



Published in final edited form as:

*Sex Dev.* 2015 ; 9(5): 239–259. doi:10.1159/000441988.

## The Genetic and Environmental Factors Underlying Hypospadias

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### Abstract

Hypospadias results from a failure of urethral closure in the male phallus and affects 1 in 200–300 boys. It is thought to be due to a combination of genetic and environmental factors. The development of the penis progresses in 2 stages: an initial hormone-independent phase and a secondary hormone-dependent phase. Here, we review the molecular pathways that contribute to each of these stages, drawing on studies from both human and mouse models. Hypospadias can occur when normal development of the phallus is disrupted, and we provide evidence that mutations in genes underlying this developmental process are causative. Finally, we discuss the environmental factors that may contribute to hypospadias and their potential immediate and transgenerational epigenetic impacts.

### Keywords

Disorders of sexual development; Hypospadias; Penis; Phallus; Urethra

Hypospadias is a congenital anomaly in which the urethral opening is not correctly positioned at the tip of the penis. It is the second most common congenital disorder in boys after cryptorchidism and the most frequent malformation of the penis [Ságodi et al., 2014], affecting one in 200–300 boys [Blaschko et al., 2012]. The severity of hypospadias can vary from minor to severe depending on where the urethral opening is located (fig. 1) and often requires surgery [Snodgrass et al., 2011]. Given the frequency and on-going implications, hypospadias is an important health issue and can be a substantial burden on health-care resources. Indeed, because of a high risk of complications, such as recurrent stenosis or fistulae, the most severe cases often require several surgeries. In addition, a significant percentage of patients suffer from cosmetic or functional difficulties that can affect urinary

and sexual function [Aulagne et al., 2010]. In the last consensus on definition of disorders of sexual development (DSDs), hypospadias was included as a form of 46,XY DSD [Hughes et al., 2006]. Numerous DSDs have a genetic cause [Eggers and Sinclair, 2012], and a number of genes have been broadly implicated in hypospadias; it is thought that a combination of environmental influences and genetic susceptibility cause this anomaly [Kalfa et al., 2011a; Marrocco et al., 2015]. Only 30% of hypospadias cases have a clear genetic cause [Ságođi et al., 2014]. In this review, we will discuss the current knowledge of the genetic contribution to hypospadias in humans and consider candidate genes implicated through mouse studies. Finally, we discuss the role of the environment and epigenetics in hypospadias risk.

## Hypospadias-Associated Malformations and Syndromes

In the majority of cases, hypospadias presents as an isolated condition, but it can occur in association with other abnormalities, especially in the urogenital tract. The most common associated abnormalities are: uni- or bilateral cryptorchidism, a condition where one or both of the testes have not descended properly into the scrotum, and micropenis, where the dorsal length of the neonate penis is less than 2.5 cm. The occurrence of these co-morbidities often indicates an androgen deficiency, which can be tested by hormone assays [Snodgrass et al., 2011]. In addition, hypospadias has been described in over 200 other syndromes [Kalfa et al., 2011a]. Among these, are WAGR [Wilms' tumour (WT), aniridia, genitourinary abnormalities and mental retardation], Denys-Drash syndrome (genitourinary malformations and susceptibility to WT) and Smith-Lemli-Opitz syndrome (malformations of the heart, lungs, kidneys, gastrointestinal tract, and genitalia). In these syndromic cases, there are often well-defined genetic causes [reviewed in Shih and Graham, 2014] such as mutations in *WT1* in Denys-Drash syndrome or in *DHCR7* for Smith-Lemli-Opitz. In this review, we will concentrate on the molecular genetics underlying non-syndromic hypospadias.

## Isolated Hypospadias and Known Genetic Causes

Numerous lines of evidence suggest an important genetic component in hypospadias with the presence of an affected family member being the biggest risk factor so far identified [Thorup et al., 2014]. Overall, the heritability of hypospadias is suggested to be between 57 and 77% [Stoll et al., 1990; Schnack et al., 2008], with equal transmission through both maternal and paternal lines [Schnack et al., 2008]. In non-syndromic (isolated) forms of hypospadias, most cases are idiopathic [Carmichael et al., 2012]; however, familial aggregation can be found in ~ 10% of cases [Kalfa et al., 2011a]. For example, the brother of a male with hypospadias has a 9–17% risk of also having the condition [Schnack et al., 2008]. Heritability appears to vary with severity, with several studies showing that anterior or middle hypospadias occurs more frequently in familial clusters than proximal hypospadias [Fredell et al., 2002; Brouwers et al., 2010]. However, it is hard to formally distinguish this from a lack of fecundity or appropriate sexual function in the most severe cases. Recent advances in massively parallel sequencing technologies and linkage studies using large cohorts are uncovering novel hypospadias-associated genes and potentially causative variants [Achermann et al., 2015; Kon et al., 2015]. In addition, the use of animal models has shed light on the molecular genetics underlying many aspects of normal and abnormal penile development.

## Normal and Abnormal Penile Development in Humans

Hypospadias is caused by abnormal or incomplete urethral closure during the early weeks of embryonic development. In humans, the development of external genitalia occurs in 2 phases: an early hormone-independent phase (5–8 weeks of gestation) and a later hormone-dependent stage (weeks 8–12) [Blaschko et al., 2012] (fig. 2).

During the hormone-independent phase, the external genitalia are indistinguishable between males and females as sexual differentiation of the gonads is not yet complete. During the 5th week of gestation, mesodermal cells spread along the cloacal membrane to form a pair of swellings – the cloacal folds. These fuse along the midline, anteriorly to the cloacal membrane, to form the genital tubercle (GT) [Schoenwolf et al., 2009]. These cloacal folds then divide into urogenital folds flanking the urogenital sinus and anal folds posteriorly. Labioscrotal folds then develop on each side of the urogenital folds. The GT consists of lateral plate mesoderm, surface ectoderm and endodermal urethral epithelium that derives from the urogenital sinus (fig. 2). The distal most part of this urethral plate epithelium (DUE) acts as a signalling centre to stimulate GT outgrowth and differentiation. From week 8 onwards, when the gonads have differentiated into testes in XY males, the hormone-dependent phase of sexual differentiation begins. The presence of testosterone produced by the testis causes the GT to elongate and the urethral groove appears, laterally defined by the urethral folds. The distal portion of the urethral groove terminates in a solid epithelial plate, called the urethral plate, which extends into the glans penis. Fusion of the urethral folds is the key step in the formation of the penile urethra. Eventually, in males, the GT gives rise to the glans and the labioscrotal folds fuse to form the scrotum (fig. 2). Finally, the penile skin, including the foreskin, develops from ectodermal tissue externally covering the entire penile length, and the corporeal bodies arise from mesenchymal condensations. Closure of these structures on the ventral midline gives rise to a ridge of tissue referred to as the median raphe.

Perturbation of the genes or signalling pathways involved in phallus development and urethral closure can result in hypospadias, which typically refers to an anomaly of closure of the ventral aspect of the penis. This results in an abnormal location of the urethral opening on the ventral aspect of the penis (fig. 1). This anomaly can also be associated with (1) a variable degree of ventral curvature of the penis, known as chordee, and (2) an abnormal foreskin, usually incomplete, referred to as hooded foreskin. Although the criteria used to evaluate the severity of hypospadias are not well defined, it is generally accepted that the position of the urethral meatus alone is not sufficient and that the level of division of corpus spongiosum, an inner component of the penis, should also be taken into account. For this reason, a definitive classification can only be completed during surgery [Snodgrass et al., 2011]. Indeed, during surgery, a portion of closed but hypoplastic urethra between the proximal division of the corpus spongiosum and the position of the urethral meatus may be uncovered and require refashioning [Snodgrass et al., 2011]. Nevertheless, hypospadias is usually classified solely on the position of the urethral opening into distal (anterior), midshaft (middle) or proximal (posterior), with some subcategories further defining the position of the malformation (fig. 1). Anterior forms of hypospadias represent 70% of cases, midshaft and posterior cases account for the remaining 30% [Carmichael et al., 2012].

## Genes Implicated in Early Patterning of the GT and Hypospadias

Development of the external genitalia during mammalian evolution was crucial to enable internal fertilisation and colonisation of the land. All therian mammals have external genitalia, and development of the GT is often compared to that of the limbs [Yamada et al., 2006; Cohn, 2011]. Indeed, the morphogenesis of both structures appears to involve similar genetic pathways including canonical Wingless-type MMTV integration site family (WNT) signalling, Hedgehog (HH) signalling, bone morphogenetic protein (BMP) signalling and Hox genes.

During embryonic development, the DUE is thought to be the major signalling centre, and regulates the growth of the GT via several signalling pathways [Haraguchi et al., 2000; Perriton et al., 2002]. Animal models are useful in understanding the potential role of these pathways. However, interpretation of mouse hypospadias phenotypes is confounded by differences in morphology between the mouse and human penis [Cunha et al., 2015]. As opposed to the human penis, which is fully developed at birth, rat and mouse penis development occurs both pre- and postnatally [Cunha et al., 2015]. The undifferentiated GT of embryonic or newborn mice is immature and subtle malformations in the external genitalia are often not manifest until several weeks after birth [Cunha et al., 2015]. Recently, however, a detailed annotation of the adult mouse penis has provided a better understanding of its development and relevance to humans [Phillips et al., 2015]. This important resource will enable an accurate comparison of murine and human hypospadias phenotypes in future studies [Phillips et al., 2015].

In humans, the majority of hypospadias association studies have either been mutation analyses in key genes or association/linkage studies in large affected cohorts. While these do not prove pathogenicity, they are valuable. Some expression analysis has also been performed, but not always in the affected tissue (i.e. in foreskin tissue removed from hypospadias patients and not the urethral epithelium). Below, we draw evidence from both animal models and human studies for the involvement of several different signalling pathways and genes in normal GT development and hypospadias.

### The Hedgehog Signalling Pathway

The Hh signalling pathway plays numerous important roles during embryogenesis [Tang and Cheng, 2014]. In mammals, 3 *HH* genes can activate this pathway, including sonic hedgehog (*SHH*), desert hedgehog (*DHH*) and Indian hedgehog (*IHH*). In mice, *SHH* plays a role in both the initial development of embryonic external genitalia and also in genital masculinisation [Haraguchi et al., 2001; Perriton et al., 2002]. In the hormone-independent phase, *SHH* from the urethral plate epithelium is required for outgrowth, patterning, and cell survival in the developing GT [Perriton et al., 2002] (fig. 2). Mice with a targeted deletion of *Shh* show initiation of the genital swellings, but outgrowth is not maintained, meaning that external genitalia are absent [Haraguchi et al., 2001]. It is thought that *SHH* signalling modulates the balance between apoptosis and proliferation [Haraguchi et al., 2001] (fig. 2). The *SHH* signal is mediated through its receptor Patched which is upregulated by *SHH* in the DUE, and the pathway activator Smoothed which controls the activity of the *GLI* transcription factors (*GLI1-3*). *GLI1* and *GLI2* are thought to be the main transcriptional

activators for the pathway. In *Gli2*, knockout mice outgrowth of the GT is initiated, but urethral formation was lacking [Miyagawa et al., 2011]. The critical role of SHH signalling during this hormone-independent phase is relayed either directly or indirectly by the expression of its targets *Fgf8* and *Bmp7* in the DUE or *Fgf10*, *Bmp2*, *Bmp4*, *Hoxa13*, and *Hoxd13* in the mesenchyme (fig. 2).

In the human foetal penis, *SHH*, *PTCH1*, *SMO*, and *GLI1* are all expressed around the time of urethral closure [Shehata et al., 2011], indicating that the role of this pathway is conserved. Variants in both *SHH* itself, and also in downstream components of its signalling pathway, have been associated with hypospadias in humans. In a large Californian cohort of patients with hypospadias, several SNPs in *SHH*, *GLI1*, *GLI2*, and *GLI3* were associated with an increased risk [Carmichael et al., 2013a] (table 1). Interestingly, some SNPs showed an association only among Caucasian patients and some SNPs in *GLI1* were associated with a decreased risk [Carmichael et al., 2013a] (table 1).

### The WNT Signalling Pathway

Like SHH, signalling by the WNT pathway is required for numerous processes during embryo development, and the 2 pathways are often intricately linked. WNT signalling has a role in both early hormone-independent GT development and later during the hormone-dependent signalling phase (see below). In mice, *Wnt5a* is highly expressed in the genital primordia from 10.5 dpc, and its expression is graded along the outgrowing axis of the GT, with the highest levels distally. In the GT, *Wnt5a* is a transcriptional target of SHH signalling [Perriton et al., 2002] (fig. 2). In *Wnt5a* knockout mice, the GT appears to be specified; however, outgrowth is affected, meaning that the GT is stunted or absent [Yamaguchi et al., 1999].

Many WNT ligands bind to their receptor Frizzled to activate canonical WNT signalling controlling the stabilisation of the transcription factor  $\beta$ -catenin. *Frizzled1* is expressed in the urethral epithelium [Li et al., 2006]. Mice with  $\beta$ -catenin (*Ctnnb1*) loss of function have an abnormal prepuce with no fusion at the ventral midline. WNT/ $\beta$ -catenin signalling appears to be required in the endodermal urethra to activate and maintain the expression of *Fgf8*, directing GT outgrowth (fig. 2).  $\beta$ -Catenin is also required in the mesenchyme to promote cell proliferation and in the ectoderm to maintain tissue integrity. Knockout of  $\beta$ -catenin in any of these tissues causes severe hypospadias in adult mice [Lin et al., 2008].

In humans, mutations in *WNT5A* have been found in patients with Robinow syndrome, who often have hypospadias [Person et al., 2010]. However, there is no report of mutations in patients with isolated hypospadias. This may be due to lethality associated with the disruption of such a vital signalling pathway, and searches may be better focused on regulatory regions or pathway effectors.

### The Fibroblast Growth Factor Signalling Pathway

Signalling by the fibroblast growth factors (FGFs) is suggested to regulate epithelial to mesenchymal interactions during GT growth. In mice, *Fgf8* is expressed in the DUE during early development under the control of WNT and SHH signalling [Haraguchi et al., 2000; Perriton et al., 2002] (fig. 2). This may be through its indirect upregulation by Homeobox

A13 (*Hoxa13*) [Morgan et al., 2003]. FGF8 is required for GT proximal-distal outgrowth, as abolishing FGF8 or its receptors leads to GT agenesis in mice [Morgan et al., 2003]. It is thought that while WNTs and FGF8 signalling work in a linear fashion to direct proximo-distal outgrowth, SHH signalling works in parallel to control growth [Lin et al., 2008] (fig. 2). *Fgf10* is expressed in the mesenchyme of the GT, also under the control of SHH signalling [Perriton et al., 2002]. In *Fgf10*-deficient male mice, the fusion of the urethral folds fails, leading to an open urethral groove [Haraguchi et al., 2000; Yucel et al., 2004]. In addition, mutations in the FGF receptor *Fgfr2IIIb*, that binds FGF10 in mice, cause severe hypospadias [Petiot et al., 2005]. Variants in *FGF8* and *FGFR2* have been associated with hypospadias [Beleza-Meireles et al., 2007a] (table 1). However, no mutations in the *FGF10* gene have yet been associated with hypospadias in humans.

### The BMP Signalling Pathway

Various BMP signalling molecules are expressed in the DUE and surrounding tissues, where they regulate different epithelial-mesenchymal interactions during GT formation [Suzuki et al., 2003] (fig. 2). *Bmp7* is expressed in the urethral epithelium in mice [Morgan et al., 2003], where it is upregulated by HOXA13. A second ligand, *Bmp2*, is expressed in the urethral epithelium and the proximal mesenchyme and *Bmp4* is expressed in both the mesenchyme and the urethral epithelium [Suzuki et al., 2003] (fig. 2). All of these BMP molecules appear to have a role in GT development. Exogenous BMP4 downregulates the expression of several other genes implicated in GT formation, such as *Fgf8* and *Wnt5a*. Null mice for *Bmp7* have an abnormal urethral plate and urethral opening [Wu et al., 2009]. BMP receptor 1a (*Bmpr1a*) mutant mice have decreased apoptosis and increased *Fgf8* expression in the DUE – causing GT hyperplasia [Suzuki et al., 2003]. Thus, BMP signalling may act to negatively regulate GT outgrowth and control apoptosis (fig. 2).

In humans, screening in hypospadias patients revealed mutations in *BMP7* [Chen et al., 2006; Beleza-Meireles et al., 2007a] (table 2) and SNPs in *BMP7* associated with a 2-fold increased risk of hypospadias, regardless of the severity [Carmichael et al., 2013a] (table 1). In addition, exome sequencing revealed 3 missense mutations in *BMP4* (table 2) in middle or proximal hypospadias patients [Chen et al., 2006]. These mutations cause changes to highly conserved amino acids. In contrast, no link between SNPs in *BMP4* and hypospadias was found in a large cohort [Carmichael et al., 2013a].

### WT1 and Its Associated Pathway

WT1 is a transcriptional regulator with various functions including signalling in both the embryonic kidneys and gonads. Mutations in this zinc finger transcription factor are associated with Denys-Drash or Frasier syndromes, which result in a broad range of malformations including hypospadias. While WT1 plays an important role in indifferent gonad development, and later in testicular differentiation, it may also play a more localised role in the external genitalia. Studies in human foetal samples showed that WT1 and an androgen receptor (AR) are co-expressed in the mesenchyme surrounding the urogenital sinus at 7 weeks. In vitro, WT1 can modulate AR expression [Köhler et al., 2007]. Indeed, mutations in *WT1* have been described in boys with isolated hypospadias [Wang et al., 2004]. In a large hypospadias cohort, SNPs in *WT1* were associated with a 2-fold increased



risk of severe hypospadias [Carmichael et al., 2013a]. In addition, SNPs in *WTAP*, a nuclear protein contributing to WT1 function [Little et al., 2000], are suggested to be associated with hypospadias [Carmichael et al., 2013a].

### Homeobox Genes

Homeobox (or Hox) genes convey positional information in developing embryos and are involved in numerous congenital malformations [Acampora et al., 1989; McGinnis and Krumlauf, 1992]. In mice, *Hoxa13* and *Hoxd13* are expressed in the mesenchyme of the GT. As mentioned above, *Hoxa13* regulates the expression of various morphogens such as *Fgf8* and *Bmp7* (fig. 2). These decrease in the urethral plate epithelium in *Hoxa13* knockout mice, causing hypospadias [Morgan et al., 2003]. *Hoxa13/Hoxd13* double-knockout mice fail to develop a GT [Warot et al., 1997].

In humans, *HOX* genes have been implicated in both syndromic and isolated hypospadias. Beside its involvement in hand-foot-genital syndrome [Halal, 1988], where hypospadias is a frequent feature, *HOXA13* is a candidate gene for isolated hypospadias. *HOXA13* expression is increased in the prepuce and urethral plate of hypospadias patients, relative to the position of the urethral meatus [Wang C et al., 2013]. In addition, 2 missense mutations in *HOXA4* have been found in cases of isolated hypospadias [Chen et al., 2006] (table 2), and recent genome-wide analyses showed increased *HOXA4* expression in a hypospadias risk allele [Geller et al., 2014]. Although mutations in *HOXB6* have been found in Chinese boys with hypospadias [Chen et al., 2006] (table 2), a recent study failed to establish a linkage between *HOXB6* and hypospadias [Carmichael et al., 2013a].

### Eph-Ephrin Genes

EfnB2 is a bidirectional signalling molecule that mediates cell adhesion and patterning events occurring at the midline, including scrotal bulge fusion, urethral closure and palate fusion (which happen by an analogous process to urethral closure) [Baskin et al., 2001]. Deletion of *EfnB2* in mice is embryonic lethal at E11.0 due to severe patterning defects [Wang et al., 1998]. However, mutations that specifically disrupt *EfnB2* reverse signalling in mice result in a severe penoscrotal hypospadias phenotype [Dravis et al., 2004]. Similarly, mutations in the *EfnB2* ligands, *EphB2* and *EphB3* result in severe hypospadias [Yucel et al., 2007]. *EFNB2* has also been identified as the key causative gene responsible for hypospadias and genitourinary malformations in 13q-deletion syndrome in humans, confirming its role as key mediator of urethral closure [Garcia et al., 2006].

### Additional Genes

Diacylglycerol kinase K (DGKK) is an enzyme that phosphorylates diacylglycerol, converting it to phosphatidic acid. In mice, *Dgkk* is expressed in the differentiating squamous epithelial cells of the urethral plate/groove and the epidermis during development [Shen et al., 2015]. While little is known about the function of this gene during this time, in humans there is a strong association between DGKK and hypospadias risk [van der Zanden et al., 2011; Carmichael et al., 2013b]. Individual genotyping of 2 SNPs in DGKK showed strong association in various European groups [van der Zanden et al., 2011] (table 2), and expression of DGKK in preputial tissues is significantly lower in carriers of the risk allele

[van der Zanden et al., 2011]. However, the risk may depend on ethnicity as these variants have not been found in a Chinese cohort [Ma et al., 2014].

The transcriptional regulator CHD7 binds to enhancer elements in the nucleus and can positively regulate the biogenesis of ribosomal RNA [Zentner et al., 2010]. Beside its implication in CHARGE syndrome, where signs of hypogonadism can be seen, exome sequencing of a male patient with penoscrotal hypospadias identified a variant in a regulatory region for this gene (table 2). This variant, however, remains of uncertain significance [Baxter et al., 2015].

## Genes Implicated in Testis Development, Masculinisation and Hypospadias

Proper formation of the penis through masculinisation relies on the secretion of male hormones, such as testosterone, from the testis. Mutations that affect the ability of the gonads to produce these hormones, or the cell's sensitivity to these hormones can result in anomalies of male development including hypospadias.

### Testis Signalling Pathways

Sex-determining region Y (*SRY*) is the Y-linked transcription factor that sits at the top of the testis developmental cascade and is sufficient to trigger male differentiation [Berta et al., 1990; Sinclair et al., 1990; Koopman, 1999; Tanaka and Nishinakamura, 2014]. While mutations in *SRY* have not been shown to directly cause hypospadias [Wang et al., 2004], sex chromosome anomalies have [Moreno-Garcia and Miranda, 2002]. In addition, genes that regulate *SRY* expression have been implicated. *MAP3K1*, also called *MEKK1*, is a mitogen-activated protein kinase [Schlesinger et al., 2002]. This gene and its pathway regulate numerous processes including upregulation of *SRY* in the undifferentiated gonad [Warr et al., 2014]. While mutations in this gene lead to complete sex reversal in 46,XY individuals [Baxter et al., 2015], one missense pathogenic variant in *MAP3K1* has been identified in 4 boys with hypospadias [Das et al., 2013] (table 2).

*SRY* activates the transcription of another *SRY*-box-related gene, *SOX9*, in the developing gonad. *SOX9* is required for testis differentiation, as its loss results in 46,XY gonadal dysgenesis. Following this, several other important male pathway regulators are activated. These include *FGF9*, which works in both a feedback loop with *SOX9* and also acts to repress the ovarian development pathway. Mutations in these essential male genes tend to cause gonadal dysgenesis and sex reversal rather than milder phenotypes such as isolated hypospadias. Consistently, in a Chinese cohort of patients with hypospadias, no mutation was found in *SOX9* [Wang et al., 2004]. *SOX9* is responsible for the proper differentiation and function of the Sertoli cells, the first cells to differentiate in the testis. Sertoli cells secrete the anti-Müllerian hormone (AMH), which triggers Müllerian duct regression. Sertoli cells are also required for the specification of the hormone producing Leydig cells. This requires several genes including *DHH*. *DHH* knockout mice have a failure of foetal Leydig cell differentiation, and in humans, mutations in *DHH* can cause 46,XY gonadal dysgenesis with hypospadias [Clark et al., 2000]. Much of the steroidogenic pathway takes place in the Leydig cells, which secrete testosterone – a key step in the masculinisation of



the external genitalia. Below, we highlight the genes involved in the hormone-dependent stage of penile development and how their disruption results in hypospadias.

### **Nuclear Receptor Subfamily 5 Group A Member 1**

The gene nuclear receptor subfamily 5 group A member 1 (*NR5A1*), which encodes steroidogenic factor 1, plays a major role in the development of both the kidneys and urogenital system [Eggers et al., 2015]. Steroidogenic factor 1 acts in the gonads to positively regulate the expression of several key testis genes including *SOX9* and *AMH* [Allali et al., 2011] as well as many of the genes involved in steroidogenesis [Scott et al., 2009]. Mutations in *NR5A1* have been reported in association with a wide spectrum of 46,XY DSD phenotypes: from complete testicular dysgenesis to undescended testes and isolated hypospadias [Köhler et al., 2009; Allali et al., 2011]. Heterozygous mutations in *NR5A1* have been described in several families and individuals with hypospadias [Warman et al., 2011; Wu et al., 2013], and loss-of-function mutations in *NR5A1* tend to be associated with more severe forms of hypospadias [Köhler et al., 2009] (table 2). However, the relative importance of different *NR5A1* polymorphisms in hypospadias may depend on ethnicity. Variants in *NR5A1* were frequently associated with hypospadias in Chinese patients, but not in Swedish Caucasians [Adamovic et al., 2012]. Two novel *NR5A1* mutations with aberrant biological activity were also found in Egyptian patients [Tantawy et al., 2014] (table 2).

### **Mastermind-Like Domain-Containing 1**

Mastermind-like domain-containing 1 (*MAMLD1* or *CXORF6*) is an X-linked transcriptional co-activator. It is expressed in mice foetal Sertoli and Leydig cells around the critical period for sexual development (E12.5–E14.5) [Miyado et al., 2012], where it is probably regulated by *Sfi* [Fukami et al., 2008]. *MAMLD1* has been implicated in various forms of human DSDs, including hypospadias. Mutations in *MAMLD1* were initially described in severe forms of hypospadias but have subsequently been described in milder forms [Fukami et al., 2006; Kalfa et al., 2008a, 2012], including intronic variants [Chen Y et al., 2010] and splice errors [Igarashi et al., 2015]. In silico modelling has predicted pathogenicity in at least 2 of these variants [Kalfa et al., 2011b]. Importantly, the role of *MAMLD1* in hypospadias may be restricted to some ethnic groups as an association was not found in a Chinese population study [Zhuang et al., 2012]. *MAMLD1* is thought to regulate the expression of steroidogenic pathway genes, such as *Nr0b1* and *Cyp17a1* in Leydig cells to permit production of a sufficient amount of testosterone for male sex development [Ogata et al., 2009; Nakamura et al., 2011]. *Maml1* null male mice have normal male development and fertility, but show a mild decrease in the expression of the Leydig-associated genes *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Insl3*. It is interesting to note that *MAMLD1* is also implicated in hypospadias in both a horse [De Lorenzi et al., 2010] and a dog [Switonski et al., 2012].

## **Androgens and Hypospadias**

### **Androgen Production**

Mutations that specifically affect androgen synthesis in the testis can lead to hypospadias. In males, the enzymatic steps that contribute to steroidogenesis mainly take place in the Leydig

cells of the differentiated testis. Firstly, cholesterol is converted into pregnenolone by the cyto-chrome P450 side-chain cleavage enzyme (CYP11A1) (fig. 3). Mutations in *CYP11A1* are thought to cause severe hypospadias with late-onset adrenal insufficiency [Rubtsov et al., 2009; Parajes et al., 2012] (table 2). Following this, 3 $\beta$ -hydroxysteroid dehydrogenase 2 (HSD3B2) catalyses the oxidation and isomerisation of 5- $\beta$ -hydroxysteroid precursors into 4-ketosteroids (i.e. pregnenolone into progesterone) (fig. 3), thus leading to the genesis of all classes of steroid hormones. *HSD3B2* is expressed almost exclusively in the adrenal glands and gonads [Lachance et al., 1991]. A deficiency in this enzyme (usually caused by homozygous or compound heterozygous mutations) can lead to acute adrenal crisis, salt wasting and male pseudohermaphroditism [Mendonça et al., 1994; Russell et al., 1994]. Although uncommon, heterozygous [Codner et al., 2004] and a putative pathogenic homozygous mutation [Kon et al., 2015] have been found in patients with hypospadias (table 2). Androstenedione is converted to testosterone in the Leydig cells by several enzymes. The *AKR1C3* gene encodes one of the 4 human aldo-keto reductases, which function in vitro as 3 $\alpha$ -hydroxysteroid dehydrogenases. They can convert potent sex hormones into their inactive metabolites or act in the reverse direction to produce potent hormones by oxidising inactive forms (fig. 3). Tissue distribution studies in human showed a strong expression of *AKR1C3* in genitourinary tissues [Azzarello et al., 2008]. One mutation has been described in the *AKR1C3* gene in hypospadias (table 2). In silico studies predicted this mutation to damage protein function or structure [Söderhäll et al., 2015]. *HSD17B3* encodes testosterone 17 $\beta$ -dehydro genase 3, another enzyme required for conversion of androstenedione into testosterone. A reduced activity of this enzyme can lead to male pseudohermaphroditism and gynecomastia. While the contribution of HSD17B3 to these cases of DSDs is well characterised, its association with hypospadias is not yet well understood. In a study of 633 hypospadias cases, significant association of 4 SNPs and 2 haplotype blocks in *HSD17B3* was found [Carmichael et al., 2014] (table 1). In a Japanese study of 89 hypospadias patients and 291 controls, the researchers found that in subjects carrying homozygous *HSD17B3*+913A alleles (289S) the risk of hypospadias was significantly higher, including the risk of severe hypospadias [Sata et al., 2010] (table 1). They found that the mRNA expression levels of this form of *HSD17B3* were lower, suggesting this polymorphism may be a risk modifier for hypospadias [Sata et al., 2010]. Following HSD17B3 bio-synthesis of testosterone, the enzyme steroid 5 $\alpha$  reductase type 2 (SRD5A2) converts testosterone into dihydrotestosterone (fig. 3). Dihydrotestosterone binds to the AR with higher affinity than testosterone and is therefore a more potent masculinisation signal for the external genitalia [Manson and Carr, 2003]. In human foetal GT samples, the expression of *SRD5A2* is localised to the stroma surrounding the urethra, especially the urethral seam area in the ventral portion of the remodelling urethra [Kim et al., 2002]. Like HSD17B3, mutations in *SRD5A2* can lead to a variety of phenotypes [Akcay et al., 2014], and the role of SRD5A2 in 46,XY DSD has been studied for almost 40 years. Indeed, various mutations in this gene have been linked with an increased risk of hypospadias [Silver and Russell, 1999; Wang et al., 2004; Samtani et al., 2011; Wang R et al., 2013; Carmichael et al., 2014] and one hypospadias-associated variant, the SRD5A2 V89L polymorphism, is less active in vitro [Thai et al., 2005]. Differences in the activity of SRD5A2 variants may explain why some are associated with hypospadias [Silver and

Russell, 1999], while others are reported to reduce the risk of both mild and severe hypospadias [Thai et al., 2005] (tables 1, 2).

### Androgen Receptor and Associated Genes

Following secretion from the testis, testosterone and dihydrotestosterone both bind the AR to activate a transcriptional response within the developing external sex organs. AR is expressed throughout the developing human penis including the urethra [Kim et al., 2002]. In human foetal genitalia between 12 and 14 weeks of gestation, AR is expressed in the epithelial cells of the urethral plate in the glans, the tubular urethra of the penile shaft, and stromal tissue surrounding the urethral epithelium. Later, between 16 and 20 weeks' gestation, the density of AR expression is greatest in urethral epithelial cells, where it is stronger along the ventral aspect than the dorsal aspect [Kim et al., 2002]. Administration of an AR antagonist to pregnant mice leads to hypospadias in the male offspring [Kojima et al., 2002; Uda et al., 2004]. Hemizygous mutations (as AR is X-linked) have been reported in hypospadias patients in several studies [van der Zanden et al., 2012], predominantly in proximal hypospadias and often associated with other features of under-masculinisation, such as micropenis or bifid scrotum [Hiort et al., 1994; Alléra et al., 1995; Sutherland et al., 1996; Nordenskjöld et al., 1999; Wang et al., 2004; Thai et al., 2005] (tables 1, 2). The contribution of several polymorphisms in AR are yet to be confirmed, with some authors reporting that an expanded CAG repeat could be relevant in isolated hypospadias [Lim et al., 2001; van der Zanden et al., 2012], whereas others found an association between the length of GGN repeats and hypospadias, especially in proximal forms [Aschim et al., 2004; Radpour et al., 2007]. These reported polymorphisms may change the ability of AR to bind testosterone rather than the levels of AR, as mouse studies have reported no direct association between changes in AR mRNA expression and the presence or absence of hypospadias [Agras et al., 2006].

Additional genes that work in conjunction with AR or downstream of it are also implicated in hypospadias. The FK506-binding protein-52 (FKBP52), encoded by the *FKBP4* gene, is a co-chaperone of AR and regulates its activity [Cheung-Flynn et al., 2005; Yong et al., 2007]. Targeted ablation in mice results in ectopic urethral seam fusion [Chen H et al., 2010] and a proximal urethral opening [Yong et al., 2007]. In humans, FKBP52 is expressed in the foreskin of prepubertal boys, and while this gene has been suggested as a candidate for hypospadias, no difference in the expression of this gene was observed between boys with hypospadias and controls [Beleza-Meireles et al., 2007b]. In addition, mutation screening in 91 boys with hypospadias did not identify any mutations, and association studies in a larger cohort failed to show a link between polymorphisms in FKBP52 and hypospadias [Beleza-Meireles et al., 2007b].

### FGF/WNT and HH Signalling

Unlike FGF8, which plays an early role in GT formation, FGF10 and its receptor (FGFR2 IIIb) appear to be involved in the hormone-dependent phase [Petiot et al., 2005]. FGF10-deficient mutant male mice have a failure in ventral fusion of the urethral plate, consistent with hypospadias [Yucel et al., 2004]. AR antagonism leads to a loss of FGF10 expression in the urethra and an associated hypospadias phenotype in mice [Petiot et al., 2005], suggesting

that this gene is a target of AR signalling. In a large association study, 5 SNPs for *FGF10* were associated with a 3- to 4-fold increased risk of hypospadias, regardless of severity. Four of these SNPs were restricted to Caucasians [Carmichael et al., 2013a]. *FGFR2* has also been associated with hypospadias, although results are contradictory [Beleza-Meireles et al., 2007a; Carmichael et al., 2013a]. *Fgfr2IIIb* is thought to be a downstream target of AR, and antagonism of AR may cause hypospadias through a loss of its expression [Petiot et al., 2005]. However, this mouse study only analysed the phenotype during the embryonic and neonatal period, without verification of its persistence into adulthood.

HH signalling is a key factor not only for initial development, but also for masculinisation of the external genitalia in males. During development, SHH expressed in the urethral plate epithelium regulates GT mesenchymal differentiation through *GLI2* [Miyagawa et al., 2011] and *Gli2* mutant mice have defective urethral formation and are feminised [Haraguchi et al., 2001; Yamada et al., 2003]. It is thought that HH signalling facilitates the masculinisation processes by regulating androgen responsiveness by its regulation of AR [Miyagawa et al., 2011]. In marsupials, SHH also plays an important role during these processes, and recent studies suggest that SHH plays a role in the early hormone-independent patterning, followed by a hormone-dependent role [Chew et al., 2014]. In addition to FGF and HH, mouse studies have also shown that the WNT/ $\beta$ -catenin pathway interacts with the androgen pathway to regulate masculinisation of the GT in the hormone-dependent phase [Miyagawa et al., 2009].

## Oestrogens and Hypospadias

### Oestrogen Production

Oestrogens (oestrone, oestradiol) are synthesised in the gonads from androstenedione or testosterone by the P450 aromatase (*CYP19A1*) enzyme. Aromatase insufficiency can result in virilisation of female patients. A novel homozygous mutation has recently been described to cause aromatase deficiency in a 46,XX newborn (with enlarged GT and fusion of labioscrotal folds) and hypospadias and cryptorchidism in the 46,XY sibling [Bouchoucha et al., 2014]. Like reduced testosterone levels, elevated oestrogen levels may also be a risk factor for hypospadias [Staib et al., 1994]. The source of oestrogen can be external, and the risk of hypospadias increases in male babies of women exposed to diethylstilbestrol, a potent synthetic oestrogen, while in utero [Klip et al., 2002].

Indeed, it appears that oestrogens play a critical role in both penile and clitoral development, and maintaining the balance between androgen and oestrogen levels is essential for normal penile development. Studies in hyena, in which females have extreme masculinisation of the external genitalia, have shown that oestrogens play a critical role in the positioning of the urethral orifice, determining elasticity of the urethral meatus, and facilitating epithelial-epithelial fusion events during distal urethra/urogenital sinus and prepuce formation [Cunha et al., 2015].

### Oestrogen Receptors and Associated Genes

In humans, there are 2 oestrogen receptors, *ESR1* (ER- $\alpha$ ) and *ESR2* (ER- $\beta$ ), which interact to form homo- or heterodimer ER complexes. ER bound by oestrogen translocates to the

nucleus to activate oestrogen-responsive genes. *ESR1* and *ESR2* are expressed in most cells of the male urethra [Dietrich et al., 2004], and studies in human foetal penile smooth muscle cells have shown that active ER is expressed [Crescioli et al., 2003]. Interestingly, while testosterone can masculinise the female foetus, oestrogen cannot generally feminise. Various polymorphisms in *ER* genes have been reported in hypospadias. Watanabe et al. [2007] showed a strong association of one haplotype (covering 5 SNPs) of *ESR1* with hypospadias (table 1). Interestingly, while some variants of *ESR1* have been reported to decrease the risk of hypospadias, others have been described to increase it [Ban et al., 2008; Tang et al., 2011]. Various SNPs in *ESR1* are associated with an increased risk of hypospadias relative to severity [Choudhry et al., 2014]. While SNPs in *ESR2* are not associated with an increased risk of hypospadias in this same cohort [Choudhry et al., 2014], a different study found that prolonged CA repeats or heterozygous mutations in *ESR2* increased the risk of hypospadias [Beleza-Meireles et al., 2006, 2007c]. In a Norwegian population, no association was found between hypospadias and 2 polymorphisms of *ESR2* [Aschim et al., 2005]. The expression of both *ER* genes is reduced in foreskins from hypospadias patients versus controls [Qiao et al., 2011], consistent with aberrant oestrogenic effects playing a role in the aetiology of hypospadias.

Factors that affect the level or activity of ER may also play a role. In boys with congenital genitourinary tract masculinisation disorders, including hypospadias, 1.35% of cases showed a de novo duplication of a region of the X chromosome containing the *VAMP7* gene [Tannour-Louet et al., 2014]. Transgenic mice with an increased dose of *VAMP7* phenocopied the defective urogenital traits such as cryptorchidism and hypospadias as well as reduced penile length and subfertility. *VAMP7* is a member of the SNARE family of proteins and is involved in vesicle trafficking from early endosomes to lysosomes [Advani et al., 1999]. *VAMP7* may control the processing/turnover of select proteins, including *ESR1*, and *VAMP7* positively regulates *ESR1* levels meaning its duplication results in an increase in the transcription of oestrogen-responsive genes including *Atf3*, *Ctgf* and *Cyr61*. These genes have all been shown to be upregulated in the foreskin of individuals with hypospadias [Wang et al., 2007]. It will be interesting to see if *VAMP7* or other vesicle-trafficking proteins are associated with isolated cases of hypospadias in the future.

### Oestrogen-Responsive Genes

Like androgens, oestrogen via its receptors can up-regulate target genes in the developing external genitalia. The expression of various oestrogen-responsive genes, including *CYR61*, *GADD45β*, and *ZEB1*, is increased in the foreskin of patients with hypospadias [Wang et al., 2007]. Oestrogen activates the expression of *ZEB1*, a transcription factor [Qiao et al., 2011], and patients with severe hypospadias have increased expression of *ZEB1* in the basal layer of the foreskin epidermis [Wang et al., 2007]. *Zeb1* mutant mice exhibit various developmental defects, although no defect of the sex organs has yet been described, nor have variants in this gene been associated with hypospadias. Like *ZEB1*, the transcription factor *ATF* is an oestrogen-responsive gene that is strongly upregulated in the penile skin of patients with hypospadias [Liu et al., 2005; Wang et al., 2007; Zhou et al., 2009; Gurbuz et al., 2010; Karabulut et al., 2013]. Furthermore, it is overexpressed in the urethral plate surrounding the ectopic orifice of the urethra in human foetuses with hypospadias [Kalfa et

al., 2008b]. Genomic variants of *ATF3* accounted for 10% of patients with hypospadias in one cohort [Kalfa et al., 2008b]. However, other reports showed that ethnicity may have an influence, as upregulation of *ATF3* was not found in tissues from Japanese patients [Takahashi et al., 2013]. Finally, 2 variants in *ATF3* have been found in 2 patients with moderate forms of hypospadias [Beleza-Meireles et al., 2008] (table 2).

Connective tissue growth factor (CTGF) is a mitogen secreted by vascular endothelial cells and plays a role in chondrocyte proliferation and differentiation as well as cell adhesion. CTGF enhances FGF-induced DNA synthesis. The expression of *CTGF* is significantly upregulated in the tissues of patients with hypospadias when they are exposed to oestradiol, compared to a control group [Zhou et al., 2009], an effect relative to the severity of hypospadias [Wang et al., 2007]. Indeed, it has been proposed that *CTGF* may be a candidate gene for hypospadias via the oestrogen regulation pathway [Zhou et al., 2009]. While null mice die at birth, mice with CTGF over-expression have small testes and reduced fertility [Nakanishi et al., 2001]. Polymorphisms in this gene are yet to be linked to hypospadias in humans.

## Candidate Hypospadias Genes

Several other candidate hypospadias genes have been described. *Midline 1 (MID1)*, encodes an E3 ubiquitin ligase involved in Opitz G/BBB syndrome, which can include hypospadias in its phenotype [Lu et al., 2013]. Mild changes in the MID1 protein may cause hypospadias alone, and one study found a SNP associated with hypospadias [Zhang et al., 2011]. Although of unknown function, basophilin 2 (*BNC2*) is evolutionary conserved and expressed in human periurethral tissue. Mutations have been found to be associated with hypospadias [Bhoj et al., 2011] (table 2). Finally, a human gene expression array in hypospadias and control penile tissues found 24 genes to be upregulated. Apart from *ATF3* and *CYR61*, already reported and discussed earlier, genes were involved in apoptosis (*FOS*), apoptosis and signalling (*NR4A1*), metabolism (*PTGS2*), protein binding (*RTN4*), receptor activity (*CD69*), signalling (*DUSP1*, *SOCS3*, *NR4A2*, *EGR1*, *RGS1*, *HBEGF*, *CD9*), transcription (*FOSB*, *JUN*, *JUNB*, *IER2*, *ZFP36*, *KLF2*, *BTG2*, *HNRNPUL1*), translation (*EIF4A1*), and transporter activities (*SLC25A25*) [Karabulut et al., 2013]. The sample size, however, was small, and the significance will need to be confirmed in a larger cohort.

## Environmental Factors and Hypospadias

### Exogenous Endocrine-Disrupting Chemicals and Oestrogen-Like Compounds

The process of urethral closure is known to be exquisitely responsive to the hormonal environment. Oestrogenic and anti-androgenic compounds are well established to induce hypospadias in humans and mice. The increasing incidence of hypospadias, particularly in developed countries has led to the hypothesis that our elevated exposure to oestrogenic and anti-androgenic environmental factors may contribute to its aetiology [Lund et al., 2009; Kalfa et al., 2011a]. This may include exposure to molecules that interfere with synthesis, transport or metabolism of endogenous hormones, such as xenoestrogens or endocrine disrupting compounds (EDCs) [Mar-rocco et al., 2015]. Indeed, a recent prospective study has shown that exposure to EDCs during foetal life significantly increases the risk of



developing hypospadias [Kalfa et al., 2015]. One type of EDCs are phthalates, and an association between phthalate levels in patients and hypospadias has been demonstrated [Choi et al., 2012], perhaps due to their ability to repress testosterone biosynthesis [Giordano et al., 2010]. Phthalates can act in a variety of different ways; dichlorodiphenyltrichloroethane (DDT) and its metabolites block AR and linuron reduces expression of the luteinising hormone (LH) receptor, affecting, in turn, testosterone production. Dioxin is suspected to lower LH secretion, repressing *StAR* and *CYP17*.

Many other chemicals have been identified as toxic for the urogenital tract, such as phytoestrogens, mycoestrogens, epichlorohydrin, atrazine, and furans [Nassar et al., 2010], and many of them have pro-oestrogenic or anti-androgenic effects [Luccio-Camelo and Prins, 2011]. In a recent study, the expression of *WT1*, *LHR*, *17βHSD3*, and *SRD5A2* were downregulated after exposure to bisphenol A [Liu et al., 2015].

### Contraceptives and Assisted Reproductive Technologies

Although they are high in oestrogens, oral contraceptives have not been associated with an increased risk of hypospadias [Nørgaard et al., 2009]. Conversely, an increased risk of hypospadias may be associated with both intracytoplasmic sperm injection and in vitro fertilisation – the former higher than the latter – compared to natural conception [Bonduelle et al., 2005]. However, the data is controversial, and paternal subfertility, as well as epigenetics, such as methylation of the *AR* gene have been suggested as confounding factors [Fauser et al., 2014]. Questions also exist regarding the risk of oestrogen treatment used in these medical interventions [Carmichael et al., 2005; Sørensen et al., 2005].

### Diet, Lifestyle and Maternal Environment

Vegetarian diet with iron supplementation in pregnant women has been associated with a higher risk of hypospadias, perhaps due to greater exposure to phytoestrogens [North and Golding, 2000] or to the increased ingestion of pesticides and herbicides [Giordano et al., 2008]. Iron supplementation itself has also been suggested as a risk factor [Brouwers et al., 2007]. Similarly, an increased risk of genital malformations has been found in greenhouse workers or farmers [Kalfa et al., 2011a] and hairdressers [Ormond et al., 2009]. These findings are however contradictory [Brouwers et al., 2007]. Regarding parental exposure to pollutants, offspring of mothers working in the leather industry [García and Fletcher, 1998] or military personnel [Araneta et al., 2003] have an increased risk of hypospadias. As for the paternal side, some jobs carry increased risks such as vehicle operators, police officers or fire fighters [Schnitzer et al., 1995] and men exposed to dust from grinding metals [Brouwers et al., 2010]. For people living close to hazardous waste-disposal sites, the risk was first described as minor [Elliott et al., 2001], but this has also been refuted [Morris et al., 2003].

A strong association between low birth weight and hypospadias exists, especially in the proximal forms [Giordano et al., 2010]. This has been attributed to in utero growth retardation [Hussain et al., 2002]. Moreover, placental dysfunction may lower chorionic gonadotropin production and influence external genitalia differentiation [Fujimoto et al., 2008]. Maternal smoking is typically associated with low birth weight [Varvarigou et al.,

2009], and one study has suggested an alteration of the Sertoli cell-specific gene *DHH* by maternal smoking [Fowler et al., 2008]. Some studies, however, associate maternal smoking with a negative risk of hypospadias [Håkonsen et al., 2014], perhaps through the anti-oestrogenic effect of tobacco smoke [Michnovicz et al., 1988]. Maternal hypertension during pregnancy, preeclampsia and preterm delivery is also associated with hypospadias [van der Zanden et al., 2012]. An increased risk of hypospadias has been found in overweight and obese mothers [Marengo et al., 2013], which is attributed to an increased level of free circulating oestrogens or larger quantities of chemicals stored in the fatty tissue. Again, however, some reports are contradictory [Adams et al., 2011] and low maternal weight has also been associated with an increased risk of hypospadias [Rankin et al., 2010].

Consumption of several drugs, such as valproate [Rodríguez-Pinilla et al., 2008], loperamide [Källén et al., 2008] or paroxetine [Reis and Källén, 2010] may confer an increased risk of hypospadias in offspring.

One must note, however, that in most cases it is difficult to establish direct association between hypospadias risk and these factors as confounding elements such as the effect of genetic or ethnic background on susceptibility may interfere.

### Environmental-Genetic Interactions and Epigenetics

Gene polymorphisms seem to play a role in the impact of EDCs on the pathophysiology of hypospadias. As many pollutants are metabolised by detoxifying enzymes, allelic variations in these enzymes may change the impact of chemicals and their metabolites on the developing urogenital tissue [van der Zanden et al., 2012]. Cytochrome P4501A1 (or CYP1A1) metabolises various toxins, and an association between CYP1A1 variants and mothers of boys with hypospadias has been shown [Kurahashi et al., 2005]. Similarly, glutathione S-transferases (*GSTM1* and *GSTT1*) are involved in toxin metabolism, and an association between hypospadias and deletion of these 2 genes in mothers has been described [Kurahashi et al., 2005; Shekharyadav et al., 2011].

Environmental factors can change transcriptional activity via epigenetic modifications to the genome. A genome-wide DNA methylation study of foreskin samples from 12 patients and 8 controls identified 14 CpG islands associated with hypospadias. Differentially methylated CpG islands were identified in association with the *SCARB1* and *MYBPH* genes, suggesting these as potential candidates for hypospadias [Choudhry et al., 2012]. DNA methylation of the *AR* was also found to be higher in fore-skin samples from hypospadias patients than in controls [Vottero et al., 2011]. Diethylstilbestrol (DES), thought to be an anti-abortive medication and used until 1971, is associated with an increased risk of hypospadias in males exposed in utero, and this effect extends into the second generation [Klip et al., 2002; Brouwers et al., 2007]. However, for the effects of DES to be considered truly transgenerational, an increased risk of hypospadias must persist into the F3 generation as the F2 are derived from germ cells directly exposed to DES in utero themselves. While this data is not yet available, it does 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. It is well known that DES interferes with histone methylation [Baccarelli and Bollati, 2009],

thus suggesting an epigenetic mechanism for the action of DES [Bredfeldt et al., 2010]. Oestrogen receptor activation is also well known to alter the epigenetic landscape [Wong and Walker, 2013], suggesting that oestrogenic compounds known to cause hypospadias are equally likely to be making long-lasting changes to the transcriptional environment.

## Future Directions

While the studies highlighted above have suggested a role for both genetics and environment in hypospadias, further research is needed to better understand this complex disorder. Identifying candidate genes and regulatory regions is confounded by the complex interaction between environmental exposures and gene polymorphisms in the manifestation and severity of hypospadias in humans. The advances in massively parallel sequencing and their reduced costs will be extremely important for the discovery of novel pathogenic variants or genes associated with hypospadias. In addition, large-scale epigenetic screens in the affected tissues will shed light on the interplay between environment and genetics. Given that numerous morphogenic pathways are involved in external genitalia development, screening for mutations in these genes is unlikely to identify mutations that cause hypospadias alone because of their numerous developmental roles. Instead, focus on the regulatory regions that control the external genitalia-specific action of these genes, as well as identification of genital-specific effector proteins, will be important. Animal models are essential for our understanding of the development of the external genitalia. Advances in genome editing, such as CRISPR technologies, will allow rapid screening for novel genes and mutations that cause hypospadias. Together, these technologies will allow a more comprehensive understanding of the environmental, epigenetic and genetic factors that contribute to hypospadias and provide avenues for prevention or intervention.

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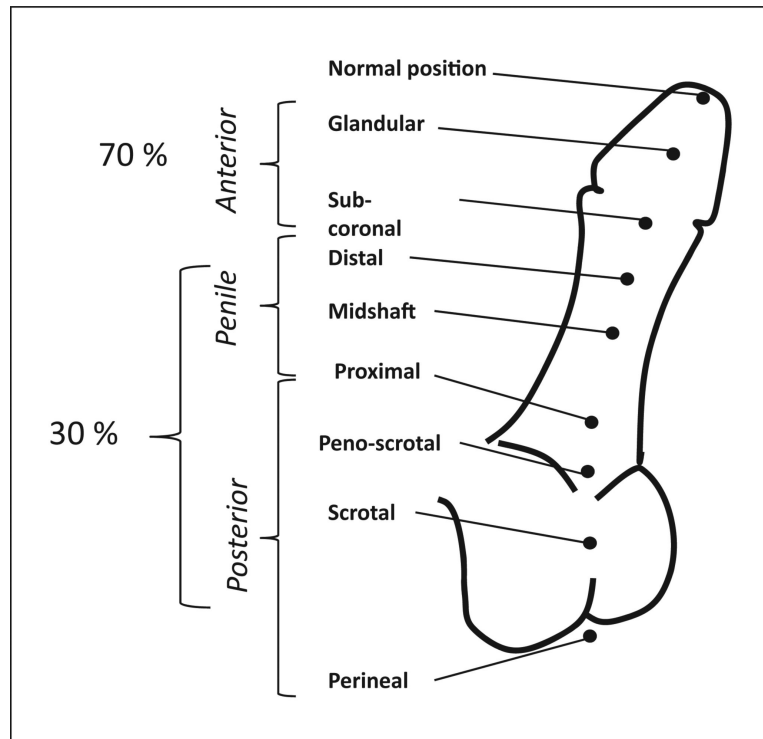
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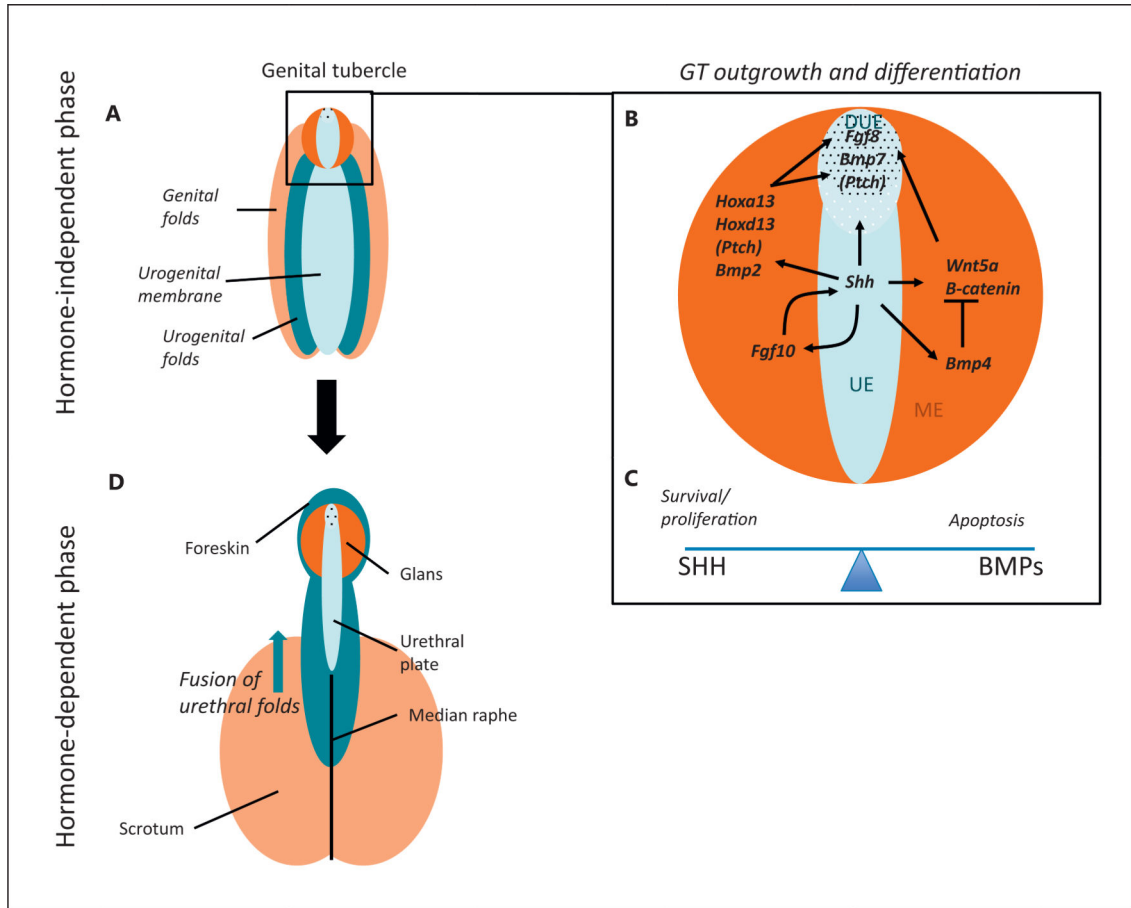
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**Fig. 1.** Clinical classification of the severity of hypospadias. Hypospadias severity is classified according to the position of the urethral meatus. Anterior hypospadias is also referred to as distal or minor, and posterior as proximal or severe. The respective prevalence for each form is noted.

**Fig. 2.**

The molecular pathways underlying the development of the male external genitalia. **A–C** The hormone-independent phase where the urogenital sinus is indifferent. This phase occurs following the division of the cloacal membrane into the urogenital sinus and anal membrane. **A** At this stage the tissue is made up of the urethral folds (dark blue), the urethral epithelium (light blue), the labioscrotal swellings (light orange), and the mesenchyme of the GT (dark orange). **B** The GT (enlarged in box) consists of a urethral plate epithelium (UE), including the distal urethral epithelium (DUE), surrounded by a bilateral mesenchyme (ME). Numerous signalling molecules contribute to GT outgrowth and differentiation during this first phase. A central cue is SHH, which is expressed in the UE, and activates its pathway via its receptor Patched (PTCH). In the bilateral mesenchyme the HH pathway activates the expression of *Hoxa13* and *Hoxd13*, *Wnt5a* and its pathway (including  $\beta$ -catenin). SHH signalling also activates *Fgf8* and *Bmp7* in the DUE. SHH has a positive feedback loop with FGF10 in the mesenchyme. SHH signalling is required for both GT outgrowth and cell survival. SHH targets *Fgf8* and *Wnt5a* are also necessary for outgrowth. Opposing this, BMP4 (and other BMPs) repress outgrowth, perhaps via their repression of *Wnt5a* and *Fgf8*. **C** BMP4 also represses proliferation while activating cell apoptosis, a balance that is counteracted by SHH signaling. **D** The later hormone-dependent stage of development after masculinisation has occurred. During masculinisation, testosterone secreted by the testis causes the GT to elongate and urogenital membrane to transform into urethral groove.

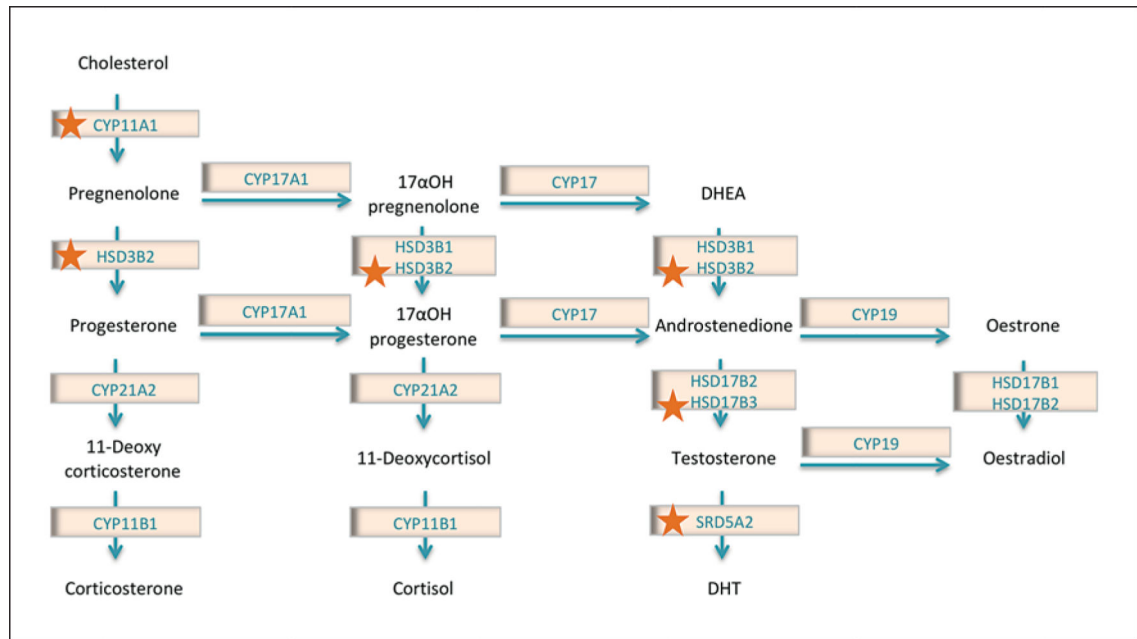
Following this, all components of the penis start to close ventrally, giving rise to the median raphe. A balance between testosterone and oestrogen signalling regulates these processes. The colour of each tissue represents its origin in the previous stages.

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**Fig. 3.**

The steroidogenic pathway. The synthesis of androgens occurs in the Leydig cells of the testis via the steroidogenic pathway. This pathway converts cholesterol into sex hormones, especially testosterone and dihydrotestosterone (DHT). These are necessary for the hormone-dependent stage of penis development, and disruptions in their production can cause hypospadias. Enzymes marked with a star have been implicated in human hypospadias.

**Table 1**

Polymorphisms associated with hypospadias in humans

	Gene	Variants	Effect	Reference
Hedgehog pathway	<i>SHH</i>	rs9333613 (A:G)	↑ (M)	Carmichael et al., 2013a
	<i>GLI1</i>	rs10783827 (T:G), rs4760259 (C:T), rs2228226 (C:G) rs3825077 (G:A), rs3782126 (G:A), rs2292657 (C:T)	↑ (M) ↓	
	<i>GLI2</i>	rs4848125 (A:G), rs4848126 (G:A) rs4143116 (G:A)	2 ↑ (M, Cauc) 2 ↑ (M)	
	<i>GLI3</i>	rs6974655 (G:T) rs9886211 (A:G) rs3801223 (C:T)	↑ (A) ↑ (P) ↑ (M)	
FGF pathway	<i>FGF8</i>	rs3218238 (G:A)	↑	Beleza-Meireles et al., 2007a
	<i>FGF10</i>	rs16901816 (T:G), rs2973644 (T:C), rs2973646 (C:A) rs6892212 (A:C)	2–4 ↑ (M, Cauc) 3 ↑ (M, Cauc)	Carmichael et al., 2013a
	<i>FGFR2</i>	c.382 + 52→G, c.550 + 27C>T, c.727 + 180T>G M186T (hetero)	↑ ↑ (M)	Beleza-Meireles et al., 2007a
BMP	<i>BMP7</i>	rs6070007 (C:A) rs6127978 (A:G), rs6127985 (G:A) rs6127980 (G:A)	↑ (M) ↑ ↑ (P)	Carmichael et al., 2013a
WT1	<i>WT1</i>	rs12293750 (C:A) rs1799937 (T:C), rs5030277 (T:A), rs16754 (A:G), rs5030234 (C:A), rs385449 (G:A)	4 ↑ (A) ↑ (P)	Carmichael et al., 2013a
Additional genes in GT patterning	<i>DGKK</i>	rs1934179 (A), rs7063116 (A)	2 ↑ (A, M)	van der Zanden et al., 2011
		rs1934179, rs7063116, rs4143304, rs9969978, rs1934190, rs1934188	↑ (A, M)	Ma et al., 2014
		rs5961179, rs7882950, rs12556919, rs17003341, rs1934190, rs4143304, rs1934188, rs17328236, rs9969978, rs1934179, rs5961183, rs7876567, rs7063116	↑ (A, M)	Carmichael et al., 2013b
		rs1934183, rs6614511	↓	
Androgen production and signalling	<i>HSD3B1</i>	rs6203(C:T)	2 – 3 ↑ (M, Hisp)	Carmichael et al., 2014
	<i>HSD17B3</i>	rs12552648 (C:T) rs8190566 (A:G) rs8190557 (C:T) rs2026001 (C:A)	↑ (M) 2 ↑ (P) ↑ (P) ↓ (A)	Carmichael et al., 2014
		rs2066479	3 – 4 ↑ (P)	Sata et al., 2010
	<i>SRD5A2</i>	rs1042578 (G:A), rs9332975 (A:G), rs2281546 (T:G), rs28383032 (C:T)	1, 5 ↑ (M) ↑ (M)	Carmichael et al., 2014



	Gene	Variants	Effect	Reference
		rs6543634 (T:G), rs2268794, rs7562326 (T:C) rs519704 (G:A) rs725631 (C:A), rs765138 (C:A)	3 – 4 ↑ (P) ↓ (P)	
		V89L	↓	Thai et al., 2005
		V89L (G:C)	3 ↑ (P)	Sata et al., 2010
	<i>AR</i>	GGN repeats >22	↑ (M)	Radpour et al., 2007 Aschim et al., 2004
		GGN repeats >24	↑ (M)	
Oestrogen production and signalling	<i>ESR1</i>	rs 926779 (G:A), rs 3020364 (A:G), rs 6932902 (G:A), rs 3020371 (C:T), rs 3020375 (C:A)	haplotype AGATA strongly associated	Watanabe et al., 2007
		rs11155820 (A:G), rs3798573 (A:G), rs9479190 (A:G), rs1709183 (A:G), rs3020371 (C:T), rs2347923 (A:C), rs750686 (G:A), rs3798758 (G:T), rs3778099 (T:C), rs3020407 (A:G), rs726281, (A:G), rs7743290 (T:G), rs9397463 (C:T), rs3020364 (A:G), rs3020375 (A:C), rs6932902 (G:A), rs926777 (C:A), rs9479188 (G:A), rs11155819 (T:C), rs9340931 (T:C), rs2459107 (G:A), rs2347867 (G:A), rs6901451 (G:A), rs13203975 (G:A), rs13219476 (T:G), rs3798568 (G:A), rs3822990 (C:T), rs3778090 (C:T), rs12664544 (G:C), rs985695 (C:T), rs9340995 (G:C), s7755185 (A:G), rs2982683 (C:T)	↑ (Cauc and Hisp)	Choudhry et al., 2014
		rs2234693 (T:C)	2 ↑ (M)	Ban et al., 2008
		rs9340799 (A:G)	↓ (M)	
	<i>ESR2</i>	rs944050 (A:G)	↓ (M)	Ban et al., 2008
		rs944050 (A:G), rs3832949 (266_267insC)	↑	Beleza-Meireles et al., 2006
		↑ CA repeat	↑	
		rs2987983 (T:C), rs10483774 (A:G), (CA) <sub>n</sub>	↑	Beleza-Meireles et al., 2007c

Genes are grouped with their respective pathways. In many cases the reference SNP number and the associated allele or nucleotide change is shown. The effect on hypospadias risk (increased or decreased), fold, and associated severity are shown. The number indicated the fold change. ↑ indicates an increased and ↓ indicates a decreased risk of hypospadias. A = Associated with anterior hypospadias; Cauc = the association was found only in Caucasians; Hisp = the association was found only in Hispanic population; M = associated with penile hypospadias; P = associated with proximal hypospadias. The reference for each study is shown.

**Table 2**

Variants identified in hypospadias in humans

	Gene	Variants	Phenotype	Reference
BMP pathway	<i>BMP4</i>	c.619C>G (het), c.668G>A (het) c.751C>T (het)	penoscrotal penile	Chen et al., 2006
	<i>BMP7</i>	c.907C>T (het) c.597G>A (het), c.1567A>G (het) c.1465T>A (het)	glandular penile penoscrotal	
WT1	<i>WT1</i>	N130N (het) A131T (het) S159S (het)	penoscrotal or penile penile glandular	Wang et al., 2004
Homeobox	<i>HOXA4</i>	c.385G>T (het) c.869C>G (het)	penoscrotal penile	Chen et al., 2006
	<i>HOXB6</i>	c.367T>C (het) c.124C>A (het)	penile scrotal	
Gonad development and signalling	<i>MAP3K1</i>	c.2416G>A (het)	penoscrotal	Das et al., 2013
	<i>CHD7</i>	not specified	penoscrotal	Baxter et al., 2015
	<i>NR5A1</i> (SF1)	c.319C>T (het), c.103-3C>A (het), c.31G>T (het)	penoscrotal	Köhler et al., 2009
		p.Arg313Cys	glandular	Allali et al., 2011
		c.184C>T, c.361delGAGACAGG, c.460G>A p.Arg62Cys (het), p.Glu121AlafsX25 (het), p.Ala154Thr (het)	penile penile	Tantawy et al., 2014
		W279X (het), g3314-3317delTCTC	perineal	Warman et al., 2011
		p.Y25C (het)	penoscrotal	Wu et al., 2013
	<i>MAMLD1</i>	E124X (hemi), Q197X (hemi), R653X (hemi)	penoscrotal	Fukami et al., 2006
		V432A (hemi), L121X (hemi) CAG 10 > 13	proximal coronal	Kalfa et al., 2008a
		S143X, P384L	proximal	Kalfa et al., 2012
p.P286S, p.V432A, p.N589S, p.531ins3Q, p.Q529K		not specified	Chen Y et al., 2010	
	p.K609fsX1070	not specified	Igarashi et al., 2015	
Steroidogenesis	<i>HSD3B2</i>	p.S213T (het), p.S284R (het)	proximal	Codner et al., 2004
		p.A10T (hom)	proximal	Kon et al., 2015
	<i>CYP11A1</i>	p.R360W, p.R405X (comp het)	penoscrotal	Parajes et al., 2012
		L222P (hom)	midshaft	Rubtsov et al., 2009

	Gene	Variants	Phenotype	Reference	
	<i>AKR1C3</i>	p.Ala215Thr (het)	penile	Söderhäll et al., 2014	
Androgen production and signalling	<i>SRD5A2</i>	R246Q (hom), Q6X (hom), G203S + L224H (het), 656delT (het)	scrotal	Wang et al., 2004	
		R227Q (hom), G203S (hom)	penile or scrotal		
		R227Q (het)	glandular		
		L113V (het), H231R (het)	proximal	Silver and Russell, 1999	
		G196S (het)	midshaft	Thai et al., 2005	
	<i>AR</i>	1991C>T (hemi)	glandular	Wang et al., 2004	
		2577C>A (hemi)	penile		
		2519G>A (hemi), 2525T>C (hemi), 2564G>A (hemi)	perineal, micropenis, bifid scrotum	Thai et al., 2005	
		Q798E (hemi)	scrotal		
		V870A (hemi)	penoscrotal		Hiort et al., 1994
		G566V (hemi)	perineal		Alléra et al., 1995
		P546S (hemi)	midshaft		Sutherland et al., 1996
		S597T (hemi)	severe		Nordenskjöld et al., 1999
Oestrogen pathway	<i>ATF3</i>	A90G (het), c.817C>T (het)	moderate	Beleza-Meireles et al., 2008	
		C53070T, C53632A Ins53943A	various	Kalfa et al., 2008b	
		L23M (het)	anterior		
Others	<i>BNC2</i>	p.P306A (het), p.P579L (het), p.Q152R (het), p.E240G (het) p.R283G (het)	not detailed	Bhoj et al., 2011	

Gene variants and mutations proposed to be pathogenic in hypospadias. Genes are grouped into signalling pathways. The cDNA or amino acid changes and the inheritance are indicated based on the original publication. The phenotype of the patient(s) carrying these variants is indicated. The references are shown for each study. hemi = Hemizygous; het = heterozygous; hom = homozygous.