Endocrine disrupting chemicals in the pathogenesis of hypospadias; developmental and toxicological perspectives

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ABSTRACT

Hypospadias is a defect in penile urethral closure that occurs in approximately 1/150 live male births in developed nations, making it one of the most common congenital abnormalities worldwide. Alarming, the frequency of hypospadias has increased rapidly over recent decades and is continuing to rise. Recent research reviewed herein suggests that the rise in hypospadias rates can be directly linked to our increasing exposure to endocrine disrupting chemicals (EDCs), especially those that affect estrogen and androgen signalling. Understanding the mechanistic links between endocrine disruptors and hypospadias requires toxicologists and developmental biologists to define exposures and biological impacts on penis development. In this review we examine recent insights from toxicological, developmental and epidemiological studies on the hormonal control of normal penis development and describe the rationale and evidence for EDC exposures that impact these pathways to cause hypospadias. Continued collaboration across these fields is imperative to understand the full impact of endocrine disrupting chemicals on the increasing rates of hypospadias.
Introduction

Endocrine disruptors are "substances that alter one or more functions of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO, international Programme on Chemical Safety). There is unequivocal data pertaining to worldwide levels of human exposure to a range of endocrine disrupting compounds. Currently there are over 85,000 known chemical products (Street et al., 2018). The vast majority of these have not undergone stringent toxicology screenings to assess their effects on human health. Despite this, already more than 1000 of these chemicals have been demonstrated to be potentially harmful endocrine disrupting chemicals (EDCs) and can disrupt hormonal signalling in a wide range of tissues (Street et al., 2018). Most people are exposed to multiple EDCs through the environment, diet and occupational exposures (Parron et al., 2014). Exposure to EDCs can also involve non-monotonic dose–response curves with low doses resulting in significant impacts (Vandenberg et al., 2012). This makes the study of EDCs very complex, particularly when trying to make predictions about their potential health impacts.

EDC exposures are particularly deleterious during embryonic development when the hormonal environment is tightly regulated and when tissues are rapidly growing and patterning. Many EDCs can cross the placenta and interfere with the normal development of the reproductive, nervous, immune and hormonal systems, ultimately altering the normal function of the exposed individual and impacting future generations. The mechanisms through which EDCs exert specific actions are dependent on the cell and tissue type and are influenced by other factors such as developmental stage and sex of the embryo. Recently, an Expert Consensus Statement identified ten key characteristics of EDCs to describe the properties of chemicals that alter hormone action and improve hazard identification (La Merrill et al., 2020). These characteristics recognise not only antagonism of hormones and their receptors, but also alteration of hormone synthesis, transport, metabolism and signal transduction.

Over the last decade, exposure to EDCs has emerged as a global environmental risk and is now considered an urgent threat to public health by organisations such as the Endocrine Society and WHO (Gore et al., 2015a; WHO/UNEP, 2012). Multiple studies have been conducted examining trends in human reproductive health and the impact of EDCs on wildlife reproductive health. These have been conducted alongside in vitro and in vivo experimental data to further refine our understanding of how these chemicals and exposures impact our development and reproductive function (Bonde et al., 2016).

Development of the human penis and hypospadias

The development of the genitalia is one of the most hormone sensitive processes during sexual development. The external genitalia of both males and females develops from the sexually indifferent genital tubercle (GT) in the early embryo. Outgrowth and development of the GT into a penis in males is triggered by a surge of androgens produced from the fetal testis shortly after sex determination. This results in a structure with an internalised urethra that terminates at the tip of the glans (Carlson, 1994; Hamilton et al., 1972). Hypospadias, a misplacement of the urethral meatus, results from a failure of urethral closure during development and can have both underlying genetic and hormonal causes. Hypospadias requires surgical intervention and individuals and their families often experience significant psychological trauma, resulting in a substantial burden to health care (Attina et al., 2016; Paulozzi et al., 1999; Yu et al., 2019). Hypospadias currently impacts ~1/150 live male births in developed countries (Baskin, 2000; Nassar et al., 2007) and is predicted to be increasing still at a rate of 1% PA (Nassar et al., 2007; Paulozzi, 1999; Yu et al., 2019). Despite the common occurrence of hypospadias worldwide, definitive mechanistic links between EDCs and hypospadias are yet to be defined. This has posed a major barrier to our understanding of the impacts of EDCs to male reproductive health, the development of policies regulating our exposures to these chemicals and the development of methods to mitigate the impacts of EDCs.

The mouse as a model for human penis development and hypospadias

Mice develop a genital tubercle using identical hormonal and genetic pathways to humans (Haraguchi et al., 2000; Phillips et al., 2015). Although the mouse and human penis look morphologically...
different, all the structures are homologous (Phillips et al., 2015). Penis outgrowth and urethral closure are primarily androgen driven, and occurs in the early embryo, as in humans. Interestingly, estrogen has recently been shown to play a key role in the urethral closure process in mice, particularly mediating distal urethral closure (Baskin et al., 2001; Cripps et al., 2019; Govers et al., 2019b; Seifert et al., 2009, 2010). As in humans, any disruption to hormonal signalling in early male mice development can cause hypospadias, indicating that urethral closure is a tightly regulated process. The conserved hormonal and molecular mechanisms governing penis development make it susceptible to EDC exposures in all animals, not just mice and humans. Many studies on EDC exposures have occurred in aquatic animals with relatively less in terrestrial mammal species (Bjerregaard et al., 1998; Cook et al., 2020). However, these studies demonstrate that the findings on the impacts of EDCs from rodents and humans are likely to have broad implications for impacts these chemicals may have on all vertebrates.

Establishing the link between EDCs and hypospadias

The possible health threats posed by EDCs were officially outlined by the Endocrine Society (Gore et al., 2015a) and the WHO/UNEP (WHO/UNEP, 2012), with the conclusion that exposure to EDCs is related to a multitude of diseases, including impaired reproduction. However, systemic reviews and meta-analyses focusing on human studies have demonstrated inconsistent results (Kahn et al., 2020; Lee, 2018). While the ability of EDCs to promote adverse modifications of endocrine homeostasis in human and wildlife raises serious concerns about their potential health impact, uncertainty remains around a definitive link between EDCs and reproductive disorders and mechanistic links between the two (Bergman et al., 2013, 2015; Combarnous and Nguyen, 2019; Dietrich et al., 2013; Gore et al., 2013; Kortenkamp et al., 2012; Lamb et al., 2015; Rhomberg et al., 2012; Zoeller et al., 2014). Swaen et al. (Swaen et al., 2018) argue that profound changes in human reproduction have a major impact on the incidence of reproductive disorders.

Increased rates of several reproductive disorders were compared to changes of reproductive factors within a Dutch population from 1955 to 2015. A combination of decrease in parity or family size and an increase in maternal age at first pregnancy accounted for a 34% increase in the prevalence of hypospadias. Although this raises the possibility that changes in human reproductive factors can partly explain fractions of reported increases in hypospadias, there are numerous studies that demonstrate a strong association between increasing rates of hypospadias and exposures to endocrine disruptors. Yu et al examined the rate of hypospadias during 1980–2010 from 27 surveillance programs around the world, analysing changes over time in international total prevalence of hypospadias, prevalence for each specific program and prevalence across different degrees of severity of hypospadias. They concluded a consistency in observed trends across programs and in severity, indicating that the prevalence of hypospadias continues to increase internationally (Yu et al., 2019).

Exogenous estrogens and anti-androgens can adversely impact external genital development in humans (Fernandez et al., 2016; Kalfa et al., 2015) mice (Ma et al., 2009; Yang et al., 2010; Yucel et al., 2003; Zheng et al., 2015) and rats (Christiansen et al., 2009; Lee, 1998; Metzloff et al., 2007; Sinclair et al., 2017; Vorherr et al., 1979), resulting in hypospadias. Exogenous estrogens and estrogenic EDCs are among the most pervasive chemicals in our environment and, along with anti-androgenic EDCs, are potential causative agents of hypospadias (Fig. 2). Both estrogenic chemicals and those that antagonise the androgen receptor or inhibit fetal testis steroidogenesis, reduce testosterone production and/or its action to cause male reproductive tract malformations, including hypospadias. However, estrogen receptors are also present in the penis and isolated penis cultures have demonstrated that estrogen and estrogenic EDCs can directly activate these to cause hypospadias, independent of altered hormonal outputs from the gonads (Fig. 3) (Govers et al., 2020, 2019b). Thus, the penis also appears to be a direct target of EDCs in the development of hypospadias. Importantly, the ability of EDCs to affect penis development both directly and indirectly highlights the susceptibility of penis development to EDC exposure.

![Fig. 2. EDC sources and their direct target tissues in the male. The top panel shows known sources of EDC that impact male reproductive development and cause hypospadias. These include plastics, flame retardants, pharmaceuticals, phytoestrogens, pesticides and medical devices. Many of these chemicals can act as estrogen receptor agonists while others block estrogen action or can have pro or anti androgen functions. The lower panel shows the direct targets of EDCs in the male reproductive system. The testes and in particular the germ cells, Leydig cells and Sertoli cells are all sensitive to the hormonal environment and express both androgen and estrogen receptors. EDCs impact cell fate decision in the developing Sertoli cells, alter steroid output from the Leydig cells and alter the epigenome of germ cells leading to multigenerational and transgenerational transmission of disease. EDCs also directly target the penis. The penis has a broad distribution of androgen and estrogen receptors and both androgen and estrogen levels are critical for normal patterning. Experiments on isolated genital tubercles have shown estrogen can directly target the penis to cause hypospadias.](image-url)
Interestingly, recent research has shown that a loss of estrogen signalling in the penis can also cause hypospadias (Gripps et al., 2019; Govers et al., 2019b). Distal hypospadias (the most common form seen in humans) was identified in mice deficient for estrogen signalling, with mutations in either Erα or the Cyp19a1 gene (which encodes aromatase). Distal hypospadias are also the most common phenotype caused by exogenous estrogen signalling in mice and humans. Thus, a tightly maintained balance between androgen and estrogen signalling is critical for normal penis development. Any disruption causing either too much or too little estrogen signalling can result in hypospadias. Disruption of this tightly regulated balance can occur from exposure to EDCs that interact with either androgen or estrogen and their associated receptors (Baskin and Ebbers, 2006; Fernandez et al., 2016; Kalfa et al., 2015, 2011b; Wang and Baskin, 2008; Willingham and Baskin, 2007) and most likely contributes to the prevalence of hypospadias.

While many epidemiological studies indicate an increased risk of male reproductive disorders following prenatal and postnatal exposure to EDCs (Bonde et al., 2016; Gore et al., 2015b; Skakkebaek, 2016), the evidence specifically linking hypospadias to EDC exposure is more limited. Bonde et al. (2016) undertook a rigorous, systematic meta-analysis of the epidemiological evidence on prenatal and postnatal EDC exposure and male reproductive disorders. They analysed 33 papers and found a shared prenatal etiology of male reproductive disorders, but also a heterogeneity of EDCs in terms of the potential effects on male reproduction. They also highlighted the limitations of observational epidemiological studies, urging caution be applied to the interpretation of such studies and emphasising the need for high quality epidemiological studies to add weight to the EDC hypothesis. Similarly, Raghavan conducted a literature review and concluded that pharmaceuticals have been associated with hypospadias risk and data on dichlorodiphenyltrichloroethane and hexachlorobenzene pesticides, as well as non-persistent pollutants, particularly phthalates, also suggests associations with hypospadias (Raghavan et al., 2018). However, they also cautioned that improvements in assessment of EDC exposure, timing during development and the use of clear identification and classification schemes are required to increase the evidence of a link between EDC exposure and male reproductive health. It is difficult to establish a cause-effect relationship between EDCs and hypospadias due to a number of confounding factors. EDCs are ubiquitous within our environment but vary in their concentrations and mixes and their subsequent impact, making it difficult to assign phenotypes to an isolated EDC. Within epidemiological studies, control groups are not always available or included, and genetic causes and susceptibilities are not often accounted for. Metabolism, body composition, lifestyle choices such as smoking and genetic susceptibility can all make the effects of EDCs highly variable between individuals (Kalfa et al., 2015). It is clear that there needs to be collaboration between epidemiological, toxicological and developmental studies to establish a definitive causative role and uncover mechanistic links between EDCs and hypospadias.

Impacts of EDCs can differ between occupational and environmental exposures, involving differences in the route, dose, duration and frequency of exposure (Garcia et al., 2017). Limited data exists on the risk of hypospadias with environmental exposures, in contrast to numerous studies evaluating associations between occupational exposure during pregnancy and hypospadias (Estors Sastre et al., 2019; Morales-Suarez-Varela et al., 2011; Nassar et al., 2010). Analysis of the relationship between risk of hypospadias and maternal occupation with regard to EDC exposure found little evidence for a correlation, but the authors cautioned that the exposure classification was crude and warranted further studies (Vrijheid et al., 2003). Kalfa et al. (2015) studied a cohort of isolated hypospadias without genetic confounders and found that occupational and environmental exposures to EDCs during fetal life was a significant risk factor for hypospadias. Detergents, pesticides and cosmetics accounted for 75% of the cases, and maternal exposure was a greater risk factor than paternal exposure. Furthermore, severity of hypospadias and exposure levels were not a linear dose–effect relationship, indicating that even low exposure may have potent effects.

Nassar et al. (2010) assessed occupational exposure to EDCs in both parents in a job-exposure matrix. They demonstrated an association between maternal occupation exposure to heavy metal and phthalates and hypospadias, and an association between paternal occupation exposure to polychlorinated organic and bi-phenolic compounds. Giordano et al. (2010) also confirmed that a risk of hypospadias was associated with perinatal maternal occupational exposures to EDCs. Although the number of cases was below 100, serum from mothers of infants with hypospadias and controls was analysed for dichlorodiphenyldichloroethylene, hexachlorobenzene, and several polychlorinated biphenyl congeners, with evidence for an association between maternal EDC exposure and hypospadias. Furthermore, prevalence rates of hypospadias are significantly greater in agricultural areas with high use of pesticides (Garcia et al., 2017; Kraft et al., 2010; Kristensen et al., 1997; Morales-Suarez-Varela et al., 2011). In a case-control study of over 1600 newborn males, Gaspari et al found male genital malformations, including cryptorchidism, micropenis and hypospadias, were significantly associated with pesticide exposures (Gaspari et al., 2011).

EDCs associated with hypospadias

Over the last 5–10 years there has been an exponential increase in research into the effects of potential EDCs on male reproductive health. Here, our focus is on recent studies of potential associations between exposure to EDCs and hypospadias and investigating the molecular impacts these exposures have on penis development (Table 1).

Pharmaceutical agents

Pharmaceutical EDCs are a heterogeneous group of compounds with mostly estrogenic and anti-androgenic properties. Estrogenic

Fig. 3. EDCs impact penis development through direct and indirect mechanisms. EDCs impact development of the testis and many alter its hormonal output (green arrow). In particular, estrogenic EDCs are known to suppress androgen output from the testis. This leads to downstream impacts on the virilization of the penis and can cause hypospadias (blue arrow). In addition, EDCs can directly target the penis to cause hypospadias (green arrow). Thus, the penis is impacted both directly and indirectly (via altered hormonal output from the testis) by EDCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 1

EDCs described in this review and their impacts on hypospadias. EDCs listed represent the most extensively studied EDCs that impact male reproductive development and in particular are associated with increased rates of hypospadias. It should be noted that this is not an exhaustive list and many other EDCs exist with plausible links to hypospadias but require further study to confirm a definitive link. It should also be noted that although chemicals are often classified as either estrogen agonists or anti-androgens, estrogen agonists also suppress androgen output from the testis.

| EDCs       | Sources                          | Mechanism         | Impact on urethra development | References                                                                 |
|------------|----------------------------------|-------------------|-------------------------------|-----------------------------------------------------------------------------|
| Atrazine   | Herbicide                        | ER agonist        | Association with hypospadias  | (Agopian et al., 2013; Garcia et al., 2017; Govers et al., 2019; Kraft et al., 2010; Kristensen et al., 1997; Morales-Suarez-Varela et al., 2011) |
| BPA        | Polycarbonate plastics, epoxy     | ER agonist        | Association with hypospadias  | (Choi et al., 2012; Fernandez et al., 2016; Liu et al., 2015)             |
| Clomiphene, | Pharmaceuticals                   | ER agonist/       | Mixed results - Moderate and severe hypospadias increased | (Carmichael et al., 2005; Meijer et al., 2006; van Rooij et al., 2013) |
| progestins, oral contraceptives |                          | antagonist        |                               |                                                                            |
| DES        | Historical pharmaceutical        | ER agonist        | Hypospadias                   | (Kalfa et al., 2011a; Klip et al., 2002; Mahawong et al., 2014; Palmer et al., 2005; Vorherr et al., 1979) |
| DDT        | Contaminated water, soil, fish,  | ER agonist        | Association with hypospadias  | (Bornman et al., 2010; Fernandez et al., 2007)                             |
| crops      |                                    |                   |                               |                                                                            |
| Genistein  | Phytoestrogen in legumes and     | ER agonist        | Hypospadias                   | (Michikawa et al., 2019; Moule, 1961; North and Golding, 2000; Padilla-Banks et al., 2012; Ross et al., 2011; Vilela et al., 2007) |
|            | clover                           |                   |                               | (Fernandez et al., 2016)                                                 |
| Parabens   | Cosmetics and pharmaceutical     | ER agonist        | Possible association          | (Koren et al., 2019; Poon et al., 2018)                                     |
|            | products, toothpaste and food    |                   | with hypospadias              |                                                                            |
|            | preservatives                     |                   |                               |                                                                            |
| PBB, PBDE  | Flame retardants                 | ER agonist/       | Association with hypospadias  | (Jiang et al., 2016; Nasar et al., 2010; Ormond et al., 2009; Qian et al., 2020; Raghavan et al., 2018; Sathyaranayana et al., 2017; van den Driessche et al., 2017; Vrijheid et al., 2003; Zhu et al., 2009, 2020) |
|            |                                  | antagonist        |                               |                                                                            |
| Phthalates | Contaminated food, PVC plastics   | ER agonist/       | Strong association            | (Amato et al., 2018; Gray et al., 1999; Kelce et al., 1994; Vilela et al., 2007; Yang et al., 2019) |
|            | and flooring, personal care,     | antagonist        | with hypospadias              |                                                                            |
|            | medical devices                   |                   |                               |                                                                            |
| Vinclozolin| Fungicide                        | ER agonist        | Hypospadias                   | (Amato et al., 2018; Gray et al., 1999; Kelce et al., 1994; Vilela et al., 2007; Yang et al., 2019) |
|            |                                  |                   |                               |                                                                            |

Genistein

Genistein is an isoflavone phytoestrogen that is commonly found in all legumes, with specifically high levels in soybeans and their products (Fukutake et al., 1996). Along with other isoflavones, genistein is also abundant in a key feed crop for ruminant animals; subterranean clover (Curnow, 1954; Wang et al., 2008). Genistein is able to cross the placenta (Balakrishnan et al., 2010), has a similar structure to human estrogen (Barnes, 2004) and has a high relative affinity for ERα, 150–300 fold higher to ERβ than other isoflavones (Scioppo et al., 2004). Genistein consumption has been associated with increased rates of hypospadias in humans (Michikawa et al., 2019; North and Golding, 2000), mice (Padilla-Banks et al., 2012; Ross et al., 2011; Vilela et al., 2007) and in sheep (Moule, 1961). As the consumption of soy products increases in western populations, understanding the role of genistein in inducing hypospadias becomes even more important (Huang et al., 2016).

Vegetarian and vegan mothers, whose intake of genistein is higher than the general population, have a 4-fold elevated risk that their sons would be born with hypospadias (North and Golding, 2000). However, Carmichael et al. associated an increased soy intake with a decrease in hypospadias risk (Carmichael et al., 2013a). A prospective birth cohort study of Japanese mothers found an association between low genistein intake and hypospadias (Michikawa et al., 2019). However, neither of these studies included first degree ‘mild’ hypospadias, which account for a significant majority of phenotypes. Several smaller studies have concluded that maternal ingestion of either legumes or soy based products was not associated with either an increased or decreased risk of hypospadias (Giordano et al., 2008; Pierik et al., 2004), however both of these studies were limited in their heterogeneous EDC exposure and retrospective self-reporting of genistein consumption. In utero exposure to genistein induces hypospadias and other genital abnormalities in mouse models (Padilla-Banks et al., 2012; Ross et al., 2011; Vilela et al., 2007). Hypospadias was observed in approximately 25% mice that were fed a diet comprising genistein but was not observed in a...
control group with a soya-free diet (Vilela et al., 2007). Genistein exposure contributes to hypospadias in mice by altering pathways of tissue morphogenesis, cell proliferation and cell survival (MAPK, TGF-beta, FOXO, HOX and ER) (Ross et al., 2011).

**Bisphenol A (BPA)**

One of the most common EDCs that humans are exposed to is the plasticiser Bisphenol A (BPA). BPA binds to both ERα and ERβ (Acconcia et al., 2015), along with several residue binding sites for AR and PGR (Rehan et al., 2015). Gestational exposure to BPA has been associated with a wide range of developmental abnormalities, including male reproductive tract abnormalities and fertility (Meli et al., 2020; Santoro et al., 2019). While only a few studies have analysed the association of BPA exposure with hypospadias in humans, these indicate that mothers with high concentrations of BPA in urine, blood or placenta have male offspring with significantly higher risks of developing hypospadias (Choi et al., 2012; Fernandez et al., 2016). A genome-wide screen using human foreskin fibroblast cells derived from hypospadic patients identified inhibition of a specific matrix metalloproteinase MMP11, a known effector of development (Qin et al., 2012).

Exposure to BPA during sexual differentiation in male mice induced hypospadias along with reduced AGD, reduced testicular weight, decreased testosterone levels and elevated estradiol. This was combined with decreased expression of androgen dependent genes related to male sexual development - WT1, LHR, 17BHSF3 and SRD5A2 (Liu et al., 2015). Gestational exposure to BPA in rats did not induce hypospadias, although this was only at relatively low doses (2–200 ug/kg/day) (Howdeshell et al., 2008). Indeed, studies into the effect of low dose BPA exposures on reproductive health show inconsistent results and require further investigation (Hong and Yang, 2017).

**Phthalates**

Phthalates belong to a group of compounds known as non-persistent organic pollutants, EDCs that have a short residence time in the environment and short half-lives in the human body (Gregoraszczuk and Ptak, 2013). They are ubiquitous in synthetic products as they are widely used as plasticisers and are one of the most widely studied EDCs in relation to male reproductive health effects (Czubacka et al., 2021; Foster et al., 2017). The primary phthalate pollutants in the environment are Σ4-2(ethylhexyl)-phthalate (DEHP) and Σ dibutyl phthalate (DBP) (Gao and Wen, 2016; Szewczynska et al., 2020).

Phthalates accumulate within the body and their metabolites are capable of crossing the placenta, affecting embryo development (Qian et al., 2020). Phthalate metabolites activate ERα, ERβ and AR, making them prime candidates for disrupting penile development (Engel et al., 2017; Takeuchi et al., 2005). Many reproductive abnormalities may be induced by phthalate exposure during pregnancy, including shortened anogenital distance, cryptorchidism and hypospadias (Czubacka et al., 2021; Qian et al., 2020; Skakkebaek et al., 2016). Phthalate exposure disrupts testicular androgen production in mice, lowering sperm quality and reducing fertility (Barakat et al., 2017, 2019). Multiple studies in humans have linked maternal blood concentrations of DEHP and the metabolite MEHP to higher incidences of male offspring with reduced anogenital distance and reduced overall penis size, and a reduced volume of the penis at birth, similar in nature to BPA (Bustamante-Montes et al., 2013; Suzuki et al., 2012). Replication of these studies in rats also indicated that maternal exposure of DEHP over the critical window of hormone dependent development of the GT had a higher instance of undescended testis and reduced anogenital distance (Ema and Miyawaki, 2001).

Occupational exposure studies have indicated an association between exposure to estrogenic or anti-androgenic phthalates and hypospadias. A high rate of hypospadias was observed in children whose mothers were exposed to phthalates, particularly mothers working in the hairdressing, beauty, or cleaning industries, during pregnancy (Nassar et al., 2010; Ormond et al., 2009; Vrijheid et al., 2003).

Cryptorchidism and hypospadias are associated with increased amniotic concentration of INSL3, a testis specific biomarker impacted by phthalate endocrine disruptors, during gestation (Anand-Ivell et al., 2018). The authors conclude that phthalates may be altering the dynamics of testicular development and hormone production, leading to hypospadias. Sathyanarayana et al also examined the relationship between phthalates and estrogen and testosterone levels during early fetal development (Sathyanarayana et al., 2017). They found that DEHP induced lower testosterone levels resulted in a higher prevalence of male genital abnormalities, including hypospadias. Similarly, hypospadias were also observed after DBP-induced androgen deprivation during the masculinizing window in rats (van den Driesche et al., 2017). Although these studies only addressed the indirect effects of EDC exposure on the penis, the estrogenic and antiandrogenic properties of phthalates also have the potential to directly impact development of the GT leading to a hypospadias phenotype (Govers et al., 2019b).

More recently, studies have started to uncover the mechanism behind phthalate induced hypospadias. In prenatally DBP exposed rats, Jiang et al found a potential correlation between decreased testosterone levels, reduced AR and Srd5a2 and hypospadias (Jiang et al., 2016). Significantly decreased expression of key signalling molecules in GT formation was observed in DBP induced hypospadias in newborn rats, including sonic hedgehog, fibroblast growth factor and transforming growth factor-beta family members (Zhu et al., 2009). Furthermore, in rats, DBP causes hypospadias via its oxidative stress effect, significantly increasing calcium concentrations and inhibiting epithelial to mesenchymal transition (EMT) in urethral epithelial cells, blocking the fusion process of the urethral groove (Zhu et al., 2020). Prenatal exposure to DBP induces abnormal hedgehog signalling and autophagy in uroepithelial cells which may have an important role in hypospadias development (Li et al., 2017; Zhao et al., 2018, 2019). In mice, an association between miRNAs and mice has been uncovered. Male mice with hypospadias have significantly higher miR-494 and lower Nedd4l expression, a target gene of miR-494. Down regulation of miR-494 exerted protective effects on urethral epithelial cells by impeding cell proliferation and inducing cell apoptosis, indicating that downregulation of miR-494 inhibits TGF-β1/Smad signalling, preventing the development of hypospadias through upregulation of Nedd4l (Tian et al., 2020).

Interestingly, postnatal exposure to DEHP from birth in rats led to the complete failure of preputial separation, where the glans of the penis remained fully attached to the prepuce in the dorsal region, forming a ‘hood’ at adulthood (Moore et al., 2001). This ‘hood’ foreskin phenotype is consistent with the known effects of in vivo estrogen exposure on male embryos in both mice and humans (Phillips et al., 2015; Zheng et al., 2015).

**Parabens**

Parabens are another group of well described estrogen agonists (Lemini et al., 2003; Routledge et al., 1998) that also act as anti-androgens and can induce a variety of reproductive effects in vivo, in both male and female offspring during developmental exposure (Böberg et al., 2020). An association between exposure to parabens and both cryptorchidism and hypospadias has also been implicated (Fernandez et al., 2016). It should be noted that there were confounding factors identified in this study and no other studies have assessed a direct association between hypospadias and parabens. However, a number of earlier studies indicated no significant effects were seen on male reproductive outcomes following exposure to parabens (Janjua et al., 2007; Meeker et al., 2011). Interestingly, in animal
studies, delayed prepartum separation was observed in rats exposed to butylparaben, a paraben that has more than one modality, including estrogenic and anti-androgenic actions (Bobberg et al., 2020; Maske et al., 2020; Zhang et al., 2014). An early disruption in prepartum separation and mild hypospadias was also observed in aromatase knock-out mice, demonstrating a requirement for correct estrogen signalling for normal prepartum development and urethral closure (Cripps et al., 2019). Exposure to butylparaben in rats also resulted in delayed testicular descent, and decreased sperm count, motility and daily sperm production (Maske et al., 2020). Significantly perturbed expression of a number of testicular genes was also observed, along with increased testosterone and LH levels, resulting in subfertility (Maske et al., 2020). Numerous studies in rats and mice have also shown effects of parabens on spermatogenesis (Guerra et al., 2017; Oishi, 2001, 2002; Zhang et al., 2014) and a reduced AGD (Bobberg et al., 2016; Kang et al., 2002; Yang et al., 2016; Zhang et al., 2014). Therefore, the estrogenic/anti-androgenic actions of parabens observed in rats and mice, in combination with the suggested association with hypospadias (Fernandez et al., 2016), points to a need for further animal studies to understand their impacts on male reproduction, including development of the penis.

**Flame retardants**

Accumulating evidence suggests that exposure to flame retardants, such as polybrominated diphenyl ether (PBDEs) and polybrominated diphenyl ethers (PBDEs), during critical windows of development may be associated with impacts on male reproduction and fertility (Hales and Robaire, 2020). PBDEs persist in the environment and ability to bioaccumulate, along with their adverse impacts on human health, led governments to restrict their use worldwide (Govaci et al., 2011). Gestational exposure to eight PBDEs was measured in mothers of boys diagnosed with hypospadias. Total PBDE levels were significantly higher (up to 48%) among mothers of infants with hypospadias than among controls (Koren et al., 2019; Poon et al., 2018). A similar study found that maternal PBDE levels were not significantly different between women with infants with hypospadias and women with unaffected infants, however only 20 hypospadias infants were included in this study (Carmichael et al., 2010). Similarly, despite effects on other male genital developmental processes, no significant association was seen between PBDE exposure and either cryptorchidism and hypospadias in humans (Small et al., 2009). A novel study that used a combination of epidemiological and in vivo studies showed that PB153 exposure altered the epigenome by disrupting methyltransferase activity leading to defects in imprint establishment causing altered gene expression, therefore having the potential to contribute to reproductive, developmental and endocrine issues in offspring (Greeson et al., 2020). Inconsistencies between epidemiological studies into flame retardants as outlined above point to a need for further studies combining animal experimental models with epidemiological studies to define their mechanistic link with hypospadias.

**Solvents**

Trichloroethylene (PCE) is a solvent used in dry cleaning, textile processing, and metal degreasing (Aschengrau et al., 2008). Studies of residents in the USA exposed to PCE contaminated drinking water showed that mothers with high levels of PCE exposure had increased risk of having a child with hypospadias (Aschengrau et al., 2018). These findings support previous studies that found an increased risk of birth defects following occupational prenatal exposure to solvents (Chervier et al., 2006; Destroisiers et al., 2012) and highlight the risk of environmental contaminants inducing birth defects, including hypospadias, over several decades.

**Agricultural chemicals**

Atrazine is a commonly used pesticide throughout Australia and the USA and is a persistent organic pollutant that has a long half-life in the environment, up to 228 days in soil (Solomon et al., 2008). Due to the widespread use of atrazine, several lakes, subterranean water stores and human drinking supplies have become contaminated (Benotti et al., 2009; Solomon et al., 2008). A higher prevalence and risk of hypospadias was observed in children of parents in areas of high pesticide exposure (Garcia et al., 2017), confirming previous studies that identified an association between prenatal pesticide exposure and hypospadias prevalence (Kraft et al., 2010; Kristensen et al., 1997; Morales-Suarez-Varela et al., 2011).

The endocrine disrupting effects of atrazine have been well documented across amphibians (Hayes et al., 2011), fish (Van Der Kraak et al., 2014), reptiles (Solomon et al., 2008) rodents (Cook et al., 2019; Jin et al., 2013) and mammals (Cook et al., 2020). Atrazine can have a wide range of effects resulting in a disruption in the fine balance of hormones in the reproductive tract. Atrazine increases the expression of Cyp19a1, leading to an increase in expression of the enzyme aromatase which is responsible for the conversion of testosterone to oestradiol (Holloway et al., 2008). Atrazine also suppresses Srd5a2 in rats leading to a reduction in the conversion of testosterone into dihydrotestosterone (DHT) (Holloway et al., 2008; Jin et al., 2013; Kniewald et al., 1995). Atrazine exposure during pregnancy induces a shortening of penis structures and increases the incidence of hypospadias in mice (Govers et al., 2019). An association between hypospadias and estimated medium levels of maternal atrazine exposure has been identified in humans, particularly for more severe, posterior hypospadias (Agopian et al., 2013). However, a subsequent study showed only a very weak association between drinking water atrazine and hypospadias (Winston et al., 2016). Both of these studies highlight the need for further research using improved exposure characterization to confirm an association and define a mechanism of atrazine action.

Exposures to antiandrogens such as vinclozolin, a fungicide, also cause hypospadias in both mice (Vilela et al., 2007) and rats (Gray et al., 1999; Kelce et al., 1994). Vinclozolin is regularly used to induce hypospadias in mouse models to investigate the mechanism of antiandrogen action on the development of the urethra (Amato et al., 2018; Yang et al., 2019). Vinclozolin also affects the sperm epigenome in rats, resulting in long lasting impacts on spermatogenesis across multiple generations (Beck et al., 2017; Ben Maamar et al., 2018; Nilsson et al., 2018; Schuster et al., 2016; Skinner et al., 2019). However, there is still no definitive evidence for vinclozolin induced hypospadias in humans.

Dichlorodiphenyltrichloroethane (DDT) is a well known and controversial pesticide originally used worldwide. Mothers exposed to household DDT spraying had a 33% greater chance of having a son with urogenital malformations, with 11% of boys developing either hypospadias or cryptorchidism (Borman et al., 2010). In a case control study in Spain, Fernandez et al found an association between high pesticide use, including DDT, and male urogenital malformations, including hypospadias (Fernandez et al., 2007).

Although risk assessments of pesticides are typically based on the no observed adverse effect levels (NOAELs) for single chemicals, exposures are more often to a mixture of several EDCs (Blount et al., 2000). Exposure of rats to a mixture of low doses of five pesticides caused nipple retention and increased incidence and severity of genital malformations, including hypospadias, in male offspring. These effects were severe at dose levels where the individual pesticides caused smaller or no effects when administered alone (Hass et al., 2012).

**EDC mixtures**

Typically, EDC exposures In humans occur to more than one chemical at any given time and several anti-androgenic chemicals have been
identified as being present as mixtures (MIX) in humans (Blount et al., 2000; Swan et al., 2005), making it crucial to understand the effects of exposure to combined EDCs. Rats exposed to anti-androgens vinclozolin, flutamide and procymidine in a MIX had an increased rate of hypospadias at doses where each of the individual chemicals caused no observable effects (Christiansen et al., 2008). Similarly, the effect of exposure to a MIX of four anti-androgens - DEHP, vinclozolin, prochloraz and fenisteride - was synergistic in male rats, resulting in malformation of external sex organs (Christiansen et al., 2009). These studies confirm that anti-androgens act in an additive way with the ability to cause a very high frequency of male reproductive malformations, including hypospadias, when interacting with other similarly acting anti-androgens (Hass et al., 2007; Metzdorf et al., 2007). These MIX findings highlight that risk assessments based on NOAELs for single anti-androgens underestimate the risk of hypospadias.

Anti-androgenic chemicals have the capacity to alter the androgen signalling pathway via multiple mechanisms. In utero exposure of rats to DEHP and DBP, both of which inhibit fetal testis hormone production, caused additive effects on differentiation of the male reproductive tract, including hypospadias (Howdeshell et al., 2007). Mixtures of benzyl butyl phthalate (BBP) and linuron (Hotchkiss et al., 2004), or procymidine and DBP (Gray et al., 2004), anti-androgens which act through different mechanisms, also induced hypospadias in a dose-additive manner, indicating that the effect on hypospadias was dose additive, even though the mechanisms of action were different. Similarly, a mixture of seven anti-androgens that alter androgen signalling through diverse mechanisms caused hypospadias in a cumulative dose-additive manner (Rider et al., 2008). In an even more complex study, a mixture of ten chemicals (four pesticides and six phthalates) that elicit their anti-androgenic effects through AR antagonism or inhibition of androgen synthesis, caused hypospadias in a cumulative dose-additive model (Rider et al., 2010). Taken together, these studies suggest that the effects of EDCs on development of the male reproductive tract must take into consideration both exposures to individual chemicals and the cumulative effect of EDCs that are present during the critical period of urethral closure.

Transgenerational impacts of EDC exposures

EDCs impact not only the individual exposed but have the potential to impact the germ line causing effects on subsequent generations. These effects are mediated through epigenetic mechanisms and are termed epigenetic transgenerational inheritance. Epigenetic alterations induced by EDCs include DNA methylation, non-coding RNAs and histone modifications, which together alter the chromatin structure. Transgenerational impacts on male reproductive health following EDC exposure have been identified (Anway et al., 2006; Anway and Skinner, 2008; Guerrero-Bosagna et al., 2012; Nilsson et al., 2018). These findings raise the concern that certain environmental exposures may not only induce hypospadias in the exposed individual, but also create long-lasting epigenetic changes that continue to increase the hypospadias risk for generations to come.

Several studies in both humans and mice have observed an association between maternal exposure to DES, a synthetic estrogen, and increased rates of hypospadias in the next two generations (Kalfa et al., 2011a; Mahawong et al., 2014). This demonstrated that estrogenic exposures can alter the epigenome to impact male reproductive health in subsequent generations. However, no data yet exists on the potential for these effects to be transgenerational. Transgenerational, effects must persist into the F3 generation, since a single exposure to EDCs in utero exposes the fetus and the germ cells which give rise to the F2. As yet, few studies have addressed the question of transgenerational inheritance of hypospadias and the potential for EDC exposure to create persistent epigenetic changes in multiple generations.

DES interferes with histone methylation (Baccarelli and Bollati, 2009), thus suggesting a potential epigenetic mechanism for the action of DES (Bredfeldt et al., 2010). Estrogen receptor activation is also well known to alter the epigenetic landscape (Wong and Walker, 2013), suggesting that estrogenic compounds known to cause hypospadias are equally likely to be making long-lasting changes to the transcriptional environment. Importantly, DNA methylation changes have been identified in hypospadias patients in the androgen receptor (Vottero et al., 2011), along with SCARBI and MYBPH (Choudhry et al., 2012). A further 25 CpGs were identified in a genome-wide association study of 45 hypospadias cases and these were associated with germ layer differentiation, beta-catenin signaling, androgens and reproductive traits (Richard et al., 2020). Epigenetic mechanisms of transgenerational impacts of vinclozolin exposure on spermatogenesis have been extensively studied in rats. Epimutations in DNA methylation, non-coding RNA expression and histone retention have all been observed in germ cells of F3 males following an ancestral exposure to vinclozolin (Beck et al., 2017; Ben Maamar et al., 2018; Nilsson et al., 2018; Schuster et al., 2016; Skinner et al., 2019). Evidence that both vinclozolin, an anti-androgen, and DES, a synthetic estrogen, cause epigenetic changes implies a broad range of EDCs can impact the germ line to cause ongoing health impacts long after the chemical exposure.

Genetic pathways altered by EDCs

Understanding the impacts that EDCs have on genetic pathways regulating urethral closure has also proven challenging. However, some critical penis development genes have been identified which seem to be especially sensitive to the impacts of hormonal disruptions. Among these are the hedgehog family members SHH and IHH. HH signalling is critical for penis development and patterning (Perriton et al., 2002). Mutations in SHH and its downstream signalling pathway mediators are associated with hypospadias and genital abnormalities in humans (Carmichael et al., 2013b) and mice (Perriton et al., 2002). Hormonal manipulations in wallabies and mice impact SHH expression and demonstrate that it is under control from both androgen and estrogen signalling. SHH is suppressed by androgens during normal penis development (Chen et al., 2018c) and is upregulated by estrogens (Cripps et al., 2019). Similarly IHH is a downstream target of androgen signalling in the development of the penis (Zheng et al., 2015). Given the fundamental role of HH signalling in the development and outgrowth of the penis, it is likely that this pathway is a major target of both estrogenic and anti-androgenic EDCs in the development of hypospadias.

The WNT, FGF and BMP signalling pathways play fundamental roles in the development and patterning of many tissues across the body, including the penis. These pathways are interconnected with HH signalling and there is considerable crosstalk between their gene networks. Not surprisingly then, several members of the WNT, FGF and BMP signalling pathways are sensitive to hormonal perturbations during development and are known to be associated with hypospadias in both humans and mouse models (Fig. 4) (Beleza-Meireles et al., 2007; Haraguchi et al., 2000; Li et al., 2006; Person et al., 2010; Petitot et al., 2005; Yamaguchi et al., 1999). Hormonal manipulation in the wallaby has shown that, like HH signalling, these pathways can be regulated by both androgen and estrogen signalling (Chen et al., 2018b) again highlighting the range of EDCs which can then impact these pathways during development. Among the most impacted genes are Wnt4 which is activated by androgens, Wnt5a which is suppressed by androgens, while Wif1 and Wnt7a are suppressed by exogenous estrogen in the developing penis (Chen et al., 2018b). Interestingly Fgf2 appears to be suppressed by endogenous estrogens during normal penis development in mice, and was activated in the development of distal hypospadias in the estrogen receptor alpha

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knockout mouse (Cripps et al., 2019). Exogenous estrogen also suppresses Bmp5, and its neighbouring Inc-BMP5 during penile development in the tammar wallaby suggesting some of the impacts of estrogen might be mediated by regulatory IncRNAs (Chen et al., 2018a).

Several other genes have been identified that have key roles in penises development and are also hormonally responsive, and sit outside of the HH, FGF and WNT signalling pathways. These are VAMP7 and the keratin genes (Fig. 4). VAMP7 copy number variation is associated with hypospadias in humans (Chavez-Lopez et al., 2020; Tannour-Louet et al., 2014) and gene duplications cause an upregulation of estrogen-responsive genes (Tannour-Louet et al., 2014). Similar to FgfR2, endogenous estrogen in normal male penis development appears to upregulate Vamp7 levels, while in the absence of estrogen signalling, Vamp7 was significantly increased. Together, these studies illustrate a potential feedback mechanism between VAMP7 and estrogen receptor levels which is required for normal penis development. It also appears that endogenous estrogen in the developing penis is required to regulate keratin gene expression, with both Krt6a and Krt8 significantly impacted by a loss of estrogen signalling in the developing penis. In mice, this leads to a shift in the developmental timing of critical delamination events in the developing penis and an eventual distal hypospadias phenotype (Cripps et al., 2019).

Conclusions

Development of the male urethra and patterning of the penis is an exquisitely hormonally sensitive process. The identification of hormonally regulated genes has been critical for our understanding of normal penile development as well as the manifestation of hypospadias. Many of these hormonally sensitive genes sit in fundamental patterning pathways and appear to be regulated by both androgen and estrogen signalling. Perhaps then it is not so surprising that chemicals that interfere with androgen and estrogen levels during development can easily result in hypospadias, providing a plausible causative agent for the incidence and increasing levels of this disease over recent decades. Hypospadias would be expected to result from estrogen agonists as well as antagonists, and androgen agonists as well as antagonists. Alarming, these EDCs also have the ability to alter the epigenetic landscape, resulting in hypospadias in successive, unexposed generations. As the number of potential EDCs continues to increase there is an urgent need to improve assessment of exposures, define critical exposure windows and use consistent methodologies and hypospadias classification schemes. The collaboration of toxicological, developmental and epidemiological scientists is crucial to allow the collation of data across studies and enable the identification of clear dose-response relationships.

CRediT authorship contribution statement

Deidre M. Mattiske: Writing - original draft, Writing - review & editing. Andrew J. Pask: Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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