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Developmental Biology 270 (2004) 1–18
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Review

Molecular and genetic regulation of testis descent and external genitalia development

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Received for publication 11 September 2003, revised 18 January 2004, accepted 17 February 2004

Available online 12 April 2004

Abstract

Testicular descent as a prerequisite for the production of mature spermatozoa and normal external genitalia morphogenesis, and therefore facilitating copulation and internal fertilization, are essential developmental steps in reproduction of vertebrate species. Cryptorchidism, the failure of testis descent, and feminization of external genitalia in the male, usually in the form of hypospadias, in which the opening of the urethra occurs along the ventral aspect of the penis, are the most frequent pediatric complications. Thus, elucidating the molecular mechanisms involved in the regulation of testis descent and the formation of external genitalia merits a special focus. Natural and transgenic rodent models have demonstrated both morphogenic processes to be under the control of a plethora of genetic factors with complex time-, space-, and dose-restricted expression pattern. The review elucidates the molecular mechanisms involved in the regulation of testis descent and the formation of external genitalia and, wherever possible, assesses the differences between these rodent animal models and other mammalian species, including human.

Keywords: Testis descent; Genitalia development; Molecular regulation; Genetic regulation

Introduction

Testis descent and the formation of external genitalia are part of an essential developmental continuum between the onset of sex determination in utero and the emergence of a reproductively competent adult male (Fig. 1). Caused by genetic or extrinsic endocrine-disrupting compounds, *cryptorchidism*, the failure of the testis to descent into the scrotal sac, and *hypospadias*, a urethral tube dysmorphogenesis with the ectopic ventral opening of the urethra along the penis, scrotum, or perineum, are the result of dysharmony of these orchestrated multistep and multifactorial developmental processes. Regarded as the most frequent complications in newborn males, both ailments are of significant pediatric relevance. Despite important species-specific anatomical and endocrine differences, findings from a variety of animal models have provided deeper insights to allow a better understanding of factors controlling these critical steps in reproductive development in the human.

Aside from male sexual differentiation and the development of testicular tissue, the descent of the testes from an intra-abdominal into the scrotal position is an essential prerequisite for spermatogenesis to occur in humans and many other mammals. Cryptorchidism (Greek: hidden testis) is regarded the most frequent pediatric complication, which affects some 3% of the mature male newborns. While this decreases to 1% in boys aged 1 year, the prevalence rate is 30% in premature boys. Conditions like prematurity, low birth weight, small size for gestational age, twinning, and maternal exposure to estrogens during the first trimester are all associated with a higher frequency of undescended testis. There is a considerable involvement of genetic factors as demonstrated in inherited X-chromosome-linked anomalies associated with cryptorchidism (Table 1; Saifi and Chandra, 1999). Cryptorchidism is also frequently encountered in domestic animals and shows a significantly higher incidence in some breeds (Cox et al., 1978; Hayes, 1986; Rothschild

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doi:10.1016/j.ydbio.2004.02.018

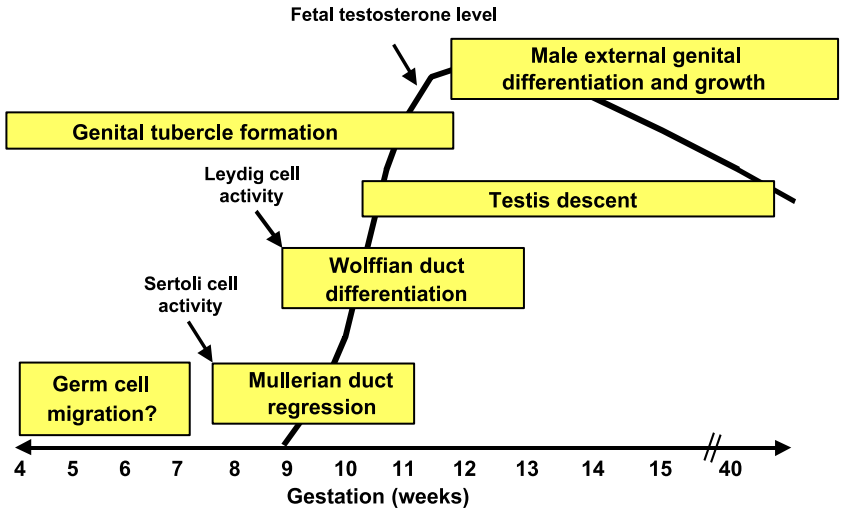


Fig. 1. Testis descent and external genitalia formation/differentiation are late developmental steps during human ontogeny.

et al., 1988). If left untreated, cryptorchidism may lead to disturbed spermatogenesis, impaired fertility, and a higher incidence of testicular cancer in later life (15-fold for unilateral, 33-fold for bilateral cryptorchidism), which is likely a result of exposure of the testis to the increased intra-abdominal temperatures (Benson et al., 1991; Chilvers and Pike, 1989).

Multistaged testicular descent in the human and in animal species can be subdivided into two separate phases, an intra-abdominal and inguino-scrotal phase (Fig. 2; Hutson, 1985; Hutson and Donahoe, 1986). Despite these similarities, there are remarkable differences in the morphology of the testicular suspensory apparatus and the timing of testis descent between species. Testis descent in rodents, lagomorphs, and dogs takes place postnatally, whereas in the human, pig, horse, cattle, and sheep, testis descent is normally completed before birth (Table 2; Barteczko and Jacob, 2000; Rüsse and Sinowatz, 1998; Heyns et al., 1986;1993; Wensing, 1973a,b, 1968).

Table 1
Chromosomal and clinical summary of syndromes associated with cryptorchidism in the human

Chromosome	Clinical syndrome associated with cryptorchidism	Chromosomal location
Chromosome X	Testicular feminization	Xq11–q12, AR
	FG syndrome	Xq12–q21.31
	Dandy–Walker syndrome	Xq25–q27
	Aarskog–Scott syndrome	Xp21.1, *FGDY*
	Torticollis, keloids, renal dysplasia, cryptorchidism	Xq28
	X-linked Kallmann syndrome	Xp22.31
	Arthrogryposis multiplex congenita	Xp11.3–q11.2
	Frontometaphyseal dysplasia	Xq28
	Ichthyosis	Xp22.32, *ARSC1*
	Lowe oculocerebrorenal syndrome	Xq26.1, *OCRL1*
	Mental retardation, X-linked dysmorphism	Xp21.1–p11.22
	Lenz dysplasia	Xq27–q28, *ANOP1* Xp11.4–p21.2, *ANOP2*
Chromosome 1	Zellweger syndrome 2	1p22–p21, *PXMP1*
Chromosome 3	Fancomi anemia	3p22–p26
Chromosome 11	Denys–Drash syndrome	11p13
Chromosome 11	Genitourinary dysplasia component of WAGR syndrome	11p13
Chromosome 15	Prader–Willi syndrome	15q11–13
Chromosome 16	Rubinstein–Taybi syndrome	16p13

INSL3 mediates transabdominal testicular descent

The developing gonads are attached to the abdominal wall in a pararenal position by a cranial and caudal ligamentous apparatus derived from the genital mesenteries. Derived from the cranial portion of the former gonadal and mesonephric ligament (Van der Schoot, 1993), the cranial suspensory ligament (CSL) connects the upper gonadal tip and the attached genital duct with the posterior abdominal wall. Caudally, a ridge-shaped condensation of mesenchymal cells projects from the caudal pole of the developing testis to the future intra-abdominal inner ring of the inguinal canal. This primitive gubernaculum testis further develops into a rather inconspicuous upper intra-abdominal gubernacular mesenchymal cord and a larger caudal segment. During the first phase of testis descent, this caudal gubernacular segment, which is rich in mesenchymal cells, develops into an intra-abdominal segment connecting with the inner inguinal ring, and a larger extra-abdominal part that traverses the inguinal canal and protrudes into the scrotal sac (Barteczko and Jacob, 2000). Associated with the gubernaculum are two structures, the cremaster muscle and the processus vaginalis, which extends into the gubernaculum as a peritoneal diverticulum. The processus vaginalis, together with muscular and fascial layers of the body wall, evaginates into the scrotal swelling, forming the inguinal canal. In the human fetus,

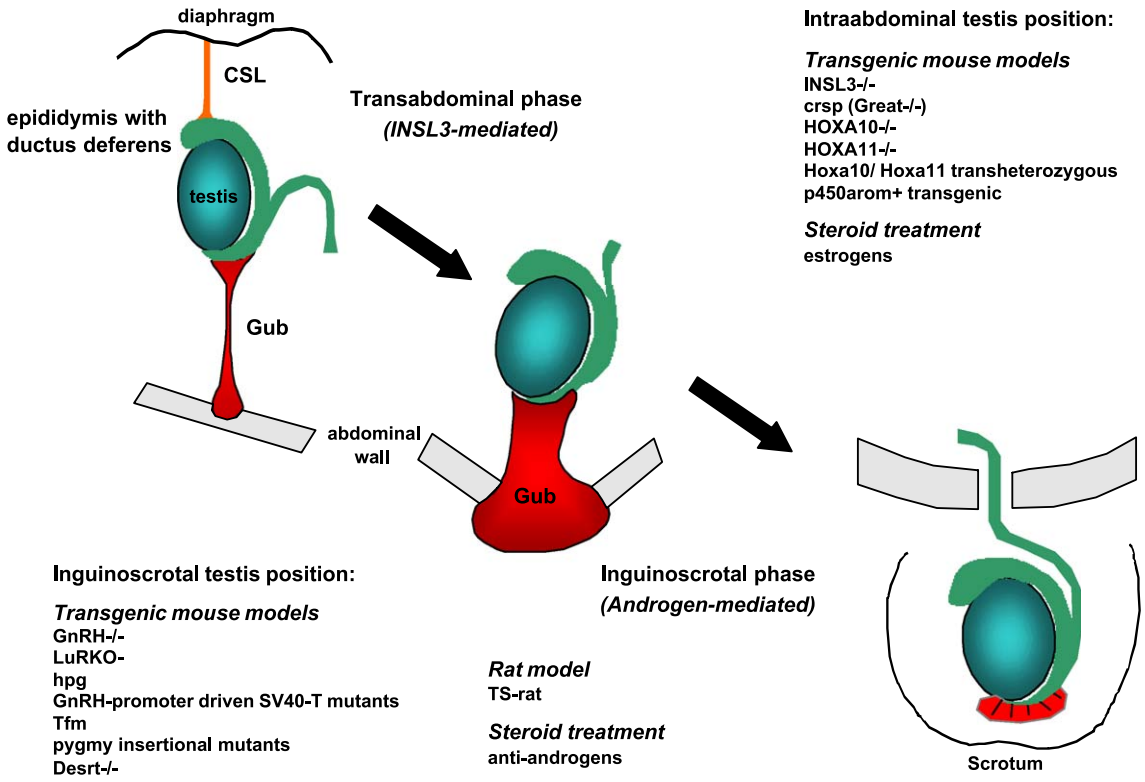


Fig. 2. Two critical phases of testis descent transabdominal (left), and inguinoscrotal (right) travel are essential to move the testes into the scrotum. The intermediate stage is shown in the middle. Molecular factors affecting or regulating the migration of the testis from the abdominal and the inguinoscrotal positions are also summarized. For references, see text.

intra-abdominal testicular descent to the inner inguinal ring is initiated at about 10–14 weeks of gestation and lasts to about weeks 20–23 (Table 2; Barteczko and Jacob, 2000; Costa et al., 2002; Rajfer and Walsh, 1997).

First evidence for the molecular mechanisms involved in the first phase of testis descent came from pioneering experiments on dog puppies some 20 years ago, which demonstrated that uni- and bilateral castration caused abnormal gubernaculum development (Wensing, 1973c). Later, a low molecular weight factor extracted from porcine testicular tissue was shown to stimulate gubernacular mesenchymal cells during the transabdominal phase of testicular descent. This proliferative response was not mediated by androgens (Fentener van Vlissingen et al., 1988; 1989; Heyns et al., 1990; 1986; Visser and Heyns, 1995). This elusive low-molecular-weight factor likely resembles the insulin-like peptide hormone INSL3 which is structurally closely related to relaxin (Adham et al., 1993; Pusch and Ivell, 1996). Regarded a marker of mature testicular Leydig cells of the descended testis in the human as in other species (Hombach-Klonisch et al., 2000; Ivell, 1997; Klonisch et al., 2003), testicular INSL3 is produced in large quantities and can be detected in the serum of the adult male (Boockfor et al., 2001). Bilateral cryptorchidism in INSL3−/− mice results from small, undifferentiated gubernacula without a central core of mesenchyme. Thus, testes remain located high in the

Table 2
Species-specific variation in the stages at which critical phases of testis development and migration occur, shown in both days and expressed as percentages of gestational length in brackets

Species	Genital ridge formation	Testis formation	Beginning of transabdominal phase	Beginning of inguinoscrotal phase	Testis in scrotum
Human (270)	49 (18%)	56 (21%)	70 (26%)	182 (68%)	245 (91%)
Pig (115)	21–22 (18–19%)	27 (24%)	55 (48%)	85–90 (74–78%)	around birth
Horse (336)	30 (9%)	34 (10%)	45 (13%)	ca. 310 (92%)	around birth
Cattle (281)	30–32 (11%)	41 (15%)	80–90 (29–32%)	112 (40%)	around birth
Sheep (149)	22 (15%)	31 (21%)	60–65 (40–44%)	72–75 (48–50%)	around birth
Dog (65)	23–24 (35–37%)	29 (45%)	42 (65%)	4–5 dpp (106–107%)	35–40 dpp (154–162%)
Mouse (20)	9.5 (48%)	12 (60%)	15.5 (78%)	6 dpp (130%)	21 dpp (205%)
Rat (22)	9.5 (43%)	12 (55%)	16 (73%)	6 dpp (127%)	19 dpp (186%)

(Mean length of gestation in days in days postcoitum); dpp = days postpartum; % = percent of gestation.

abdominal cavity close to the kidneys (Nef and Parada, 1999; Zimmermann et al., 1999). Further proof of the essential role of INSL3 for the development of a male-like gubernaculum came from transgenic INSL3−/− male mice overexpressing INSL3 in pancreatic beta-cells, which resulted in normal transabdominal testis descent (Adham et al., 2002). In the mouse testis, INSL3 expression is developmentally regulated and INSL3 transcripts were first detected at embryonic day 13.5, shortly before the induction of gubernacular mesenchymal cell development and gubernaculum outgrowth at embryonic days 15.5 to 17.5 (Adham et al., 2000; Zimmermann et al., 1997). In female mice, gubernacular development is not induced since INSL3 expression in the ovary is initiated only at day 6 after birth (Table 2; Zimmermann et al., 1998). However, transgenic female mice overexpressing INSL3 displayed descended ovaries and inguinal hernia but normal fertility (Adham et al., 2000; Koskimies et al., 2003). The leucine-rich G protein-coupled receptor LGR8 has been shown to specifically bind INSL3 at low nanomolar concentrations in transfected cells (Hsu et al., 2002; Kumagai et al., 2002). Mice carrying mutations in the mouse INSL3 receptor *LGR8* gene (also called *GREAT*) or INSL3 overexpressing transgenic male mice deficient for the *LGR8* gene display a similar abdominal cryptorchid phenotype as INSL3−/− mice (Table 3; Bogatcheva et al., 2003; Overbeek et al., 2001; Tomiyama et al., 2003). Kubota et al. (2002) detected the presence of functional LGR8 receptors in rat gubernacula explants and demonstrated a strong growth response induced by INSL3 alone or in synergy with dihydrotestosterone (DHT), with DHT alone only giving a mild effect. Despite all these findings, only a minority of patients with cryptorchidism show mutations in the INSL3 gene with two important residue changes, Pro^{23} to Leu and Arg^{102} to His or Cys, both located within the C-peptide (Canto et al., 2003; Ferlin et al., 2003; Koskimies et al., 2000; Krausz et al., 2000; Lim et al., 2001; Marin et al., 2001; Tomboc et al., 2000). Just five cases of cryptorchidism all with a heterozygous mutation within the ectodomain of the LGR8 receptor (Thr^{222} to Pro) have so far been described resulting in abolished relaxin-like-induced cAMP production (Ferlin et al., 2003; Gorlov et al., 2002). This suggests a significant involvement of factors other then INSL3 and LGR8 in human spontaneous cryptorchidism, which, among others, may include the insulin receptor family (Nef et al., 2003). Currently, information is lacking on the role of INSL3 in patients with familial cryptorchidism. The reported lack of mutations of the LGR8 gene in Finnish patients with a family history of cryptorchidism further endorses the view that mutations of the LGR8 gene only play a minor role in both nonfamilial and familial cryptorchidism (Roh et al., 2003).

Estrogen-like compounds can affect testicular descent (Fig. 2) and are believed important factors contributing to the testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001). It has long been known that in utero exposure to excess estrogens can inhibit transabdominal testis descent and reduce the amount of extracellular matrix and size of the gubernaculum in pregnant rat, mouse, and oppossum (Habenicht and Neumann, 1983; Hadziselimovic and Girard, 1981; Hutson and Donahoe, 1986; Rajfer and Walsh, 1997). Recently, 17alpha- and 17beta-estradiol and diethylstilbestrol were shown to downregulate INSL3 expression in fetal mouse Leydig cells providing a molecular mechanism for estrogen-mediated inhibition of transabdominal testis descent (Nef et al., 2000). The mouse, rat, and bovine proximal *Insl3* gene promoter contain three consensus binding sites for the steroid factor-1 (SF-1) and this transcription factor has been shown to be involved in the expression of INSL3 in the mouse Leydig tumor cell line MA-10 (Zimmermann et al.,

Table 3
Rodent models associated with cryptorchidism

Rodent model	Cause of cryptorchidism	Reference
$DAX1^{-/-}$ mouse	DAX1 deficiency[a]	Caron et al., 1999
$Desrt^{-/-}$ mouse	A–T rich interaction domain (ARID) class transcription factor deficiency	Lahoud et al., 2001
GnRH-promoter-driven SV40-T transgenic mouse	LHR/ FSHR deficiency	Radovick et al., 1991
$GREAT^{-/-}$ mouse	INSL3/ relaxin receptor deficiency	Overbeek et al., 2001
hpg (hypogonadal) mouse	GnRH deficiency	Charlton et al., 1983
Hoxa $10^{-/-}$ mouse	Homeobox gene products A 10 (HOXA 10) deficiency	Satokata et al., 1995
Hoxa $11^{-/-}$ mouse	Homeobox gene product A 11 (HOXA 11) deficiency	Hsieh-Li et al., 1995
Hoxa 10/Hoxa11	Abdominal-B-related homeobox gene product transheterozygous deficiency	Branford et al., 2000
$Insl3^{-/-}$ mouse	INSL3 deficiency	Nef and Parada, 1999; Zimmermann et al., 1999
LuRKO mouse	LHR deficiency	Zhang et al., 2001
$p450AROM^{+}$ mouse	Aromatase overexpression[a]	Li et al., 2001
Pygmy transgenic mouse	HMGI protein(s) insertional inactivation	Zhou et al., 1995
Tfm (testicular feminization) mouse	Androgen receptor (Ar) mutations and/ or AR dysfunction[a]	Charest et al., 1991
$WT1^{-/-}$ mouse	WT1 deficiency[a]	Kreidberg et al., 1993
Trans-scrotal (TS) rat	CGRP receptor downregulation	Ikadai et al., 1988

[a] Factors also critical to sex determination.

1998). However, estrogens do not alter SF-1 expression or its function on the cholesterol side-chain cleavage cytochrome P450scc gene (Clemens et al., 1994) in fetal mouse Leydig cells indicating that estrogen receptor- (ER-) mediated suppression of *Insl3* gene activity involves additional unidentified regulatory factors other than SF-1 (Nef et al., 2000). A cryptorchid phenotype, similar to that seen in male offsprings of pregnant mice exposed to excess estrogens, can be mimicked by constitutive overexpression of a transgene in male mice (P450AROM+) encoding the enzyme aromatase, also named CYP19 (Li et al., 2001). In these AROM+ mice, cryptorchidism may be the result of a combined effect of the suppression of INSL3 expression, the systemic feedback inhibition of the hypothalamo-pituitary-gonadal (HPG) axis by excess estrogens and the local changes in the androgen–estrogen balance.

Androgens mediate inguinoscrotal testis descent

The second phase of testis descent is usually completed by week 35 in the human and at around day 95 of gestation in the pig fetus (Heyns et al., 1993; Fentener van Vlissingen et al., 1988; Wensing, 1968). While the morphological changes during the inguinoscrotal phase of testis descent from the inner inguinal ring through the inguinal canal into the scrotum are similar among all species, including men, they differ significantly in topography and timing (Table 2; Fentener van Vlissingen et al., 1988; Heyns et al., 1993; Wensing, 1968). After swift passage of the testis through the inguinal canal, the gubernaculum extends as far caudally as the scrotal floor without being attached to the scrotal sac. With further movement of the testis into the scrotum, the collagen content of the gubernaculum increases reflecting its gradual involution (Heyns et al., 1989).

Natural rodent mutants and transgenic mouse models have helped to elucidate the functional role of factors involved in the second inguinoscrotal phase of testis descent (Table 3). The hypothalamo-pituitary-induced production of gonadal androgens appears to be a determining factor for the second phase of testis descent (Hutson et al., 1997; Pitteloud et al., 2002). Impaired testicular descent is a familiar symptom associated with a nonfunctional hypothalamo-pituitary-gonadal (HPG) axis resulting in hypogonadotrophic hypogonadism (Giannopoulos et al., 2001). In the homozygous LH receptor knockout mouse (*LuRKO*), which is devoid of LH stimulation, and in the natural hypogonadal (*hpg*) mouse lacking the gonadotropins FSH and LH because of a mutated gonadotropin-releasing hormone (GnRH) gene, inguinoscrotal, but not transabdominal, testis descent is impaired (Charlton et al., 1983; Grocock et al., 1988; Zhang et al., 2001). Further evidence for the role of CNS-produced GnRH in testis descent came from transgenic mice expressing the viral oncogene, simian virus 40 tumor (T) antigen (Tag), driven by the promoter of human GnRH gene (−1131 to +5 base pairs). Migratory arrest of Tag-expressing neuronal cells and tumor formation along the neuronal migratory pathway caused hypogonadotrophic hypogonadism and cryptorchidism (Radovick et al., 1991). Natural *tfm* (testicular feminization) mutant mice deprived of a functional androgen receptor display a phenotype resembling that of an androgen insensitivity syndrome in humans, which phenotypically presents as incomplete development of the Wolffian duct, micropenis, hypospadiasis, and cryptorchidism (Brinkmann, 2001; Sultan et al., 2001). Importantly, both *tfm* male mice and 46 XY pseudohermaphrodite men with androgen insensitivity syndrome present a normal intraabdominal phase but a failure of inguinoscrotal phase of testicular descent (Fig. 2; Hutson, 1986).

As sexually dimorphic structures, both the gubernaculum and the cranial suspensory ligament (CSL) are target tissues of androgens. In most mammalian species, regression of the CSL is an androgen-dependent process and persistence or aberrant growth of the CSL in male animals, as in men, can result in cryptorchidism and ectopia, the aberrantly directed outgrowth of both the processus vaginalis and the testis (Kersten et al., 1996; Polani et al., 1970; Van der Schoot and Elger, 1992, 1993). Prenatal exposure of boars to the nonsteroidal androgen antagonist flutamide results in the persistence of a CSL and a higher tendency toward cryptorchidism in postnatal life (McMahon et al., 1995). Male *Tfm* rodents retain the CSL resulting in intra-abdominal retention or ectopia of testes and the genetic background (i.e., strain) appeared to affect the penetrance of this phenotype in *Tfm*-rats (Table 3; Hutson, 1986; Stanley et al., 1973; Van der Schoot and Elger, 1992; Van der Schoot and Emmen, 1996;). Interestingly, androgens are unable to suppress CSL formation in male bats with retractable testes and in testicond mammals lacking testis descent (Paenungulata, Monotremata, Edentata, Cetacea) (see, for details, Van der Schoot and Emmen, 1996).

Developmental actions of androgen during inguinoscrotal testis descent

There appears to be a critical time window of specific androgen-mediated, androgen receptor- (AR-) driven actions on the gubernaculum and the CSL early in the process of testis descent. In the rat and pig, mesenchymal cells of the gubernacular core produce immunoreactive AR, which is a prerequisite of androgen action (Bentvelsen and George, 1993; Bentvelsen et al., 1995; Heyns and Pape, 1991; Husmann and McPhaul, 1991; Oprins et al., 1988). The levels of AR are initially expressed independently of androgens in the gubernacula of both sexes, then increase in a ligand-dependent manner in the male gubernaculum, whereas, in the female gubernaculum expression of AR declines (Bentvelsen et al., 1994). In the rat and pig, gubernaculum bulb development is inhibited by the anti-androgen flutamide preventing inguinoscrotal testis descent (Fig. 2; Cain et al., 1995; Husmann and McPhaul, 1991;

Kassim et al., 1997; Spencer et al., 1991). The timing of flutamide treatment appears to be critical. In the rat, flutamide treatment has to be initiated before embryonic day 19 of gestation during the transabdominal phase of testis to inhibit testicular descent and reduce gubernacular size. These flutamide actions are reversed by simultaneous application of testosterone or dihydrotestosterone (Husmann and McPhaul, 1991; Spencer et al., 1991, 1993). Similarly, cryptorchidism is induced in the pig fetus if flutamide treatment is initiated before 74 days of gestation. In this species, inguinoscrotal testis descent occurs after 80 days of gestation and the effect of flutamide is associated with failure of gubernacular regression (McMahon et al., 1995).

The balance between AR and estrogen receptors (ERalpha and ERbeta), as opposed to androgens alone, may be an important factor for male reproductive development (McKinnell et al., 2001; Rivas et al., 2002; Sharpe et al., 2000; Williams et al., 2001). Treatment of neonatal rats with DES has been shown to suppress both testosterone levels and AR expression (McKinnell et al., 2001; Prins and Birch, 1995; Williams et al., 2001). Inhibition of androgen signaling by the anti-androgen flutamide in the prenatal period (embryonic days 14–16) has also been reported to cause a significant increase in ERalpha and ERbeta immunoreactivity in the gubernaculum and cremaster muscle in cryptorchid rats during the first 2 weeks of postnatal life as opposed to normal rats. Flutamide treatment does not appear to affect AR protein expression in both tissues (Yang et al., 2002). This may in part explain the finding that the adverse changes of high doses of diethylstilbestrol on male reproductive tract development in rats can be largely prevented by coadministration of testosterone (Williams et al., 2001).

Another mechanism by which androgens indirectly affect testicular descent is via masculinizing effects of testosterone on the sensory nucleus of the genitofemoral nerve (GFN). In males, this is in L1 to L2 of the dorsal root ganglia. Unilateral transsection of the GFN causes ipsilateral cryptorchidism (Goh and Momose, 1993; Hrabovszky et al., 2000; Momose et al., 1992; Park and Hutson, 1991). The sensory branch of the GFN acts via the neurotransmitter, calcitonin gene-related peptide (CGRP) and affects gubernacular migration during the inguinoscrotal phase. In the rat model of flutamide-induced cryptorchidism, the number of CGRP-immunopositive cell bodies in the sexually dimorphic sensory nucleus of the GFN is significantly decreased following prenatal exposure of male rats to flutamide (Goh and Momose, 1993). CGRP elicits rhythmic contractions of rodent neonatal gubernacula in culture and stimulates the growth and differentiation of neonatal myogenic cells (Momose et al., 1992; Noble et al., 1993; Park and Hutson, 1991). CGRP receptors have been localized to the developing cremaster muscle in rodent gubernacula (Yamanaka et al., 1993). The natural mutant trans-scrotal (TS) rat strain has decreased numbers of CGRP binding sites in an otherwise normally developed gubernaculum and 85% of TS rats display congenital unilateral cryptorchidism (Table 3; Hrabovszky et al., 2001; Goh and Momose, 1993; Terada et al., 1995). Frequently, these cryptorchid testes are found in abnormal locations suggesting that a GFN-derived CGRP-mediated mechanism has a role in directional migration during testis descent.

Testis descent is a multifactorial developmental scenario

DNA binding proteins associated with the transcriptional machinery have been shown to affect testicular descent. Among them are transcription factors of the *AbdB* homeobox (HOX) gene family, which possess key roles in the morphogenesis of posterior body segmental structures, including nerves and ganglia (Krumlauf, 1994). Homozygous mice with homologous recombination for *Hoxa* 10 or *Hoxa* 11 as well as *Hoxa*10/*Hoxa*11 transheterozygous males displayed uni- and bilateral cryptorchidism due to an absent transabdominal testis descent (Table 3) (Branford et al., 2000; Hsieh-Li et al., 1995; Satokata et al., 1995; Rijli et al., 1995). *Desrt* (*de*velopmentally and *s*exually *r*etarded with *t*ransient immune abnormalities), a transcription factor of the A–T-rich interaction domain (ARID) class, is also involved in testis descent (Lahoud et al., 2001). Male mice with a homozygous deletion of the *Desrt* gene are uni- or bilaterally cryptorchid. The undescended testes were in the inguinal region, suggesting that the transabdominal phase of testicular descent was unaffected but, instead, the inguinoscrotal phase was incomplete (Lahoud et al., 2001). Cryptorchidism is also caused by transgenic insertions at two pygmy gene loci ($pg^{TgN40ACha}$ and $pg^{TgN40BCha}$) in pygmy mutant mice. One of these transgenic insertions ($pg^{TgN40ACha}$) results in the deletion of the *Hmgi-c* gene, which encodes for a member of the HMGI high mobility group (HMG) DNA-binding protein family shown to be important in regulating gene expression by affecting the conformational DNA–protein transcription complex (Table 3; Benson and Chada, 1994; Zhou et al., 1995). The phenotypic manifestations common to the pygmy mouse model resembled those seen with a human dwarf syndrome, the growth hormone-resistant Russell–Silver syndrome (RSS), suggesting that the pygmy mouse is a potential animal model for the study of human RSS.

The tumor-suppressor gene product WT1, a nuclear zinc finger DNA-binding protein, has a critical role in urogenital development. *WT1*-knockout mice display failure of urogenital tract development (Kreidberg et al., 1993). In boys, somatic loss-of-function mutations in the *WT1* gene are associated with cryptorchidism and hypospadias as part of the WAGR syndrome (*W*ilms tumor, *a*niridia, *g*enitourinary anomalies, and mental *r*etardation) (Bruening and Pelletier, 1994). Male 46XY patients suffering from Denys–Drash syndrome with a dominant-negative mutation of the *WT1* gene present male pseudohermaphroditism with female or ambiguous external genitalia and hypospadias (Patek et al., 1999; Van-Heyningen et al., 1990). The critical threshold level of gene activity for *WT1* and *WT1* isoforms appears to

be lower in men than in mice, since heterozygous mutant mice show normal testis descent and external genitalia. Gene dosage affecting the phenotype has also been reported in male Dax1- and MIS-null mutants of humans and mice. Men with mutations in the dosage-sensitive sex-reversal gene, Dax1, present a complex syndrome of hypogonadotrophic hypogonadism, adrenal hypoplasia congenita, and uni- and bilateral cryptorchidism (Caron et al., 1999). By contrast, male Dax1-null mice show normal gonadotropin secretion and testosterone production during the embryonic and early postnatal period sufficient to allow for testis descent and the formation of external male genitalia. DAX1 protein would be expected to have a pleiotropic effect on testis descent in men since the *Dax1* gene is expressed in the hypothalamus, pituitary, and gonads (Guo et al., 1995; Swain et al., 1996) and, thus, would cause a combined hypothalamic-pituitary-gonadal defect (Caron et al., 1999). Although speculative at this stage, direct protein interaction between DAX1 and SF1 may negatively affect the testicular INSL3 expression and, thus, prevent the initiation of transabdominal testis descent (Nachtigal et al., 1998; Zimmermann et al., 1998). Müllerian Inhibiting Substance (MIS; also named anti-Müllerian hormone, AMH), a member of the transforming growth factor-beta superfamily and a product of the immature testicular Sertoli cells, has long been considered to play a major role during transabdominal testis descent. In the human, absence of MIS was reported to prevent the first phase of testis descent resulting in pararenal or hypermobile testes (Josso and diClemente, 1997). However, earlier findings in the pig suggested (Fentener van Vlissingen et al., 1988) and recent data in mice and rat indicate a contributing but not a critical role of MIS or its receptor MISRII in gubernacular development in these species (Bartlett et al., 2002; Kubota et al., 2002; Nef et al., 2000).

Disturbance of testicular Leydig cells differentiation by inactivation of Desert hedgehog (*Dhh*)/Patched1 signaling has been shown to critically affect not only testis descent but also the formation of male external genitalia. Mice with a deletion of the *Dhh* gene display arrested Leydig cell maturation. Consequently, these male $Dhh^{-/-}$ mice contain pararenal testes indicative of the lack of sufficient INSL3 production in undifferentiated testicular Leydig cells (Table 3) and feminized external genitalia as a result of androgen deprivation (Clark et al., 2000).

Formation of the genital tubercle and vertebrate limb buds share common developmental genetics

In mammals, delivery of male gametes by copulation and efficient internal fertilization depends on the coordinated growth and differentiation of highly developed external genitalia, and this process includes the formation of the urethral plate and the urethral tube. Constituting the anlage for penis and clitoris, the genital tubercle (GT) is the last structure to develop along the antero-posterior body axis during embryogenesis and emerges as a medial proximo-distal outgrowth of the external genital anlage ventral to the

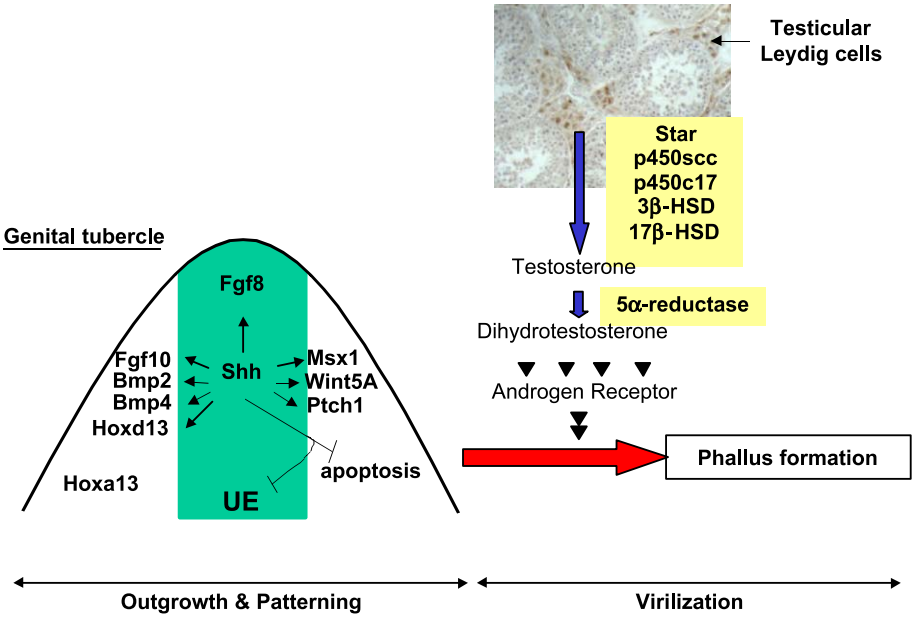


Fig. 3. Diagrammatic summary of the molecular factors involved in the formation, patterning, and virilization of the genital tubercle (GT). While Fgf8, but not Shh, seems essential for the initiation of the GT formation, Shh expressed in the urethal epithelium (UE; green) critically affects GT differentiation by (a) regulating, directly and indirectly, the expression of other factors in the GT mesenchyme and (b) affecting apoptosis. Disturbances in GT and external genitalia development are also observed in mouse mutants with deleted Hoxa13 and/ or Hoxd13 genes (see also Table 5). Expression of Hoxd13 is enhanced by *Shh* but the regulation of Hoxa13 gene activity in the GT is still unclear. Androgens are essential for virilization of the GT and developmental defects in the androgenic pathway will result in feminization of male external genitalia, such as hypospadias.

Table 4
Species-specific variation in the stages at which genital tubercle formation and male external genitalia differentiation are initiated, shown in both days on average and expressed as percentages of gestational length

Species (mean length of gestation in days)	Formation of the genital tubercle, days postcoitum (percent of gestation)	Differentiation of male external genitalia, days postcoitum (percent of gestation)
Human (270)	30 (11%)	80 (30%)
Pig (115)	20 (17%)	30 (26%)
Horse (336)	30 (9%)	45 (13%)
Cattle (281)	37 (13%)	60 (21%)
Sheep (149)	22 (15%)	43 (29%)
Dog (65)	30 (40%)	40 (62%)
Mouse (20)	11.75 (58.5%)	17 (85%)
Rat (22)	14.25 (65%)	17 (77%)

cloacal membrane (Fig. 3; Table 4). The GT consists of the lateral plate mesoderm, surface ectoderm, and endoderm urethral epithelium derived from the urogenital sinus. Similarities exist between genital and limb development, both on the morphogenic and the molecular level: (a) the GT has polarizing activity; (b) transplanted mouse GT supports digit formation in chick wings; (c) Hoxd13 and Hoxa13 gene products are critical for GT and digit development; and (d) the GT urethral epithelium acts as a molecular signaling center for the coordinated epithelial–mesenchymal interactions essential for normal genital development (Fig. 3; Dolle et al., 1991; Haraguchi et al., 2000; 2001; Izpisua-Belmonte et al., 1992; Perriton et al., 2002; Warot et al., 1997; Zakany et al., 1997).

Initial outgrowth and patterning of the early GT are an androgen-independent process and require the presence of *Sonic hedgehog* (*Shh*), which is essential for external genitalia formation in mice (Haraguchi et al., 2001; Perriton et al., 2002; Kalloo et al., 1993). Evidence for the central role of Shh during GT formation also comes from two sets of experiments. Explanted GT incubated in vitro with an anti-Shh antibody show retarded GT outgrowth and this may be the result from impaired Shh signaling, as mice with a targeted deletion of the transcription factor Gli2, a member of the Shh signaling cascade, display a similar GT phenotype (Table 5; Haraguchi et al., 2001). Administration of an alkaloid with the ability to block Shh signal transduction, dihydrojervine, to pregnant mice at 9.5 days postconceptum (dpc) also induces abnormal GT development (Table 5; Cooper et al., 1998; Haraguchi et al., 2001). Murine Shh is detected as early as embryonic day 10, shortly before the onset of GT outgrowth in the cloacal membrane. Subsequently, Shh is also expressed in the endoderm of the urogenital sinus and its derivatives, including the urethral epithelium (Perriton et al., 2002). Although the earliest initiation of paired genital swellings, a prerequisite for GT formation, is an Fgf8-mediated Shh-independent process, GT outgrowth, and differentiation depend on the ability of Shh to orchestrate, directly or indirectly, the activity of numerous genes, including its receptor Ptch1, endodermally derived Fgf8 or Fgf10, Bmp2, Bmp4, Wnt5A, Msx1, and Hoxd13 from the GT mesenchyme (Fig. 3; Haraguchi et al., 2000, 2001; Perriton et al., 2002). Inhibition of Shh signaling leads to a downregulation of these genes and, in the case of Fgf10, may attribute to a hypoplastic mesoderm, growth retardation, and ventral GT dysmorphogenesis in Fgf10 mutation mice (Haraguchi et al., 2000). It should, however, be emphasized that Fgf10 is not essential for early GT outgrowth but significantly affects morphogenesis of the most distal GT structures, the glans penis and the glans clitoridis. $Shh^{-/-}$ mouse embryos displayed a strong increase in apoptosis at 10.5 dpc before the initiation of GT outgrowth and at 11.5 dpc, which correlated with (a) decreased proliferation of both the urethral epithelium and surrounding mesoderm and (b) alterations in the intensity and cellular location of Bmp4 gene expression that suggest Shh to affect apoptosis and cell proliferation during GT formation. Expressed by the GT urethal epithelium downstream of Shh, Fgf8 was shown to augment the expression of the genes for Bmp4, Fgf10, Msx1, and Hoxd13 within the

Table 5
Transgenic mouse models with impaired external genitalia development

Transgenic/natural mouse models	Phenotype of external genitalia	Reference
Sonic hedgehog ($Shh^{-/-}$)	Agenesis of external genitalia due to lack of GT outgrowth	Haraguchi et al., 2001
Gli2 transcription factor ($Gli2^{-/-}$)	Ventral malformation with lack of urethral tube formation	Haraguchi et al., 2001
Fibroblast growth factor 10 ($Fgf10^{-/-}$)	Hypoplastic GT, altered morphogenesis of the glans penis/glans clitoridis	Haraguchi et al., 2000
Hypodactyly ($Hd^{-/-}$) mouse (frameshift mutation in exon1 of Hoxa13)	Penian bone defects; high lethality; sterile	Post and Innis, 1999
$Hoxd13^{-/-}$	Altered size of penian bone; hypofertile	Podlasek et al., 1997; Warot et al., 1997
$Hoxa13^{+/-}$; $Hoxd13^{-/-}$	Hypoplastic erectile penile tissues (corpora cavernosa); sterile	Warot et al., 1997
$Hoxa13^{-/-}$; $Hoxd13^{-/-}$	Agenesis of external genitalia due to lack of GT outgrowth; sterile	Warot et al., 1997
$Hoxd11^{-/-}$; $Hoxd12^{-/-}$; $Hoxd13^{-/-}$ ($HoxD^{del/del}$)	Reduced size of the GT and penian bone; hypofertile	Zakany et al., 1997
$Hoxa13^{+/-}$; $HoxD^{del/del}$	Severely reduced size of the penian bone; sterile	Zakany et al., 1997
Desert hedgehog ($Dhh^{-/-}$)	Defective Leydig cell differentiation; cryptorchidism and hypospadias	Clark et al., 2000; Yao and Whoriskey, 2002

mouse GT mesenchyme between 12.5 and 13.5 dpc (Haraguchi et al., 2000). However, inhibition studies with a neutralizing anti-FGF8 antibody indicate a direct regulatory role for FGF8 on Bmp4 only, suggesting the presence of additional unidentified stage-specific mediators of FGF8 action within the GT signaling from the urethral epithelium to the mesenchyme.

Of the most 5′-located Hoxa13, Hoxd11, Hoxd12, and Hoxd13 Hox gene paralogues, expression of Hoxa13 and Hoxd13, acting in a redundant manner, is critical for the induction and growth of the GT as well as limbs (Table 5; Warot et al., 1997) and, for Hoxd13, has been shown to be independent of Shh. These two genes cannot be compensated for by either paralogues Hoxd11, Hoxd12, or Hoxc13 also reported to be present in the GT. The close association of these 5′-Hox genes with external genitalia and limb development is further illustrated in male mice, which, like many male rodents, contain a penile bone known as the baculum (os priapi). The cells forming the baculum strongly express HoxD genes. Inactivation of Hoxd13 ($Hoxd13^{-/-}$) or simultaneous inactivation of Hoxd11, Hoxd12, and Hoxd 13 ($HoxD^{Del/Del}$) results in minor alterations and size reduction of the baculum, whereas male mice with a combined $Hoxa13^{+/-}$, $HoxD^{Del/Del}$ genotype have an extremely short baculum and also a reduced digit size (Table 5; Zakany et al., 1997). In the human, rare dominantly inherited distal limb malformation conditions are synpolydayctyly (SPD; MIM 18600), Hand–Foot–Genital syndrome (HFGS; MIM 140000) and Guttmacher syndrome (MIM 176305). SPD is caused by a mutation at the N′-terminus of the HOXD3 gene, resulting in an expansion of a normally 15-residue polyalanine sequence, named polyalanine tract, by additional alanines in the HOXD13 protein. In one case with exceedingly large polyalanine tract expansion, affected males of this family displayed severe limb defects and hypospadias (Goodman, 2002). HFGS is caused by *HOXA13* mutations and the urogenital malformations include hypospadias in about 50% of cases and sometimes also cryptorchidism

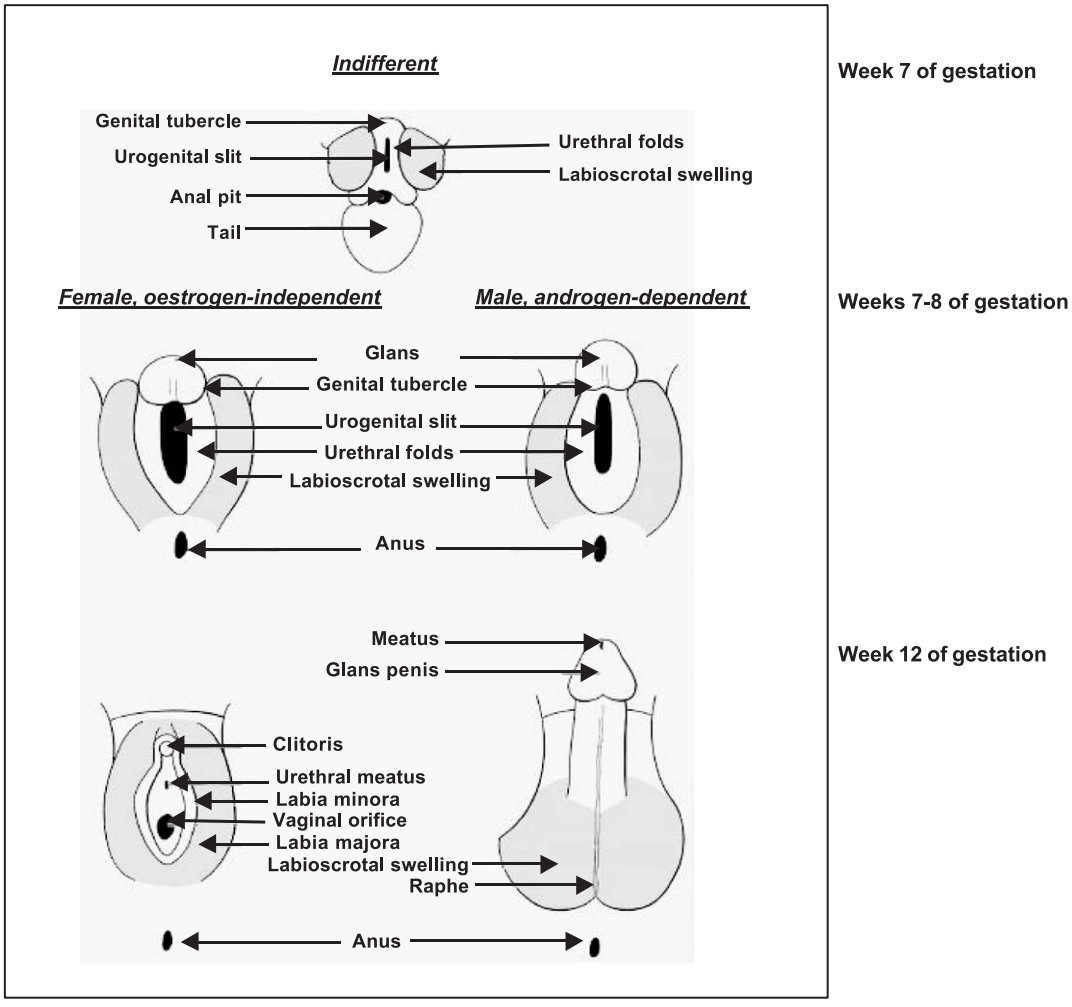


Fig. 4. Stages of genital development in the human fetus. Differentiation of an indifferent genital anlage towards a female or male genital is initiated at around 7 weeks of gestation and is dependent on the actions of estrogens and androgens. Defective ventral closure of the urethral tube during the formation of the penile urethra will result in hypospadias and may be caused by disruption of androgen signaling.

and micropenis (Goodman et al., 2000). Hypospadias is also associated with Guttmacher syndrome, which shares many other clinical features with HFGS (Guttmacher, 1993).

Androgens are essential for virilization of the genital tubercle

Upon formation of a sexually dimorphic GT, exposure to androgens masculinizes the genitalia characterized by the rapid elongation of the GT into the phallus, the closure of the opposing urethral folds to form the penile urethra and the formation of a scrotum by fusion of the genital swellings (Fig. 4). In human female fetuses, the GT elongates only slightly to form the clitoris and the urethral folds and genital swellings do not fuse but develop into the labia minora and labia majora, respectively. Disruption of androgen signaling can result in feminization of the genitalia, which frequently includes hypospadias, the most common congenital malformation in humans affecting one in every 125 male births. The incidence of hypospadias is rising dramatically in many countries (Boehmer et al., 2001; Clarkson et al., 1993; Czeizel, 1985; Toppari and Skakkebaek, 1998; Yamaguchi et al., 1991). More severe disturbances in androgen biosynthesis result in the clinical condition of pseudohermaphroditism, which is characterized by a genotypic sex masked by a phenotypic appearance closely resembling the other sex. This is opposed to the extremely rare true hermaphroditism, characterized by the presence of both ovaries and testes within the same individual.

Female pseudohermaphroditism results from excessive and inappropriate exposure to androgens during early gestation causing variable masculinization of the external genitalia. This may result from endogenous fetal androgen overproduction caused by defects in enzymes of the biosynthetic pathways toward glucocorticoides and mineralocorticoides as in congenital adrenal hyperplasia, leading to shunting of precursor molecules into the androgenic pathway. CYP21 21-hydroxylase deficiency accounts for over 90% of steroid biosynthesis defects, with an incidence of 1:10,000 to 1:18,000 live births (Donohoue et al., 1995). Rarer instances include deficiencies in the enzymes for 11beta-hydroxylase (CYP11B1) and 20,22-desmolase (Prader and Gurtner, 1955; White et al., 1991). Maternal androgen-secreting tumors such as Sertoli–Leydig cell tumor or exogenous administration of androgens and progestins to the mother can also attribute to female pseudohermaphroditism (Table 6; Sultan et al., 2001).

Table 6
Causes of male and female pseudohermaphroditism in the human

Female pseudohermaphroditism	Male pseudohermaphroditism
CYP21, 21-hydroxylase deficiency in congenital adrenal hyperplasia	Defect in the formation of functional testis
CYP11B1, 11beta-hydroxylase deficiency	Hypoplastic testicular Leydig cells
20,22-desmolase deficiency	Insufficiency of the LH ligand–receptor
Androgen-secreting tumors, e.g., Sertoli–Leydig cell tumors	p450 scc deficiency
Excessive androgen administration	3 β-hydroxysteroid-dehydrogenase type II deficiency
Excessive progestin administration	17 α-hydroxylase/17,20-lyase (P450c17 or CYP17) deficiency
	17 β-hydroxysteroid-dehydrogenase type III deficiency
	5 α-reductase
	Functional androgen receptor deficiency

By contrast, male pseudohermaphroditism is the result of defects in androgen- and AR-mediated virilization of the external genitalia in a 46 XY genetically male. The most frequently encountered congenital anomaly of the male external genitalia resembling a feminized genital phenotype is hypospadias. Deficiencies at multiple levels, which result in the failure to generate sufficient androgens, are common

Fig 6. A cross-species comparative review of the timing of key developmental events. Genital ridge formation and testis descent expressed as percentages of gestation in the human and in various animal species are shown in the left-hand panel. Plasma concentrations of anti-Mullerian hormone (AMH; only high levels indicated) and androgen levels in males are shown in the right-hand panel as a developmental function of fetal/perinatal testicular Sertoli and Leydig cells (Donahoe and Swann, 1978; Gondos, 1980; Lejeune et al., 1998; Tran et al., 1987), respectively. No published data were found for equine fetal AMH plasma concentrations. For the male dog, plasma androgen concentrations were only available until postnatal day 54 (*). Developmental data sufficient to determine the onset of a functional hypothalamic-pituitary-gonadal (HPG) axis were available for the human (185 dpc/66% of gestation), pig (81 dpc/72% of gestation), cattle (120 dpc/43% of gestation), sheep (80 dpc/47% of gestation), mouse (17 dpc/85% of gestation), and rat (20 dpc/91% of gestation) as indicated by the star. In the fetal dog, detection of immunoreactive LH in the fetal pituitary is suggestive of a canine functional HPG axis at around day 38 (63% of gestation; Sasaki and Nishioka, 1998). Significant interspecies differences in timing and endocrine profile were observed for each developmental step of testis descent and external genitalia differentiation, with the sheep displaying developmental parameters resembling most closely the situation in men. Note that the mouse is the only ascrotal species shown. Published data on fetal AMH and androgen profiles of the species investigated are as follows: Allen (2001); Baker and O'Shaughenssy (2001); Baumans et al. (1985); Beck-Peccoz et al. (1991); Clark et al. (1984); Clarnette and Hutson (1999); Colenbrander et al. (1978); Collu et al. (1983); Donahoe et al. (1976); Donahoe et al. (1997); Dominguez et al. (1988); Feldman and Bloch (1978); Ford et al. (1980); Gross and Baker (1979); Hochereau-de Reviers and Monet-Kuntz (1987); Josso et al. (1993); Kawakami et al. (1993); Kiser et al. (1975); Kuroda et al. (1990); MacArthur et al. (1967); Majdic et al. (1998); Meyers-Wallen et al. (1991); Meyers-Wallen et al. (1993); Mongkonpunya et al. (1975); Murray et al. (2000); Nemeskeri et al. (1986); O'Shaughenssy et al. (1998); O'Shaughnessy et al. (2000); Pakarinen et al. (1994); Pashen et al. (1982); Ponzilius et al. (1986); Quirke et al. (2001); Raeside (1976); Raeside and Renaud (1985); Raeside et al. (1997); Rhind et al. (2001); Rota et al. (2002); Schwarzenberger et al. (1993); Tapanainen et al. (1984); Tassemeier (2002); Terasawa and Fernandez (2001); Thomas and Brooks (1997); Tran and Meusy-Desolle (1977); Van Vorstenbosch et al. (1984); Vigier et al. (1983); Visser and Heyns (1996); Warren (1989); Warren and Haltmeyer (1973).

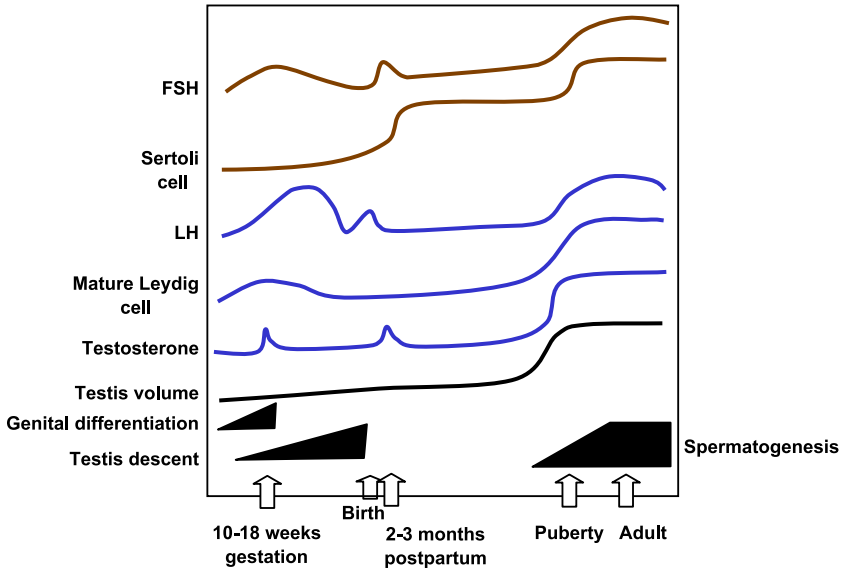
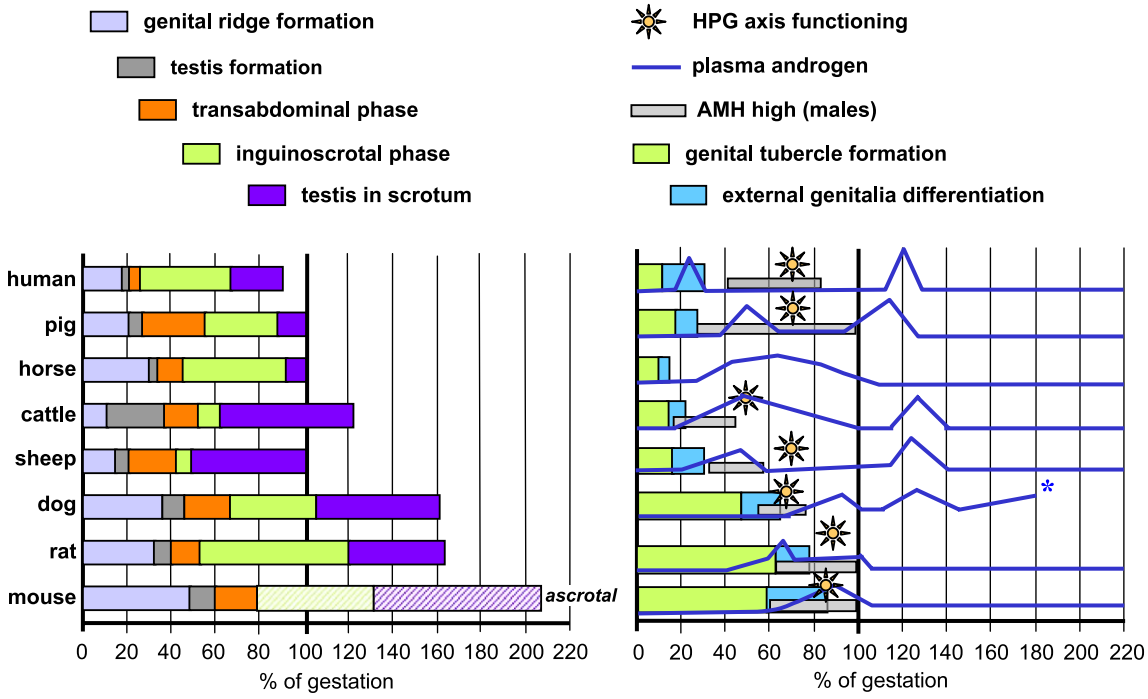


Fig. 5. Schematic diagram illustrating the correlation between morphological and endocrine developmental parameters during the course of male maturation from human fetal life to adulthood. Qualitative changes in testosterone and gonodotropin (FSH, LH) serum concentrations and changes in testicular Sertoli and Leydig cell numbers are indicated.

causes for defects in virilization of the external male genitalia. In the human fetus, DHT is the most potent androgen to cause virilization of the developing external genitalia (Table 6; Imperato-McGinley and Zhu, 2002; Imperato-McGinley et al., 1985). The lack of functional testis formation by the 12th week, complete absence or presence of functionally impaired hypoplastic testicular Leydig cells, and partial or complete insufficiency of the LH ligand–receptor system will all result in virilization defects (Boehmer et al., 2001; Clark et al., 2000; Themmen and Huhtaniemi, 2000; Yao and Whoriskey, 2002). Insufficient testosterone biosynthesis as a result of mutations in one of the enzymes converting cholesterol into testosterone (P450 side chain cleavage (P450scc), 3beta-hydroxysteroid-



dehydrogenase type II [3beta-HSDII], 17alpha-hydroxylase/17,20-lyase [P450c17 or CYP17], and 17beta-hydroxysteroid-dehydrogenase type III [17beta-HSDIII], leads to varying degrees of male pseudohermaphroditism (Table 6; Miller, 2002). In the human, but not in mice, disturbed conversion of testosterone into DHT due to deficient 5alpha-reductase activity will result in feminized genitalia at birth, emphasizing the endocrine and morphological interspecies differences in external genitalia development (Baskin, 2000; Mahendroo et al., 2001; Pakarinen et al., 2002; Takane et al., 1990). Numerous mutations of the androgen receptor (AR) gene located at Xq11–12 may affect signal transduction by both testosterone and DHT (Table 6; McPhaul and Griffin, 1999; http://www.mcgill.ca/androgendb/) but appear not to be a major cause of dysmorphogenesis of the external genitalia and hypospadias in men (Allera et al., 1995; Hiort et al., 1994).

How appropriate are animal models of cryptorchidism and hypospadias to the human?

An optimal environment for the continued production of fertile spermatozoa and an efficient delivery system of intact external genitalia are crucial landmarks in male reproduction. In the human, as in most mammalian species, the morphological and endocrine changes during testicular descent and the development of the external genitalia are orchestrated by the concerted actions of multiple genes that are believed to follow the same general principles (Fig. 5). Common phenotypes as a result of disturbances of these multistaged developmental processes are cryptorchidism and hypospadias. Natural rodent mutants and transgenic mouse models are instrumental in discovering and elucidating the functional role of numerous factors involved in testicular descent and external genitalia development (Tables 3 and 5). However, their value in determining the causes of cryptorchidism and dysmorphogenesis of external genitalia in the human and larger animals is limited because of many differences with respect to anatomical features of the reproductive tract, fetal and neonatal timing of key events, mode of endocrine actions, and the sensitivity toward certain endocrine factors (Fig. 6). With respect to anatomical structures and fetal hormone secretion, the dog, sheep, and nonhuman primates may at present be regarded suitable animal models for comparative studies on cryptorchidism because they resemble the human situation most closely. Although the pig has been employed as animal model for cryptorchidism because of similar gubernacular morphology and the timing of testis descent, pig and human do not share similar fetal hormonal milieus. Porcine gubernacular outgrowth occurs at a time of low fetal serum testosterone levels and regression of the gubernaculums takes place when circulating androgen concentrations are increasing. In contrast, gubernaculum outgrowth in the human, sheep, and dog occur at relatively high testosterone levels while regression coincides with relatively low testosterone levels (Husmann, 1998; Sweeney et al., 1997).

When compared with the human, rodents also display significant differences in urethral development, penile anatomy/morphology and pattern of androgen receptor expression. Animal models more closely resembling the anatomical and endocrine situation in the human male newborn, such as the dog, the rabbit, the immature pig, and the rhesus monkey, are frequently employed to investigate possible causes of hypospadias and other morphogenic disturbances of the external genitalia (Baskin, 2000; Bleustein et al., 2001; Dalmose et al., 2000; Herman et al., 2000; Guan et al., 1994; Lopes et al., 2001).

Conclusions and future directions

Testicular descent and the formation of external genitalia are multistep developmental processes regulated by an intricate genetic network of an ever-growing number of factors. The impressive complexity of these molecular interactions, including a degree of genetic and functional redundancy, the gene expression profiles restricted in time and body segment, and the coordinated regulation of the molecular actions demonstrate the essential role of these reproductive developmental processes for the survival of vertebrate species. It is this evolutionary fine-tuned intrinsic genetic program that is increasingly disrupted by endocrine mediators resulting in reduced reproductive capacity, particularly in those species at the end of the food chain including the human. Malfunctions induced by xenobiotic compounds exerting (anti-) estrogenic and (anti-) androgenic actions on testis descent and external genitalia morphogenesis have provided inside into the potentially destructive forces these pollutants can unleach in the reproductive tract. Future research in testis descent and external genitalia development should focus on animal species other than rodents that more closely resemble the human to understand the developmental mechanisms behind cryptorchidism and hypospadias as the two most frequent ailments in newborn males.

Acknowledgments

We are grateful to Professor C. Ashworth (Scottish Agricultural College, Aberdeen), Professor C. Aurich (University of Vienna), Dr. K.J. Barteczko (University of Bochum), Professor B. Hoffmann (Justus-Liebig-University of Giessen), Professor M. Hunter (University of Nottingham), Dr. J. Kauffold (University of Leipzig), Dr. D. Miller (University of Aberdeen) and Professor P. O'Shaughnessy (University of Glasgow) for their help tracking down some of the comparative data cited in this review.

Financial support was given in part by DFG grants Kl 1249/5-1/5-2 and HO 2319/3-1.

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