Environmental Impacts on Male Reproductive Development: Lessons from Experimental Models

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Keywords

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Abstract

Background: Male reproductive development in mammals can be divided into a gonadal formation phase followed by a hormone-driven differentiation phase. Failure of these processes may result in Differences in Sex Development (DSD), which may include abnormalities of the male reproductive tract, including cryptorchidism, hypospadias, infertility, and testicular germ cell cancer (TGCC). These disorders are also considered to be part of a testicular dysgenesis syndrome (TDS) in males. Whilst DSDs are considered to result primarily from genetic abnormalities, the development of TDS disorders is frequently associated with environmental factors. **Summary:** In this review, we will discuss the development of the male reproductive system in relation to DSD and TDS. We will also describe the experimental systems, including studies involving animals and human tissues or cells that can be used to investigate the role of environmental factors in in-

ducing male reproductive disorders. We will discuss recent studies investigating the impact of environmental chemicals (e.g., phthalates and bisphenols), lifestyle factors (e.g., smoking) and pharmaceuticals (e.g., analgesics) on foetal testis development. Finally, we will describe the evidence, involving experimental and epidemiologic approaches, for a role of environmental factors in the development of specific male reproductive disorders, including cryptorchidism, hypospadias, and TGCC. **Key Messages:** Environmental exposures can impact the development and function of the male reproductive system in humans. Epidemiology studies and experimental approaches using human tissues are important to translate findings from animal studies and account for species differences in response to environmental exposures.

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Development of Male Gonad and Reproductive Tract

In humans, development of the male gonads and the reproductive tracts takes place during 2 key phases: an initial phase of gonadal sex determination and testis dif-

ferentiation primarily driven by expression of specific genes, followed by a second phase of sex differentiation of the remaining reproductive system and the body more generally; a process driven by sex hormones primarily produced after the gonads have differentiated into testes.

Although genetic sex is determined at the point of fertilization by chromosomal pairing (46,XX for females and 46,XY for males), the developing gonad remains bipotential until approximately 6 gestational weeks (GW), after which it can develop into an ovary or a testis. The urogenital ridge, a precursor of the urinary and genital systems, is formed from approximately 4 GW from a thickening of intermediate mesoderm and proliferation of overlying coelomic epithelium [1], under regulation of a number of genes including *NR5A1* (also called *SF1*), *WT1*, *EMX2*, and *Lhx9* [2, 3]. The gonads emerge from the ventral surface of the mesonephros [4], and each mesonephros also contains the mesonephric duct (Wolffian duct) and paramesonephric duct (Mullerian duct), which are precursors of the male and female reproductive tracts [5, 6]. Primordial germ cells migrate from the yolk sac to the urogenital ridge at 5 GW.

Although the primary sex determining gene in humans and most mammals is *SRY*, several important genes and regulatory networks have been found to be required for proper differentiation of the bipotential gonads into either testes or ovaries [7–9]. In the XY gonad, the expression of *SRY* in a subset of pre-Sertoli cells at approximately 7 GW acts as the dominant “switch” in initiating testicular development [10]. The main function of *SRY* appears to be the transcriptional upregulation of *SOX9*, which in turn acts as the master regulator of early testis differentiation. In fact, *SOX9* expression is in itself sufficient to drive testis development in the absence of *SRY* [2, 11]. In the testis, *SOX9* initiates differentiation of the supporting cell lineage into Sertoli cell and prevents their differentiation into the ovarian counterpart, the granulosa cells.

The synthesis of steroid sex hormones, primarily testosterone, by the differentiated testes results in sex-specific tissue responses that determine phenotypic male sex. In addition to sex hormones originating from the Leydig cells, Sertoli cells secrete anti-Mullerian hormone (AMH) which, via BMP-like signalling pathways, causes regression of the Mullerian ducts which are the precursors for the female reproductive tracts [12]. Sertoli cells also secrete desert hedgehog which induces the development of foetal Leydig cells that, from 8 to 9 weeks of development, secrete testosterone and insulin-like peptide 3 (*INSL3*) [13]. Testosterone promotes differentiation of the Wolff-

ian duct into epididymis, vas deferens, and seminal vesicles and, along with *INSL3*, contributes to testicular descent [14]. Testosterone is converted to its more potent product dihydrotestosterone by the enzyme 5-alpha reductase in androgen-sensitive target tissues, where it acts on the androgen receptor to prompt masculinization of the external genitalia and the body more generally [15–17]. Testosterone is also important for development of secondary sexual characteristics during puberty and for supporting sperm production in adulthood. In humans and other primates, there is also a critical “backdoor pathway” where other organs such as the placenta contribute to androgen production [16, 18].

DSD and TDS

The descriptive term Differences in Sex Development (DSD) refers to a heterogeneous group of congenital conditions in which chromosomal, gonadal, or anatomical sex development is atypical [19]. Many conditions can be traced to specific gene mutations that cause the pathophysiological manifestations of a DSD.

The subcategory 46,XY DSD refers to a group of disorders characterized by an XY karyotype and includes the developmental disorders of testicular development, complete gonadal dysgenesis, partial gonadal dysgenesis, and testicular regression syndrome. Abnormal testicular development, termed testicular dysgenesis, affects the function of the gonad. Disturbed Leydig cell differentiation and function lead to decreased androgen production, ultimately affecting the development of the external genitalia and testicular descent, whilst impaired Sertoli cell function can result in the persistence of Mullerian structures due to a reduction in AMH. Disturbed Sertoli and Leydig cell function can also result in impaired germ cell maturation and subsequent infertility. In addition, individuals with 46,XY DSD have an increased risk of developing testicular germ cell cancer (TGCC) as a result of the presence of y-chromosome material [20].

Complete gonadal dysgenesis is characterized by normal female external genitalia, completely underdeveloped gonads, no sperm production, and the presence of normal Mullerian structures. Partial gonadal dysgenesis is characterized by external genitalia with mild to severe penoscrotal hypospadias with or without chordee, dysgenetic testis, reduced to no sperm production, and Mullerian structures that range from absent to fully developed uterus and fallopian tubes.

Fig. 1. Overview of TDS and DSD. Genetic (including causative mutations and epigenetic factors) and in utero environmental factors may impact on the development of these common male reproductive disorders. Note the overlap in clinical features of these 2 entities. TDS, testicular dysgenesis syndrome; DSD, Differences in Sex Development.

Causative genes involved in the developmental pathway of testicular formation have been identified in 46,XY DSD, including: *SRY* (deletion or sequence variant), *NR5A1* (*SF1*; sequence variant), *SOX9* (sequence variant), *NROB1* (*DAX1* duplication), and *WNT4* (duplication) (reviewed in [21–24]). Genetic causes of DSD may also result in additional non-reproductive phenotypes as a result of additional systemic roles of the genes involved. Due to its role in chondrocyte differentiation, *SOX9* loss of function mutations lead to a combined phenotype of 46,XY DSD gonadal dysgenesis and campomelic dysplasia, a syndrome characterized by various severe skeletal malformations [25–27]. A molecular diagnosis is typically made in <40% of cases of 46,XY DSD, although a recent report describes a molecular diagnosis in >60% of cases [24]. The impact of abnormali-

ties in genes such as *NR5A1* on phenotype can vary considerably even within families with the same mutation suggesting interplay between other factors such as modulating genes or environmental factors.

Testicular Dysgenesis Syndrome

Testicular dysgenesis syndrome (TDS) describes the observed association between poor semen quality, testicular cancer, undescended testis and hypospadias, and proposes a common link to disrupted foetal testis development or function [28]. Epidemiologic studies show that cryptorchidism, impaired spermatogenesis, hypospadias, and testicular cancer are associated as risk factors for one another [29], which may suggest a common origin. Testicular dysgenesis has been demonstrated in biop-

sies taken from the contralateral testis of men with testicular cancer and in some infertile men, which indicates a biological link between these disorders. TDS may be attributed to environmental exposure, genetic factors, intrauterine growth disorders, and lifestyle factors that affect testicular development [30]. There is evidence that the prevalence of TDS conditions are increasing, and Scandinavian databases have documented a decline in fertility and sperm quality [31] and an increasing demand for ICSI. Testicular cancer incidence has doubled every 20–30 years in many populations [32] and is associated with reduced fertility. Cryptorchidism and hypospadias are common genital birth defects, the incidence of which shows wide geographic variation and trends towards an increase in cases have been reported in several countries, as further discussed in later sections.

The overlap between clinical features of 46,XY DSD and individuals with TDS disorders suggest that there may be some common factors involved in their aetiology, and shared mechanisms that mediate disturbance of testicular development and function during foetal gonadal development. Furthermore, the phenotypic variation in individuals with the same XY DSD causing gene mutations, combined with the increasing prevalence of TDS, indicates that adverse environmental factors are likely to influence the development of DSD and TDS (Fig. 1).

Environmental Factors

Environmental factors are recognized to play an important part in the development of reproductive disorders in animals and human [33]. This may include exposure to ubiquitous environmental chemicals through direct contact or ingestion in food and drink (e.g., phthalates and bisphenols), exposures relating to lifestyle factors (e.g., maternal smoking), or exposure to medicinal agents (e.g., pharmaceuticals).

Environmental Chemicals, Pharmaceuticals, and Lifestyle Factors

Early descriptions of associations between exposure to environmental chemicals and impacts on reproductive development came largely from studies reporting effects on wildlife exposed to, for instance, persistent organic pollutants. Strong associations were demonstrated in several species of mammal, birds, and reptile (reviewed in [34]). As an example, evidence for endocrine disruption leading to male reproductive abnormalities was reported in alligators following a pesticide spill in Lake Apopka,

Florida in 1980 [35]. Alligators exposed to pesticides had a 25% reduction in penile size and a 70% reduction in testosterone levels, compared with similar sized alligators from Lake Woodruff, also in Florida. Interestingly, subsequent studies on these alligator populations revealed that the size of the phallus was not correlated to the levels of the pollutants (pesticides and polychlorinated biphenyls) in the juvenile period, leading the authors to hypothesize that reproductive disorders may be the result of exposure to pollutants during foetal development [36]. In the following years, and further evidence for this hypothesis was provided by an increasing literature in laboratory settings, relying heavily on common mouse and rat strains. In the early 1990s, endocrine disrupting chemicals were proposed to contribute to the increasing incidences of male reproductive disorders [37]. Then, at the turn of the century, the mounting evidence for a causative link between early life exposure to endocrine disrupters and male reproductive disorders lead to the elaboration of the TDS hypothesis [28].

The importance of androgen signalling during foetal life in programming male reproductive development had been appreciated since the mid-1900s following seminal work by the late Alfred Jost. A more formalized and elaborate characterization of how and when androgen action is pivotal during development was highlighted by the identification of the masculinization programming window (MPW) in rats, limiting the influence of androgen signalling to key programming windows for specific target tissues [38]. By perturbing androgen production or action during the MPW in rats, which corresponds to e15.5–e19.5 (considered to correspond to around 8–14 weeks gestation in humans), the male offspring will present with male reproductive disorders [38, 39].

The number of chemicals now having been shown to induce male reproductive disorders in animal models is high and includes phthalates, bisphenols, pesticides, and more, as shown in Figure 2. In addition to these industrial chemicals, there has also been an increasing concern about pharmaceuticals, not least non-prescription drugs. This includes analgesics; the most commonly used pharmaceutical agents during pregnancy, many of which are available over the counter [40]. Also, there are increasing concern about anti-fungal medication (the azole fungicides), as they can be potent inhibitors of steroidogenic enzymes critical for testosterone production in the human foetal testis [41, 42]. A challenge with both non-prescription analgesics and fungicides is that their use is poorly monitored, with potential high exposure doses if used excessively during pregnancy. This is of concern in

Fig. 2. Environmental factors and mechanisms of action proposed to impact male reproductive development based on epidemiologic and/or experimental evidence.

itself, but increasingly so when considering the ubiquitous background exposure to environmental chemicals which potentially contribute to a high cumulative load of chemicals substances with shared modes of action and may cause detrimental health effects [43, 44].

Whilst the majority of studies investigating the effects of environmental factors on male reproductive development have focused on environmental chemicals, the impact of maternal lifestyle has also been implicated in the development of these disorders. This includes smoking, alcohol, diet, and obesity (Fig. 2). Although some of these lifestyle factors are also “chemical exposures” (e.g., smoking [39]), they nevertheless represent a complex parameter for possible external insults to male reproductive development. Much of the evidence for lifestyle factors is derived from human epidemiological studies, but there are also a number of studies on exposure to alcohol and components of cigarette smoke on human tissues (reviewed in [45]) and animal models. Studies investigating the impact of diet and obesity largely involve in vivo exposures or epidemiologic approaches, although distinguishing between specific dietary elements, chemicals used in food packaging (e.g., bisphenols) and the metabolic consequences of obesity, itself can be challenging when trying to determine causation for male reproductive disorders.

Evidently, the potential impact of specific environmental chemicals, lifestyle factors, and pharmaceutical

exposures on male reproductive development are many and concerning. Thus, examples of studies examining effects of environmental factors and pharmaceuticals using experimental models will be discussed in more detail in subsequent sections of this review.

Models to Determine Impacts on Sex Development

Despite the similarities between human, mouse, and rat foetal testicular development and function – including processes of sex determination, sex-specific differentiation of gonads and germ cell development – there are also some important differences between rodent and human. This includes differences in regulation of foetal steroidogenesis, timing of testicular descent, and germ cell differentiation from gonocyte to spermatogonia [46–48]. It is therefore essential to validate findings from rodent studies, in relation to basic aspects of testicular development and function and consequences of exposure to environmental factors, using experimental systems that involve human tissues and cells. Several experimental models have been established in different species, including in vitro based models, such as cell lines, primary cell culture, and ex vivo culture of intact fragments of foetal testicular tissue. In addition, xenograft models can be used in which cells organoids or intact tissue fragments are transplanted

Fig. 3. Illustration of research tools, involving humans, human tissues, or animal models to investigate environmental impacts on male reproductive development. Clinical outcome measures are indicated in red, whilst histologic and biochemical measures are indicated in blue. TDS, testicular dysgenesis syndrome.

into mice [49–51]. Additionally, epidemiologic studies can be utilized to examine the impact of environmental exposures on sex development (reviewed in [52]). It is important to carefully consider the available end points and advantages and limitations for each of these approaches before deciding which to use when addressing a specific research question. Here, the available experimental and epidemiologic approaches (summarized in Fig. 3) are outlined, and examples of how they have been used to

examine various aspects of environmental impact on testicular development and function will be described.

Experimental Models

In vitro Culture Systems

Testicular in vitro culture systems include the use of immortalized cell lines and dissociated primary testicular cell populations, which are most often set-up in a 2D-system, with cells growing either attached or in suspen-

sion. Alternatively, dissociated testicular cells can be cultured *in vitro* on a supportive matrix, such as soft agar, Matrigel, or artificial scaffolds in a 3D-system [49–51]. To date, *in vitro* culture systems have not been extensively used to study basic aspects of human foetal testis development and potential impact of environmental exposures. This is mainly due to (1) the lack of available immortalized cell lines from human foetal testis and (2) the limitations related to the loss of interactions between different testicular cell types, which are considered to be essential to investigate normal testicular development and function. *In vitro* cell culture of testicular cells in rodents and humans, including germ cells, Sertoli cells, Leydig cells, and peritubular myoid cells, as well as malignant germ cells, have all been reported with some studies using co-culture of several cell types to compensate for the lack of cell interactions [53–57]. Although, promising results could be obtained using dissociated testicular cell suspensions from juvenile rodents, showing the potential of these cells to reaggregate as testicular organoids, progression of germ cell differentiation has not been reported, whilst organoid formation was not obtained using tissue samples from adult animals [55, 56].

Another difficulty, besides the lack of a functional *in vitro* system, is that no well-characterized immortalized human foetal testicular cell lines exist. This is likely due to the general notion that testicular cells do not culture well in single-cell suspension even when isolated from foetal testis that are at a developmental time point where most testicular cell types are proliferating. This is particularly evident for germ cells that are difficult to culture outside the somatic niche, most likely providing an explanation for the unsuccessful attempts to establish immortalized cell lines of non-malignant human testicular germ cells.

In contrast to the difficulty in establishing non-malignant testicular germ cell lines, a number of immortalized human cell lines from testicular germ cell tumours (TGCT) have been established (reviewed in [58]). The majority originate from embryonal carcinoma (or metastasis thereof) although few seminoma-derived cell lines exist. Consequently, these cell lines preserve some embryonic germ cell characteristics that are due to the foetal origin of testicular germ cell tumour precursor cells, termed germ cell neoplasia *in situ* (GCNIS), which are considered to be developmentally arrested foetal gonocytes [59]. Consequently, studies have primarily relied on the embryonal carcinoma-derived cell lines as a proxy for human foetal gonocytes, which may be an acceptable strategy if cautiously interpreted. Several *in vitro* studies

have examined the effects of environmental chemicals and pharmaceuticals in the embryonal carcinoma-derived cell line NTera2 as a proxy for human foetal germ cells. For example, cytotoxic effects of tributyltin, which have been used as a biocide in anti-fouling paint, have been found in NTera2 cells. This includes inhibition of cell growth and differentiation [60], induced mitochondrial fragmentation [61], and G2/M cell cycle arrest [62] after exposure to nanomolar levels. Also, the effects of arsenic exposure (0.01–5 μ M) have been examined in NTera2 cells, which resulted in altered RAR-dependent gene transcription [63]. NTera2 cells have also been used to examine effects of pharmaceutical drugs, including therapeutically relevant doses of the analgesic's paracetamol (acetaminophen) and ibuprofen. This resulted in reduced numbers of NTera2 cells, reduced transcriptional expression of pluripotency factors, and altered expression of epigenetic regulators [64].

Importantly, the use of immortalized testicular cancer cells does not require access to fresh human foetal testis tissue, which can be challenging to obtain. However, there are a number of important limitations with this type of *in vitro* culture, including in particular the lack of signalling from the other cell types present within the testicular niche, ultimately resulting in loss of original cell type characteristics in these cell lines. Moreover, immortalized cell lines are often prone to acquisition of additional genetic mutations that favour proliferation with increasing time of culture. Thus, *in vitro* cell culture of dissociated primary cells obtained from foetal testis tissue constitutes an attractive alternative strategy. With this approach, an initial experimental step to dissociate the testicular tissue into a single cell suspension is required. The cells are then either cultured directly in cell medium or subjected to an enrichment step in order to isolate or increase the number of a specific cell type within the suspension. This can be achieved by fluorescent-activated cell sorting or by specifically optimizing culture conditions to propagate the cell type of interest. Few studies have used culture of single cell suspension from foetal testicular samples, including male germ cells [65] and Sertoli cells [66]. A recent study reported a co-culture system of dissociated human foetal Sertoli cells and germ cells isolated from second trimester foetal testes [67]. However, to the best of our knowledge these types of *in vitro* culture have not been used to examine the effects of environmental chemicals and pharmaceutical drugs. Together these studies demonstrate that dissociated foetal testicular cells could be maintained in *in vitro* culture and be used to examine effects of environmental chemicals

and pharmaceuticals in future studies. However, culture of dissociated primary cells is generally considered unsustainable for longer culture periods with an overall time-dependent decay and gradual loss of unique cell characteristics in the surviving cell population, which should be considered in the experimental design.

In recent years, there has been an increased focus on in vitro generation of testicular cells (particularly germ cells) from human embryonic stem cells (ESCs) or from induced pluripotent stem cells (iPSCs). This includes the generation of human PGC-like or germ cell-like cells derived from human ESCs/iPSCs [68–72]. Also, 2 recent studies reported a model where human iPSC-derived PGC-like cells with rodent testicular cells were co-cultured [73, 74]. The study by Hwang et al. [74] reported the differentiation of hPGC-like cells into further differentiated germ cells expressing TFAP2C, DAZL, and DDX4, when co-cultured for up to 77 days with dissociated cell suspensions of foetal murine testes. Differentiation of human iPSC has also been differentiated into Sertoli-like cells [75] and PGC-like and Sertoli-like cells in co-culture [76–78]. Interestingly, Knarston et al. [79] recently reported the generation of human foetal gonad organoids from human iPSCs. These 3D gonadal structures were generated in a stepwise differentiation protocol to first obtain cells expressing bipotential cell markers and subsequently testicular Sertoli-like cells [79]. However, despite the progress made in the in vitro generation of foetal testicular-like cells from ESC/iPSC, these have so far not been used to examine the effects of environmental chemicals or pharmaceutical drugs.

Ex vivo Tissue Culture Approaches

Ex vivo culture of human foetal testicular tissue fragments has in recent years been extensively used to examine effects of environmental chemicals or pharmaceutical drugs, thereby providing important information about how these can affect human foetal testis development and function. Several types of ex vivo culture approach have been established, including culture on porous membranes, in “hanging drops,” and on agar blocks in the air-liquid interphase. These models have the same advantages and limitations overall. Limitations are mainly related to a relatively short culture period of up to 2 weeks and the need to culture small ($\sim 1 \text{ mm}^3$) tissue fragments to avoid necrosis/apoptosis in the centre of the tissue. Advantages related to the preservation of testicular morphology, spatial organization, interactions between the different testicular cell types, paracrine signalling, and endocrine function within the intact foetal testicular frag-

ments [80–85]. This ensures maintenance of the foetal testis tissue to support continued germ cell development, differentiation of the somatic cells, and endocrine function. This allows examination of functional outcomes following manipulations such as increased steroid hormone production observed when, for example, LH or hCG is added to the culture media [81, 82, 84]. Therefore, ex vivo culture approaches provide important advantages compared to in vitro culture of dissociated testicular cells and may be an attractive experimental option to examine human foetal testicular development and function following exposure to environmental chemicals and pharmaceutical drugs.

Culture of human testicular tissue on cell culture membranes has been successfully used by several laboratories to examine the effects of environmental chemicals and pharmaceutical drugs on human foetal testis development and function. This includes determining the effects of the phthalate MEHP (metabolite of DEHP) on human foetal testis tissue. No effects on testosterone production were found [82, 84], but the number of germ cells were reduced as a result of increased apoptosis [84]. Also, the effects of bisphenols have been examined using this experimental approach. One study examined the effects of bisphenol A (BPA) and found that exposure to BPA at 10^{-8} – 10^{-5} M resulted in reduced testosterone production, whilst transcription of INSL3 was also reduced when tested at a single dose of 10^{-8} M [86]. Accordingly, similar effects of the alternative bisphenols S and F on testosterone production was observed in human foetal testis cultures [87]. Also, bisphenol A (10^{-6} M) has been reported to increase germ cell apoptosis [88].

Analgesics are used by the majority (55–80%) of pregnant women, with several analgesics (e.g., paracetamol) being available without prescription in many countries [64]. Culture of human foetal testis tissue on membranes has also been used to examine the effects of analgesics in several studies, including a study investigating the effects of indomethacin, aspirin, and paracetamol [85]. Paracetamol reduced the secretion of INSL3, while no effect was observed for indomethacin, and aspirin. Unexpectedly, indomethacin and aspirin stimulated testosterone production, particularly in cultures of more immature testis samples (GW 8–9) and aspirin-stimulated AMH production [85]. A more recent study also examined the effects of ibuprofen on human foetal testis and found suppressed testosterone and INSL3 production, but only in foetal testis tissue from GW 8–9 GW in which the transcription of steroidogenic enzymes was also reduced [89]. Ibuprofen also reduced secretion of AMH

and transcription of *AMH* and germ cell markers but only in testicular samples from early first trimester [89].

Culture of human foetal testis tissue in “hanging drops” has so far mainly been used to examine signalling involved in regulation of meiosis [83] and sex-specific gonadal differentiation [90, 91]. However, a couple of studies used this experimental approach to examine the effects of pharmaceutical drugs [64, 92]. The effects of chemotherapeutic drugs (cisplatin and carboplatin) on human foetal testis development were also examined using this model and found to result in reduced germ cell numbers (affecting both the number of gonocytes and pre-spermatogonia) and reduced germ cell proliferation. The germ cell loss persisted for up to 12 weeks (after subsequent xenografting of exposed human foetal testis tissue fragments) [92]. Additionally, hanging drop culture of human foetal testis tissue was used to determine effects of analgesics (paracetamol and ibuprofen) on germ cell development [64]. This study found a reduced number of gonocytes and gonocyte proliferation after exposure, which was in accordance with results from other experimental models, including in vivo (rat), in vitro (NTera2 cells), and xenograft experiments [64].

Ex vivo culture of human testicular cells and tissue fragments maintains important paracrine signalling and cell interactions and this type of culture approach can therefore recapitulate some of the in vivo cellular processes, including cell proliferation, apoptosis and differentiation, secretion of factors from Sertoli cells, and production of steroid hormones in the Leydig cells. The model can also be used to determine the effects of modulating testicular development and function with exogenous factors and chemicals/pharmaceuticals. However, an important limitation is the failure of these systems to sustain cells over a long period. In particular, these models do not sustain germ cell differentiation and meiosis, nor do they continuously maintain the differentiation of testicular cells during development. Therefore, these culture systems do not support full spermatogenesis, which is in contrast to similar tissue culture approaches in mice where complete in vitro spermatogenesis has been achieved in several studies [93–95]. This suggests that species-specific differences exist in testicular function and germ cell differentiation, in particular related to the requirements needed to successfully support meiosis between human and mice. Indeed, all current in vitro models using human testicular cells and tissue fragments are valuable only for a limited duration and thereafter the Sertoli cells appear to de-differentiate and the germ cells are slowly but progressively lost. Methods that involve

transplantation of human testicular cells or human testicular tissue into a host animal have therefore increasingly been used when examining human testicular development and function over a longer time period.

Xenograft Models and Transplantation Approaches

Transplantation of human foetal testicular cells or tissue fragments has in recent years been used to investigate the effects of environmental chemicals and pharmaceutical drugs. This involves transplantation into immunodeficient mice. The testicular cells or tissue fragments may have been exposed prior to transplantation or can be exposed via the host mouse. The advantages of this experimental strategy mainly relate to the possibility of longer culture periods and the establishment of blood supply to grafted tissue fragments, which allows for tissue growth without the development of a necrotic core within the tissue fragment. Limitations related to this approach include the difficulty in determining the dose to which the transplanted cells or tissue have been exposed (possible metabolism in other organs) and more practically the need for access to animal facilities where immunodeficient mice can be housed.

Germ Cell Transplantation

Germ cell transplantation involves the introduction of cells into the seminiferous tubules of immunodeficient male mice through direct injection via the rete testis or efferent duct [96]. Removal of the endogenous germ cell population is required and may be achieved by treating host mice with busulfan prior to transplantation [97], or by using a host mouse strain in which germ cells are lost as a result of a genetic mutation [98]. Cells for transplantation can be prepared from a spermatogonial stem cell (SSC) line [99], or following enrichment of germ cells (or SSCs) dissociated from foetal testicular tissue. This approach overcomes an important limitation of culturing SSCs in vitro, namely, the lack of a somatic niche. Hence, signalling between different testicular cell types and an endocrine environment are preserved, while phenotypic changes induced by long-term ex vivo culture may be avoided. However, to the best of our knowledge no studies have so far reported effects of environmental factors on human foetal testis development and function using this experimental approach.

Xenografting

This experimental approach involves the transplantation of human foetal testis fragments ($\sim 1 \text{ mm}^3$) into castrated immunosuppressed mice either subcutaneously,

beneath the kidney capsule or intratesticularly (reviewed in [100]). This technique has the advantage over germ cell transplantation in which it maintains the germ cells within their normal niche, thereby maintaining cell interactions with the original somatic cells of the foetal testes. The host animal provides conditions that resemble the original milieu, which includes the establishment of blood supply to the grafted human foetal testis fragments, thereby permitting extended culture periods and the possibility to examine human testis development and function as well as effects on these following exposures to environmental factors in a situation that mimics *in vivo* conditions. Exposure to environmental chemicals and pharmaceutical drugs can be performed either prior to grafting, for example, in *ex vivo* culture followed by subsequent grafting to examine long-term effects, or be administered via the host animal. This may provide a challenge in predicting the dose that reaches the grafted testicular tissue but has been successfully used to examine the effects of both environmental chemicals and pharmaceutical drugs.

Studies using xenograft models of human foetal testis tissue to examine effects of environmental factors on human foetal testis development and function have primarily grafted tissue subcutaneously with overall high graft survival being reported [101]. Importantly, continued differentiation of both germ cells and somatic cells has been observed, and Leydig cell function is maintained as is evident by continued secretion of testosterone, which can be measured in serum or by determining the seminal vesicle weights of host mice as a read out of testosterone production [101]. The grafted human foetal testicular tissue responds to administration of hCG (to the host mice) by an increase in testosterone production, thereby demonstrating a functional response that mimics the *in vivo* situation [101, 102]. The effects of phthalates on human foetal testis development and function have been examined in several studies. Exposure to di-n-butyl phthalate (DBP) and monobutyl phthalate did not affect serum testosterone levels and seminal vesicle weight in the xenograft model, which was in contrast to DBP-mediated effects on seminal vesicle weight after xenografting of rat testis tissue [102]. Accordingly, a study by a different group found no reduction in expression of genes that regulate foetal testosterone biosynthesis in grafted human foetal testis tissue exposed to DBP [103]. Similarly, a study using a slightly different xenograft model (grafting of tissue into the renal subcapsular space) found that DBP did not affect testosterone production and weight of androgen-sensitive host organs, while these androgenic endpoints were reduced following treatment with CYP17A1-

inhibitor abiraterone acetate [104]. A recent study also examined effects of BPA exposure using a xenograft model and found no effects on hCG-stimulated androgen production as evaluated by both plasma testosterone level and seminal vesicle weight in host mice following grafting of testicular tissue from both first and second trimester [88]. The xenografting approach has also been used to examine the effects of analgesics on second trimester human foetal testis, including paracetamol which was found to reduce plasma testosterone levels and seminal vesicle weight after exposure to clinically relevant doses and regimens [105]. Also, effects of ibuprofen were examined using the xenograft model but no effects on serum testosterone levels and seminal vesicle weight was reported [89]. The effects of paracetamol and ibuprofen on germ cells were also examined using the xenograft approach, which reported reduced numbers of germ cells after exposure to paracetamol and ibuprofen for 7 days and paracetamol exposure for 1 day [64].

Environmental Influences on Specific Disorders of Male Reproductive Development

Cryptorchidism

Cryptorchidism is a condition in which one (unilateral) or both (bilateral) of the testes fail to descend from the abdomen into the scrotum. Cryptorchidism is a common male reproductive disorder with an incidence rate of approximately 1/10–50 live male births and requires surgical correction if they fail to descend in the first 6 months of life [106]. Several epidemiologic factors suggest an environmental contribution to its pathogenesis. This includes the increasing incidence in recent decades and geographical variation in incidence [33]. The process of testicular descent can be divided into 2 phases, each of which is considered to be driven by 2 key Leydig cell-derived hormones. The trans-abdominal phase is largely under the control of insulin-like 3 (Insl3), whilst the inguinal phase is driven by testosterone [107]. As a result, factors that impact on production of these hormones *in utero* can be considered risk factors for cryptorchidism.

Several epidemiologic studies have provided evidence for a direct association between environmental factors and cryptorchidism. Phthalates are one of the most frequently studied environmental chemicals and an association between *in utero* exposure to phthalates and cryptorchidism has been reported in some studies, albeit with self-reporting of exposure [108], whilst others report no association based on measurement of phthalates in amni-

otic fluid [109], breast milk [110], or maternal urine [111]. Similarly, for BPA exposure, there are conflicting results regarding association with cryptorchidism (reviewed in [45]), which may also reflect study design and method for measurement of exposure. For pharmaceuticals, association between analgesic exposure and cryptorchidism in the offspring has been described in the majority of studies (reviewed in [112], although one study did not report an association [113]. Positive associations between cryptorchidism and exposure to several other environmental agents including smoking [114], alcohol [115], and diethylstilboestrol (a synthetic oestrogen) [116] have also been reported. In addition to investigating associations between environmental exposures and cryptorchidism directly, anogenital distance (AGD) can also be used as an indicator of foetal testosterone production with several studies reporting associations between AGD and environmental exposures (reviewed in [45]). And perhaps more revealing, there are direct associations between a short AGD in boys and cryptorchidism [117, 118].

Experimental animal studies investigating the direct impact of environmental factors on cryptorchidism are restricted to in vivo approaches. The most frequently reported is the induction of a “TDS-like syndrome” (including cryptorchidism and hypospadias) in male rats exposed in utero to phthalates [119]. Animal studies may also be used to measure effects of exposure on testosterone production as an indirect indicator of potential for cryptorchidism, either by measuring testosterone or AGD in the offspring. Phthalates have been shown to reduce testosterone production in rat foetal testis in several studies [119–122]. However, conflicting results have been obtained with respect to effects of bisphenol [45] or analgesic exposures [112] on testosterone production in exposed rat foetal testis following either in vivo or in vitro exposure.

Experimental studies using human testis tissues to investigate potential association between environmental exposures and cryptorchidism primarily rely on the use of proxy measures (e.g., testosterone or *Ins13* production) for the disorder after in vitro exposure of human foetal testis tissues. To date, all studies investigating the impact of phthalate exposure on either testosterone or *Ins13* production in human foetal testis tissues have shown no effect [45], whilst effects of bisphenols on testosterone and *Ins13* production have been reported only under specific conditions, dependent on the dose, timing and duration of exposure [123, 124]. For analgesic exposures, the majority of exposure conditions investigated to date do not report a reduction in either testosterone or *Ins13* produc-

tion [85, 89, 124]. An important limitation of the ex vivo systems is the lack of a physiologic exposure to the environmental agent. Recently, xenografting of human foetal testis tissues has been used to test, the impact of exposure on testosterone production by exposing the host animal to specific environmental chemicals or pharmaceuticals. Overall, using this approach, exposure to phthalates [82, 102–104, 125] and bisphenols [88] did not affect testosterone production, reflecting the ex vivo findings described above. However, analgesic (paracetamol) exposure resulted in a reduction in testosterone production following a prolonged (7 day) exposure [105]. The impacts of environmental exposures on hormone production from human foetal testis have recently been comprehensively reviewed [45].

Hypospadias

Hypospadias is a malformation of the penis in which the urethral opening (the meatus) is located distal to its normal position on the tip of the glans. It is the second most common birth defect of the male reproductive system and is estimated to occur in 1/150–300 live male births [126], albeit the prevalence varies greatly across countries and ethnicities [128]. The majority of cases present with anterior hypospadias (a relatively small displacement of the meatus), but with other patients presenting with more severe proximal hypospadias where the meatus is located somewhere along the underside of the penile shaft and surgical intervention may be required. Boys with hypospadias also present with additional urogenital abnormalities more frequently than healthy boys, not least cryptorchidism [129, 130].

Although several gene mutations have been linked to hypospadias, the aetiology of most cases remains unknown [128, 131]. This fact, alongside a reported rise in frequency across many parts of the world, would suggest an environmental influence. Since the development of the penis is highly sensitive to sex hormones [132], EDCs have been proposed to play a major role in the development of hypospadias [126, 128, 131, 133]. In humans, several pharmaceuticals and environmental chemicals have now been associated with an increased risk of hypospadias (recently reviewed in [131]) and include pharmaceuticals such as progestins, clomiphene, analgesics, antidepressants and diethylstilboestrol, and environmental chemicals such as pesticides, paints, detergents, cosmetics, valproic acid, flame retardants, and phthalates. To further substantiate this association between foetal exposure to EDCs and the development of hypospadias in humans, a growing number of animal studies lend support to a postulated cause-

effect relationship between foetal exposure to EDCs and the development of penile malformations.

Rodent studies have established that androgens play a central role in penis development, including urethral closure [134–137]. Interestingly, animal studies have also implicated a role for oestrogens in penis development, suggesting that the androgen-oestrogen balance may be important for proper genital differentiation (reviewed in [131]). This lends support to the abovementioned chemical substances that are associated with hypospadias in humans, as many of them are not classical anti-androgens in their mode of action. This does not detract from the central role that androgens (particularly dihydrotestosterone) play in penis development, but highlight the complex interplay between endocrine signalling pathways in target tissues.

There are several rat toxicity-studies that have shown a clear link between EDC exposure and hypospadias. Most of these studies indicate an anti-androgenic mode of action since male AGD was also shorter in male offspring. AGD is a retrospective biomarker for compromised androgen signalling during the MPW [15]. For instance, exposure to flutamide [135] or finasteride [138] induces hypospadias in rats. A combined foetal exposure to linuron and benzyl-butyl phthalate induced a high rate of hypospadias in rats [139], as do the combined exposure to vinclozolin, flutamide, and procymidone [140, 141], or a mixture of DEHP, vinclozolin, prochloraz, and finasteride [142]. Hypospadias has also been induced by even more complex mixtures of pesticides [143], mixture of 7 anti-androgenic compounds [144], and mixtures of phthalates and pesticides [145]. Together, these studies suggest an anti-androgenic mode of action as the primary cause of hypospadias, but they are not conclusive. Several of the chemicals included in the various mixtures may also affect other endocrine signalling pathways and cause general disruption to the hormonal milieu. Interestingly, recent studies have also shown that loss of oestrogen signalling during mouse development can induce hypospadias [146, 147]. Previously, it has been shown that exposure to the phytoestrogen genestein can induce hypospadias in mice [148, 149], so again, these studies strongly suggest the importance of maintaining a correct androgen-oestrogen balance, as seemingly both too little and too much oestrogen signalling can perturb urethral closure.

Testicular Germ Cell Cancer

Important evidence for adverse trends in male reproductive health comes from the observed increase in the

incidence of TGCC. It is well-established that TGCC has a strong genetic component, including also a high risk of germ cell malignancies in a subset of DSD patients with gonadal/testicular dysgenesis and presence of Y-chromosome material [20]. In addition to the genetic component, there is evidence to suggest that environmental factors also contribute to the aetiology of TGCC. The rapid increase in the incidence of TGCC, initially observed in Western countries, suggests involvement of environmental and/or lifestyle factors [33, 150]. Accordingly, it has been reported in recent years that countries with a previous low incidence are now observing an increase [151, 152]. The involvement of environmental factors in the aetiology of TGCC is also supported by first-generation migrant studies indicating an increase in incidence after migration to countries with a high TGCC incidence [153, 154]. The association between environmental and lifestyle factors and the risk of developing TGCT have been investigated in a comprehensive study by McGlynn and Cook [155], although without the identification of major explanatory factors. Importantly, experimental studies examining the mechanisms, underlying the initiation, and progression of testicular cancer remain challenging due to the lack of suitable animal models, since mice and rats (or indeed any non-human species) do not develop the equivalent to human TGCT.

TGCT in humans derived from a common precursor cell, now termed germ cell neoplasia in situ (GCNIS) [156, 157]. This precursor cell has previously been known as carcinoma in situ (CIS) [156], intratubular germ cell neoplasia, unclassified (IGCNU) [158], or testicular intraepithelial neoplasia (TIN) [159]. GCNIS cells will, if left untreated within the testes, undergo malignant transformation by acquiring secondary genomic changes [160], become invasive and develop into a TGCT, although this may not occur until several years after detection in a testicular biopsy [161–163].

The hypothesis that GCNIS has a foetal origin is now widely accepted and is considered to be the result of arrest in the normal germ cell differentiation from gonocyte to pre-spermatogonia. Consequently, developmentally arrested gonocytes remain within the testis and continue to express pluripotency factors.

As mentioned above, experimental studies examining the mechanisms underlying the development of GCNIS cells and their progression to TGCT are challenged by the lack of animal models. However, ex vivo culture and xenografting of human foetal testis tissue now provide experimental options to examine the first step in the development of GCNIS cells, the arrest of germ cell develop-

ment from gonocyte to pre-spermatogonia. A recent study found that exogenous stimulation of Nodal signalling, which is normally downregulated in the transition from gonocyte to pre-spermatogonia, resulted in an increased number of gonocytes expressing pluripotency factors in ex vivo culture and persistence of gonocytes after subsequent xenografting of Nodal-stimulated testicular tissue, suggesting that dysregulation of Nodal signalling may be implicated in the pathogenesis of TGCC [91]. As mentioned above, several studies have examined effects of environmental factors, including exposure to smoking, phthalates, bisphenols, and analgesics on germ cells in human foetal testis in ex vivo and xenograft models, mainly reporting reduced proliferation and increased apoptosis of germ cells (reviewed in [45]). Thus, so far no experimental studies have established a direct link between exposure to environmental factors and the development of GCNIS cells and TGCT.

Conclusion

Testicular development and function may be influenced by environmental exposures resulting in male reproductive disorders. Understanding the role of environmental factors in the development of DSD and TDS requires appropriate experimental systems in which to test the effects of exposures, as well as the mechanisms involved. Over recent years, the range of experimental systems available has expanded from traditional methods of in vivo and in vitro studies to those that include 3D culture systems, testicular organoids, and xenografting. Combining the findings from epidemiologic and clinical studies with experimental studies to validate these find-

ings and explore mechanisms is essential for understanding the impact that environmental factors have on male reproductive health. Whilst the role of environmental factors in the hormone dependent stage of male reproductive development is frequently explored, the impact of such factors on the early stages of gonadal differentiation and the pathogenesis of TDS and DSDs is a key area for future studies.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.T.M. developed the concept for the manuscript. All authors wrote sections of the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

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