly stimulate the production of LH and FSH which ultimately leads to increased production of testosterone. Despite this initial surge of testosterone production, prolonged occupation of pituitary LH receptors results in an overall decrease in testosterone. Conversely, GnRH antagonists directly inhibit the GnRH receptor and decrease levels of LH, FSH and testosterone [128].

As previously mentioned, radiation exposure induces testicular atrophy by depleting the germ cell population. The seminiferous tubules of irradiated rats contain normal undifferentiated type A spermatogonia; rather than differentiating, these cells become apoptotic and the number of cells remains constant [127]. In addition, irradiated rats have elevated levels of intratesticular testosterone and systemic FSH compared to controls. When a GnRH analogue is administered immediately after irradiation, the seminiferous tubules maintain spermatogonial differentiation up to the round spermatids. After a recovery period allows increases in testosterone and FSH levels, the testes of irradiated rats treated with GnRH resume normal spermatogenesis for at least 12 weeks. It appears as though the transient suppression of intratesticular testosterone and FSH prevents the persistence of the radiation-induced testicular atrophy. Spermatogenic recovery is disrupted when rats are given exogenous testosterone simultaneously with GnRH treatment [127]; FSH levels are also inversely correlated with recovery. Therefore, under normal conditions, testosterone is required for spermatogenesis; however, in irradiated male rats, elevated intratesticular testosterone and FSH inhibit early spermatogonial differentiation [127].

Similarly, the testes of rats treated with the environmental toxicant 2,5-hexanedione (HD) (1%) for 3-5 weeks are atrophic 12 weeks after the start of exposure and remain so for at least 70 weeks post-exposure [127]. Histopathological examination reveals that less than 1% of seminiferous tubules contain germ cells more advanced than type A spermatogonia after this protracted HD exposure [127]. In animals treated with a GnRH agonist immediately after 3-5 weeks of HD exposure, 90% of seminiferous tubules contained advanced germ cells and greater than 80% contained elongated spermatids. Temporarily eliminating

testosterone by ablating HD-treated rats with multiple injections of EDS only temporarily increased spermatogenic recovery, indicating that suppression of testosterone alone is not enough to reverse testicular atrophy [127]. Ablating HD-treated rats with one injection of EDS followed by GnRH treatment (3x) resulted in no spermatogenic recovery. However, HD-treated rats treated 3x with GnRH and no Leydig cell ablation did lead to the restoration of spermatogenesis. These results suggest that Leydig cell factors are important in spermatogenic recovery of the atrophic testes [127].

Finally, similar experiments were conducted with aged Brown Norway rats. These animals had decreased serum and testicular testosterone, which suggests that an increase in testicular testosterone is not required for the maintenance of atrophy. Treatment of aged rats with GnRH (3x) led to a modest increase in spermatogenesis [129].

Sperm biomarkers of spermatogenic abnormalities

An emerging research area is that of human sperm mRNAs as potential biomarkers of fertility. Ostermeier *et al.* found that a genetic fingerprint of normal fertile men can be generated from mRNA present in sperm [107], and that studying ejaculated sperm is a convenient method for investigating testis-specific infertility. Wang *et al.* used microarray technology to analyze gene expression differences between testis-specific and sperm-specific genes in fertile men [130]. By examining the expression of 5 sperm motility-related genes identified in this profile by reverse transcriptase polymerase chain reaction (RT-PCR), they found the expression of two genes, TXP1 and LDHC, to be significantly altered between normal and motility-impaired semen samples. Again, their results indicated that clinical assessments of sperm quality can be made from differential sperm mRNA content patterns. Additionally, human studies have indicated that abnormal protamine ratios exist in infertile men and functional evidence has demonstrated that male protamine knockout mice are infertile [131, 132]. With this in mind, Steger *et al.* investigated the potential use of protamine ratios and Bcl2 expression as biomarkers of infertility using RT-PCR [133]. They found aberrant protamine ratios and increased mRNA expression of Bcl2 in ejaculates and in testicular biopsies of infertile men compared to controls. These results encourage the use of mRNA expression levels as predictive biomarkers of fertility status. As of yet, only one laboratory has focused on identifying biomarkers of fertility in animal models; Klinefelter *et al.* have studied expression of SP22, a sperm membrane protein, and its correlation with caudal epididymal sperm fertility after exposure to testicular and epididymal toxicants [134]. In addition, they have confirmed that post-meiotic germ cells express a testis-specific SP22 transcript. The same group is currently examining the potential use of this protein as a diagnostic marker of human infertility [134].

Epigenetic mechanisms, and specifically DNA methylation, play an important role in regulating genes during development. Methylation is a heritable yet reversible epigenetic mark that influences gene expression without altering the underlying DNA sequence. Mammalian DNA is methylated at the 5-position of cytosine residues primarily within CpG dinucleotides; this reaction is catalyzed by a family of DNA-methyltransferases. Methylation occurs almost exclusively at CpG dinucleotides in the CpG islands located in the promoter region of genes [135]. DNA methylation modifies the function of the mammalian genome, and typically results in repression of gene expression. DNA methylation is essential for normal development [136, 137]; the epigenetic reprogramming that occurs during development may be a sensitive window for disruption of the epigenome. It has been suggested that alterations in the epigenetic reprogramming processes during development can lead to adult-onset disease [13].

In the developing embryo, primordial germ cells become demethylated as they migrate along the genital ridge towards the fetal gonad. During gamete maturation, the methylation

profile is re-established in the germ line [138], resulting in a pattern of DNA methylation that reflects both inherited imprints and environmental conditions. CpG dinucleotides are under-represented in the genome, but over-represented in promoter regions. Hypomethylated promoter regions establish an open chromatin structure that allows for the initiation of gene transcription, while hypermethylated promoter regions lead to a closed chromatin structure, blocking transcription factor binding and silencing gene expression [139]. In addition to regulating gene expression, DNA methylation silences repetitive elements and is important for the stability of the mammalian genome.

Both human and animal studies have shown that abnormal methylation patterns affect fertility. Houshdaran *et al.* examined the global methylation pattern of sperm in semen samples isolated from male members of 69 couples referred for infertility analysis and found that in poor quality sperm, the methylation state of numerous sequences was elevated in the DNA [140]. They hypothesized that the mechanism behind the epigenetic change may be aberrant erasure of DNA methylation during epigenetic reprogramming of the male germ line [140]. In addition, Pathak *et al.* examined the effects of tamoxifen exposure on DNA methylation patterning in rat spermatozoa [141]. The authors measured global sperm DNA methylation, the methylation state of the Igf2-H19 imprinting control region (ICR), and embryo post-implantation loss. Although no changes in global methylation were seen, methylation was reduced at the Igf2-H19 ICR. Mating experiments showed a significant increase in post-implantation loss, which positively correlated with the reduced ICR methylation. The authors suggest that errors in paternal imprints could affect embryo development and that methylation patterns could be useful as biomarkers for evaluating male fertility [141].

Biology and toxicology of the aging male reproductive tract

After a male reaches sexual maturity, his reproductive system remains fertile and unchanged until he experiences either testicular injury or reduced reproductive system function as a result of age. Aging of the male reproductive tract brings about many changes at the regulatory, molecular, and cellular levels, which, unlike changes during fetal development, are not genetically programmed [138]. Some characteristic morphological changes of the testis associated with age are decreased Leydig cell numbers leading to a decrease in testosterone production, arteriosclerotic lesions, and thickening of the tunica albuginea [142, 143]. Male aging has also been associated with decremental changes in HPG axis function, with an amplitude decrease in GnRH and LH and an increase in pulse frequency, ultimately leading to a decrease in the production of testosterone [144].

Because these age-related changes have also been identified in rodents, rats represent a good model organism for studying the aging reproductive tract. Some rat strains are more useful than others; for example, the Brown Norway rat model may more closely resemble human reproductive tract aging, and is preferred over both the Sprague-Dawley and Wistar strains for several reasons. First, the Sprague-Dawleys and Wistars have health problems that generally accompany aging, (such as pituitary adenomas, obesity, and testicular tumors) a fact that leads to difficulty differentiating between age-related and disease-related defects in the male reproductive tract [145]. Second, the average lifespan of a Brown Norway rat is 36 months, compared to the 27 months that a Sprague-Dawley rat is expected to survive [145]. Brown Norway rats also offer a larger window for studying the age-associated decrease in testosterone production in the testis (which includes both serum and intratesticular levels); this testosterone decline occurs over an extended time of months 18-30 in Brown Norway rats, but only between months 21-24 in the Sprague-Dawley strain [145]. In addition to having lower testosterone, Brown Norway rats have decreased Sertoli cell function and numbers and a marked reduction in seminiferous tubule volume and contents [146]. In

contrast to the Sprague-Dawley and Wistar strains, Brown Norway rats live for more than a year after these changes occur in the testis, and generally remain healthy throughout the entire period of testicular aging [145]. For these reasons, the Brown Norway strain is the preferred rat model for studying human reproductive tract aging [145-147].

Research using Brown Norway rats has suggested that the age-related reduction in steroidogenesis is due not to a decrease in Leydig cell number or a change in Leydig cell responsiveness to LH [147] but rather to atrophic changes in Leydig cell size and organelle content with aging. Because testosterone production is a function of Leydig cell size [147, 148], these changes lead to dysfunction of the steroidogenic process. Decreases in testicular weight, sperm concentration, total sperm counts, plasma testosterone, LH and inhibin begin at 15 months [146]. Conversely, the proportion of regressed testes, plasma FSH levels, and germ cell loss via apoptosis increases with aging [146]. In 15 month old males, six months of LH replacement therapy did not decrease or delay any age related effects on the testis [146]. However, when 18 month old males were treated with LH, thyroxine (T₄), or a combination of both for 4 weeks, an increase in steroidogenic ability was observed [148]. The same study showed that with a daily dose of 24+5 µg dose of LH + T₄, Leydig cells can recover 100% of their volume and their steroidogenic ability [148]. These results indicate that changes in LH and thyroxine levels in serum influence the decrease in Leydig cell volume and the loss of steroidogenic potential associated with aging [148]. Decreased thyroid hormones and LH result (both directly and indirectly) from the aging of the pituitary gland, suggesting that testicular aging is correlated with pituitary aging [148].

Protection from the effects of aging-induced testicular senescence is conferred by alterations in the function of the ubiquitin system, as described above. In addition, using the Brown Norway rat model, GnRH analogue administration provides modest protection against testicular degeneration associated with aging in this model.

There is evidence that sensitivity to toxicants may differ in old age. For many drugs, these differences exist due to age-related changes in the number of receptors available, including steroid receptors, which have been shown to decrease with age [149]. Importantly, many tissues also develop a greater sensitivity to exogenous insults over time, so that the aged tissue is more susceptible to toxicants than its younger counterpart.

Distribution of the chemical in the organism can also influence relative toxicity; with age, it can change in two major ways: first, the reduction in plasma albumin that is common with aging can lead to an increase in the amount of free compound that is available in the body, increasing its toxicity. Second, an overall decrease in lean body mass among the elderly (in which the percentage of adipose tissue increases and body water decreases) changes the patterns of distribution such that water-soluble compounds have a smaller volume of distribution and lipophilic compounds, such as polychlorinated biphenyls (PCBs), have a greater volume of distribution [149]. Rates of metabolism and excretion also vary with age, as shown for TCDD comparing juvenile, adult, and senescent rats [150]. Finally, decreased renal function in the aged, can lead to a higher concentration of a chemical in the body for a longer period of time, increasing the risk of toxicity [149].

Complex exposures and low dose effects

Humans are exposed to many of the approximately 70,000 commercial and industrial chemicals currently in use on a daily basis. Because exposure to any single toxicant rarely occurs independently of other chemical exposures, it is prudent to examine toxicant effects in the context of complex mixtures or co-exposures that more adequately represent the true experience of exposure. In 1939, Bliss emphasized the importance of studying mixtures when he said that “the effect of the mixture cannot be assessed from that of individual

ingredients, but depends upon knowledge of their combined toxicity when used in different proportions. One component synergizes or antagonizes the other” [151]. By 1996, there had been enough cases of complex chemical interactions that the Food Quality Protection Act (FQPA) was passed, requiring cumulative risk testing of chemicals with similar mechanisms of toxicity. While it is possible that chemicals with different mechanisms of toxicity act independently and that the effect of their co-exposures could be simply additive, when chemicals target the same organ system interactions among the cell types targeted within that organ may lead to complex interactive effects. The interaction of chemicals with similar targets or mechanisms of toxicity can result in addition, synergism (the effect is greater than the additive effect of two or more chemicals) or antagonism (the effect is lower than the additive effect of the chemicals) [152, 153].

The developing male reproductive tract is particularly sensitive to toxicants, and especially to endocrine disruptors. This vulnerability of male reproductive development is illustrated in a study by Howdeshell *et al.*, in which fetal male rats were exposed to a mixture of two plasticizers, di-(n-butyl) phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) [154]. Mixture effects on several reproductive endpoints were demonstrated, including morphologic malformations and disruption in fetal steroid hormone production and the expression of *Ins13* and genes that are responsible for the production of steroid hormones. Howdeshell *et al.* discovered that the mixture of DBP and DEHP elicited dose additive effects, with an increase in many reproductive malformations by 50% or more [154]. In another study, Rider *et al.* examined the effects of a mixture of seven anti-androgens (vinclozolin, procymidone, linuron, prochloraz, benzyl butyl phthalate, dibutyl phthalate, and diethylhexyl phthalate) on the period of male sexual differentiation in rats [155]. It was found that with increasing doses of this mixture, male offspring of the exposed female rat dams had a dose-dependent reduction in anogenital distance, all of the male offspring had hypospadias, and there was an 80% incidence of epididymal agenesis and undescended testes in the high dose treated dams [155]. This mixture of seven anti-androgens was comprised of compounds that act via different mechanisms on the androgen signaling pathway; exposure to this mixture resulted in a dose-additive disruption of male rat reproductive tract differentiation [155]. When any of these endocrine disruptors are administered alone, the effects are far less detrimental than when they are administered as mixtures. Since we are almost never exposed to a single toxicant at a time, the dose-additivity seen with anti-androgenic mixtures speaks to the larger issue of interactive effects in combined exposures.

There is emerging evidence that adult exposure to mixtures of environmental chemicals can have harmful effects on the fully developed reproductive system. Andric *et al.* performed *in vivo* and *in vitro* studies with Aroclor 1248, a PCB mixture of congeners, in order to identify the effects of anti-androgens on the adult male reproductive system [156]. Results suggest that exposure to Aroclor 1248 causes down-regulation of testicular androgenesis through inhibition of the activity of 3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase/lyase and 17 β -hydroxysteroid dehydrogenase [156]. Markelewicz *et al.* (2004) examined the mixture effects of two testicular toxicants, 2,5-hexanedione and carbendazim, that act on the microtubule function of Sertoli cells [157]. They found that when co-administered to adult male rats, these toxicants acted together in a synergistic fashion to intensify testicular injury. Studies of mixed exposure effects in adult animals are just the beginning of an effort to understand mixtures and their effects on humans in order to inform risk assessment. Most concerning is that both mixtures of chemicals with similar and different molecular mechanisms of action are capable of producing additive or more than additive effects in adult males, whose reproductive systems are generally less vulnerable to toxicant exposure than those of developing fetal males. Because humans are predominantly exposed to

combinations of chemicals on a daily basis, it is important that studies designed to evaluate the health effects of mixtures are conducted.

In addition to the complication of mixtures risk assessment, some researchers have called for a reevaluation of the monotonic dose relationship central to the field of toxicology. Nearly all toxicological studies have assumed an increased response with increasing dose (monotonic). Recently, non-monotonic dose-response patterns with low doses showing a stimulatory effect followed by inhibition at higher doses have gained attention [158]. This “hormesis” phenomenon has been found in a wide variety of plants and animals, and is postulated to help protect the organism against subsequent stresses through beneficial responses of enhanced growth, survival, and enzyme activity, among others. However, not all low dose effects may be advantageous. A 2001 peer review report from the NTP found examples of non-monotonic dose responses following low dose endocrine disruptor exposure, with adverse outcomes such as changes to serum hormones, prostate weight, and mammary gland effects, with some occurring below the previously established NOEL [159].

While effects at low doses cannot be disregarded, the vast majority of toxicology studies to date have focused on high doses and specific endpoints, which may not be predictive of low dose effects. Depending upon the endpoint considered, the line between adaptive versus adverse responses becomes blurred [160]. In published studies that presumably show low dose effects, little is known of the potential mechanisms of such effects, and whether these effects extrapolate across life stages and species [161]. Until further mechanistic understanding emerges, acceptance of low dose effects and their applicability to human health risk assessment will remain unclear.

Transgenerational effects and the testis

Genomic imprinting during normal development

Normal development requires that both maternal and paternal genomes are properly expressed. While most genes are expressed from both parental alleles, imprinted genes represent a subset of genes that are expressed in a mono-allelic manner due to the differential methylation profiles of the alleles inherited from each parent. These imprints are established during gametogenesis or later in embryogenesis and are essential for normal development; there are several diseases that can result from improper imprinting [162]. Approximately 100 imprinted genes have been identified in mammals [163]. Genome-wide epigenetic reprogramming during gametogenesis and early embryogenesis functions to erase the maternal and paternal imprints in germline cells and re-establish these imprints according to the sex of the individual. For example, males contain one set of chromosomes with male imprints and one set with female imprints, but when these chromosomes are passed on to the next generation, both Y-bearing and X-bearing sperm must be reprogrammed to contain male imprints [164].

A wave of demethylation occurs following fertilization in the pre-implantation mouse embryo, during which most gametic methylation differences are removed. The sperm genome is actively demethylated within 4 hours of fertilization and the egg genome is passively demethylated during subsequent cleavage divisions after the two-cell stage [165, 166]. However, most imprinted genes in somatic cells escape this wave, retaining gametic methylation patterns that will remain intact throughout embryonic development in order for the imprint to translate into the monoallelic gene expression that is required for further embryonic development [167, 168]. Following erasure of methylation patterns, reprogramming or reacquisition of methylation patterns of the developing male gametes occurs in order to endow sperm with imprinted alleles. Epigenetic paternalization is an ongoing process that begins to be acquired before birth in the gonocytes or

pro spermatogonia between 15.5 and 18.5 days of gestation in the mouse, continues after birth in the spermatogenic cells as they undergo mitotic and meiotic division, and is complete by the pachytene phase of meiosis [169, 170].

Transgenerational passage of toxicant exposure effects

The reprogramming of DNA methylation that takes place in primordial germ cells is presumably acting to prevent the passage of DNA methylation defects from one generation to the next [169]. However, recent studies have raised concerns about the potential for exposures during pregnancy to cause germ-line effects on imprinted genes resulting in transgenerational passage of DNA methylation defects [171, 172]. Chemical effects on offspring due to *in utero* exposures can be defined either as multigenerational or transgenerational. An exposure is considered multigenerational if the changes are due to direct exposure, which could affect the F₀ mother, the F₁ embryo, and the F₂ germline. A transgenerational effect does not involve a direct exposure and is defined as the transmission of a phenotype between generations that persists even without additional chemical exposure. Since the germ line that generates the F₂ generation is present during a F₀ exposure, a F₂ generation phenotype is not a transgenerational phenotype. Only if this phenotype is present in the F₃ generation, the first generation that has not had direct exposure to the toxicant, can this be deemed a true transgenerational phenotype [173].

A number of studies have suggested that environmental chemicals, such as endocrine disruptors, promote a transgenerational phenotype due to embryonic or postnatal exposures through an epigenetic mechanism [172, 173]. Exposure to endocrine disruptors during the sensitive windows of testis development and epigenetic reprogramming has been hypothesized to permanently reprogram the methylation patterns of the germline, resulting in the transgenerational transmission of an altered phenotype, and further resulting in adult onset disease states. The reasoning behind this hypothesis is that due to the high frequency of adult onset diseases, and the low frequencies of DNA sequence mutations, an epigenetic mechanism that does not involve DNA sequence mutations underlies these adult onset diseases due to early exposures [172, 173]. While many DNA methylation changes are not heritable, it is thought that imprinted genes maintain methylation pattern in a heritable manner, and it has been proposed that alterations in the methylation status of imprinted genes may be a mechanism promoting these disease states [173, 174].

Vinclozolin, an antiandrogenic fungicide, and methoxychlor, an estrogenic pesticide, are endocrine disruptors that have been studied extensively for their potential to induce transgenerational effects. These studies have indicated that a persistent alteration in the epigenome can occur following *in utero* exposures [13]. The initial study that investigated the transgenerational effects of vinclozolin exposed gestating Sprague-Dawley rats to vinclozolin by daily intraperitoneal injection of 100 or 200 mg/kg from gestational days (gd) 8-14 [172]; breeding of these offspring continued for four generations. Analysis of the F₁ to F₄ generations revealed persistent reproductive effects in the males. There was a slight decrease in epididymal sperm counts and motility and, most significantly, increased spermatogenic cell apoptosis at a frequency of >90%. This frequency did not decline between the F₁ and F₄ generations, suggesting an epigenetic transgenerational effect [172]. When the vinclozolin F₂ generation males were out-crossed to wild-type untreated control females, the male progeny exhibited a similar phenotype of decreased spermatogenic capacity and male infertility, suggesting that the transgenerational phenotype is transmitted through the male germ line, likely through epigenetic changes (because the developmental period used for the endocrine disruptor exposure was during the re-methylation programming of the germ line). The authors then performed DNA methylation analysis of testicular tissue, focusing on the effects of vinclozolin on the total genome, and identified 25

different PCR products with altered DNA methylation patterns that are associated with the transgenerational phenotype [172].

Candidate DNA sequences with altered methylation were further investigated in caudal epididymal spermatozoa obtained from vinclozolin exposed F3 rats [174]. Fifteen of these sequences were found to be hypermethylated, prompting gene expression analysis; this was performed with gd16 male testis tissue in order to correlate these methylation changes to gene expression alterations. Some of the genes exhibited decreased expression, as expected with hypermethylation, whereas others exhibited increased expression. These investigations were taken a step further, and the F1 to F4 generations were analyzed for the development of adult disease states including prostate disease, kidney disease and testis abnormalities [175]. Vinclozolin appears to increase the disease prevalence, however the low sample size gives reduced confidence in these findings [13]. The authors suggest an epigenetic cause, but make no connection between epigenetic changes and the observations of increased adult disease prevalence. While some of these observations have been challenged [176, 177], the potential importance of epigenetic modifications producing transgenerational effects, and the specific example of vinclozolin, warrant further investigation.

Testis germ cell tumors and endocrine disrupting chemicals

Testicular germ cell tumors (TGCT) are most common among young men between the ages of 15-34 years old and are the predominant testis tumor type. They are characterized into subgroups based on histological characteristics: seminomas, non-seminomatous germ cell tumors, and spermatocytic seminomas [178]. In the United States, the occurrence of TGCT is slowly increasing [178]. Evidence suggests that men from European ancestry have a greater than five-fold incidence of TGCT compared to those of African descent, although the incidence of TGCT has been rising for black men as well [179]. The origins of TGCT are elusive; however, many investigators are exploring the possibility that fetal and early life endocrine disrupting chemical exposures can disrupt the critical hormonal balance during development, and in turn contribute to the formation of TGCT later in life [180].

TGCT, one of the four proposed components of TDS, has both genetic and environmental aspects associated with its occurrence. It was found that men who have some form of gonadal dysgenesis (i.e. 45X/46XY) are more likely to develop TGCT in conjunction with other male reproductive abnormalities such as hypospadias and cryptorchidism [37, 181]. TGCT is also accompanied by additional abnormalities within the seminiferous tubules [37]. Other strong associations include low birth weight [182, 183] or being born as a twin [184, 185]. These findings could be explained by altered nutritional intake or the presence of extra intra-uterine estrogens, but this is still under investigation [182-185]. Finally, lifestyle represents an additional risk factor that could impact the developing fetus: smoking, occupation, maternal habits, and socioeconomic factors should all be considered when looking for the origins of TGCT [37, 181, 186].

Loss of fertility is associated with TGCT, although further studies are needed to fully characterize this relationship [187]. Precursor cells, known as CIS cells, are thought to give rise to TGCT [186]. CIS cells do not undergo differentiation and appear similar in morphology to fetal germ cells. They are characterized by the presence of placental-like alkaline phosphatase (PLAP) [188]. Although CIS cells are similar in appearance to fetal germ cells, they lack intercellular bridges and are often found along the edges of TGCT, with the exception of spermatocytic seminoma [187, 189]. There is no known mechanism for the formation of CIS cells; however, due to their morphologic similarity with fetal germ cells, a developmental origin is one potential mechanism for TGCT induction. Because dysregulation in secretion, signaling, production, transportation and/or metabolism of

steroidogenic pathways could impair germ cell differentiation and the surrounding cellular environment (potentially initiating carcinogenesis), research into the regulation of steroidogenic pathways and their associated hormones is becoming increasingly important. Investigation into these pathways has paved the way for further exploration into environmentally ubiquitous endocrine disrupting chemicals (EDCs) that may exert anti-androgenic effects during a critical period in development, thereby predisposing the fetus to reproductive abnormalities [189].

Exposure to environmental toxicants: a potential link to testis cancer?

The mechanisms behind the development of testis cancer are still unknown, but both environmental and lifestyle factors have been associated with its development. Although the strength of the association is unclear, some EDCs have been identified that may play a role in testis carcinogenesis, including certain types of persistent organic pollutants (i.e. organochlorine pesticides) [187], and exogenous estrogens [181].

Animal studies are helpful in assessing the effects of exogenous estrogens or anti-androgenic chemicals after *in utero* or early-life exposures. Some examples of highly studied estrogenic compounds include DES, ethinyl estradiol, and bisphenol A (BPA), while anti-androgenic compounds include flutamide and vinclozolin. Results from animal studies have shown that *in utero* exposures to select EDCs can lead to the development of hypospadias, cryptorchidism and reduction in sperm volume in the majority of the animals exposed, and in some severe cases the formation of Leydig cell tumors [37]. These initial studies provide some insight on the effects of early life exposures, but the animal models have significant limitations in terms of their relevance to human TGCT.

Phthalates, which have a wide application in commercial and industrial plastics, have been shown to alter male reproductive development by acting as anti-androgens within the fetal testis of rats, as described above [37]. Phthalates induce multinucleated germ cells (MNGs) following a gestational exposure; MNGs have multiple nuclei that are contained within one cytoplasm, and are postulated to be formed from the abnormal differentiation of germ cells. Although MNGs and CIS cells share a putative origin as developmentally dysgenetic germline cells, MNGs do not appear to lead to testicular cancer in rodent models [84, 190, 191].

Congenital mouse models for exploring mechanisms behind TGCT formation

Numerous efforts have been made to develop a rodent model that could replicate the characteristics of human testis cancer. Unfortunately, no animal model to date has formed the precursor CIS cells seen in the human. Furthermore, typical model animals, such as rodents, reach puberty much more quickly than humans, which could be a constraint when investigating the harmful developmental effects of various toxicants [192].

In the 1950s, using the 129sv mouse substrain, Stevens generated the first mouse model that formed spontaneous testis tumors at a low frequency [193]. Spontaneous testis tumor formation is uncommon in other strains, suggesting that genetics play a key role. Mutations in the *Ter* gene led to tumors within weeks [194], related to a decrease in primordial germ cells initiated by the mutation [195]. A limitation of this mouse model is the formation of teratomas rather than seminomas, the more common TGCT type in humans.

An interesting tumor model more representative of a classic seminoma is found in mice that over-express glial cell line-derived neurotrophic factor (GDNF), normally expressed by Sertoli cells. Tumors begin to form at approximately one year of age, with 56% of mice presenting bilateral tumors; human males, on the other hand, tend to exhibit unilateral

tumors. Species-specific differences such as these emphasize the difficulties of extrapolating from animal models such as this to human TGCT [196].

Another important discovery in spontaneous tumor formation was made after investigating a deficiency in the p53 gene. Mouse models deficient in this gene form various types of spontaneous tumors, including testicular tumors [197, 198]. Mutation in this gene alters the normal cell-cycle control pathway, and is the probable reason for carcinogenesis formation. In about 50% of animals studies, tumors presented as early as 10 months [199]. It is clear that different genetic backgrounds are capable of predisposing rodents to varying degrees of susceptibility to tumor formation; for example, mice with a 129/Sv background tend to develop a higher proportion of testis tumors (35%) than mice with a mixed C57BL/6 × 129/Sv background (9%) [200].

Overall, the existing animal models for TGCT have significant limitations, limiting the ability to explore the relationship between EDC exposure and testis germ cell tumor induction using such systems.

Prostate diseases and endocrine disrupting chemicals

Development of the prostate

Prostate development in humans begins between the 11th and 12th weeks of gestation with the development of solid ducts from the urogenital sinus [201]. Testosterone and other androgens send messages through the androgen receptors (AR) in the urogenital sinus mesenchyme (UGM) that promote growth of these initial duct buds into branched formations throughout the UGM; these further develop into a multifaceted arrangement of ducts. Unhindered communication between the epithelial and mesenchymal tissues is critical in the developing prostate. The mesenchyme promotes ductal growth by stimulating the production of androgen receptors within the epithelium, ensuring continued propagation of epithelial tissue and related secretory proteins. In response to mesenchymal signaling, epithelial cells promote smooth muscle differentiation, further contributing to growth of the mesenchyme [202]. Due to the reciprocal nature of this signaling, dysregulation is particularly detrimental and has been implicated in the development of disease states [203]. In humans, the initial phase of prostate development is completed at birth, and resumes during puberty with increased levels of androgens. In rats and mice, prostate development follows a different trajectory; in these rodents, the prostate develops continuously from the end of fetal life until the beginning of adulthood [204].

The increasingly widespread incidence of prostate cancer has led to an explosion of research into its etiology. A dominant theory suggests that this disease, which manifests in adulthood, may have a basis in early development; for example, it is hypothesized that the use of exogenous hormones by pregnant women has led to an increase in developmental diseases in the prostate, testis, and breasts [205]. While the proposed mechanism remains unclear, data suggests that disruption of the natural hormonal balance of mother and fetus could result in a higher susceptibility to certain cancers.

Disruption of normal prostate development by exogenous estrogen exposure

Androgens were originally thought to play a central role in regulating normal and abnormal prostate progression. Androgens are well-known risk factors in certain age-related diseases, such as BPH and carcinoma [206], however, recent evidence suggests that estrogens may also play a large role in the developing prostate and that disruption of the natural androgen/estrogen hormone balance could lead to abnormal growth [207]. Elevated endogenous estrogen during late gestation can induce spontaneous squamous metaplasia or extra layers of basal epithelium cells that degenerate rapidly after birth once estrogen levels return to

normal. In aging adults, increased estradiol in conjunction with a decline in testosterone levels has been associated with the progression of prostatic diseases. Exogenous estrogens are ubiquitous in the environment, including in food (phytoestrogens) and its packaging. Natural exogenous estrogens, such as estradiol, are also found in foods, such as eggs, dairy and meat products, and can be almost 10,000-fold more potent in binding to the estrogen receptor than phytoestrogens [208].

Two of the most commonly studied estrogens include diethylstilbestrol (DES) and bisphenol A (BPA). DES, a strong exogenous estrogen, was commonly used to treat pregnant women with threatened spontaneous abortions from the 1950-1970s. In addition to functioning as an estrogen on the prostate, DES functions also has an anti-androgen effects by reducing the amount of luteinizing hormone (LH) that is secreted from the hypothalamic-pituitary axis. This reduction in LH causes a decrease in testosterone production by the testes, which affects the androgen-dependent prostate [209]. It has been reported that in male stillbirths of mothers who ingested DES during pregnancy, there was a greater incidence of prostate abnormalities, including squamous metaplasia, enlarged ducts, and enlarged utricle [210]. Yonemura *et al.* xenotransplanted human fetal prostate tissue into athymic male nude mouse hosts and treated them with DES for 1 month. Implants in treated hosts developed severe squamous metaplasia, and injuries persisted even after cessation of DES treatment and re-implantation into a non-treated host [211]. Interestingly, the DES plus testosterone co-exposed animals showed dysplastic lesions as well as the presence of carcinoma *in situ* cells [212]. These findings indicate that experiments concerning exogenous estrogen exposure during significant times in development are important to understanding disease progression in the prostate.

BPA, a ubiquitously present estrogen mimicking chemical, is a cross-linking agent used in the manufacture of polycarbonate plastics and epoxy resins. Residual, unpolymerized BPA present in the plastic may leach into food or other media, particularly after being exposed to high temperatures or continual washes. BPA, however, does not bind with high affinity to the estrogen receptors as compared to endogenous estrogens such as estradiol [213]. Early studies using animals and the subcutaneous route of exposure (bypassing first-pass metabolism by the liver) showed that low dose exposures to BPA during gestation could impact prostate growth and development by inducing an increase in cellular proliferation as well as the number of buds within the gland. These exposures also caused an apparent increase in adult prostate size without an accompanying morphological change [214].

To explore the importance of the timing of exposure to exogenous estrogens, Prins et al. developed a rodent model to determine whether “two hits” to endocrine disrupting chemicals, such as BPA, might predispose to carcinoma formation in adulthood. Briefly, low doses of either BPA (subcutaneous route) or estradiol were given to newborn male rats on neonatal days 1, 3, and 5 (one-hit), critical periods in development of the prostatic ducts. When the rats reached adulthood (day 90), a second treatment with estradiol was given by implanting silastic capsules that continuously released over 16 weeks (two-hit). Prostate intraepithelial neoplasia (PIN) lesions, the precursors to prostate cancer, were found in 100% of rats in this two-hit model of combined exposure to BPA and estradiol. In comparison, rats given corn oil during the neonatal period followed by estradiol exposure in adulthood had only 40% incidence of PIN lesions. In addition to the presence of PIN lesions, an increase in epithelial proliferation and apoptosis was noted [213, 214]. This model underscores the potential significance of early-life exposures to certain EDCs in predisposing to prostate disease later in life.

Summary

Since the term “endocrine disrupting chemical” was introduced in 1993, animal studies have aimed to define the effects of these toxicants on the male reproductive system at each stage of development – fetal, pubertal, adult, and senescent. Because these chemicals mimic the roles of endogenous hormones, they can disrupt the delicate hormonal homeostasis that protects against testicular injury. The proposed testicular dysgenesis syndrome, consisting of cryptorchidism, hypospadias, impaired spermatogenesis, and testicular germ cell tumors, is an hypothesis that ties together potential downstream effects of early life endocrine disruption of the male reproductive tract.

There are several points during reproductive tract development that are particularly sensitive to endocrine disrupting toxicants. In the fetal stage, hormones secreted by Sertoli cells and Leydig cells direct testis formation, testicular descent, spermatogenesis, and other important processes. It is during this stage that chemical insults could initiate disease processes that might not become visible until adulthood. Sexual maturation during puberty is initiated by gonadotropin releasing hormone from the HPG axis, increasing LH and FSH secretion from the pituitary gland. The resulting pubertal surge in testosterone initiates formation of seminiferous tubules and the migration and differentiation of germ cells into sperm. Formation of the blood-testis-barrier, an important immunological and reproductive toxicant barrier, occurs during the pubertal stage of development, making proper hormonal balance at this time critical for continued reproductive health. The end of puberty marks the beginning of adulthood and ultimately aging, when alterations in sperm quantity, morphology and motility, and the occurrence of reproductive tract cancers (testis germ cell cancer and prostate adenocarcinoma) might be increased if early life developmental processes were disrupted.

Many decades of research aimed at identifying and quantifying the effects of toxicants on the male reproductive system have generated a wealth of new knowledge, including initial insights into complex exposures, low dose responses, and transgenerational effects. Continuing research on male reprotoxicity, endocrine disruption, and the developmental origins of disease will yield new insights into the mechanisms responsible for disruptions of the male reproductive system throughout all life stages.

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Figure 1. Endocrine disrupting chemical effects upon the fetal testis. A) Endocrine disrupting chemicals alter both Leydig cell and Sertoli cell function, producing downstream abnormalities. B) As an example, multinucleated germ cells (arrows) are induced in gd18 fetal Fischer rat testis following 2 days of maternal exposure to 250mg/kg/day di-n-butyl phthalate. H&E staining. Bar = 50μm

Figure 2.

Cross-section of an adult rat seminiferous tubule demonstrating normal spermatogenesis. The lumen of the seminiferous tubule (*), and interstitial space (+) are identified (bar = 25µm). Along the bottom of the figure, the Individual cell types are labeled and magnified to illustrate their morphology (bar = 3µm). H&E staining.

Figure 3.
Chemical structures of model testicular toxicants: A) 2,5-hexanedione, B) 1,2-dibromo-3-chloropropane, C) ethylene-1,2-dimethanesulfonate, and D) carbendazim.