Development and descent of the testis in relation to cryptorchidism

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Abstract
The testis descends in two phases. Animal studies suggest, that the transabdominal descent of the testis depends on the insulin-like hormone 3 (INSL3). Androgens are important in the inguinoscrotal testicular descent in animals and humans. In general, the cause of cryptorchidism is unknown and the aetiology is possibly multifactorial. Histological changes in cryptorchid testes demonstrate disturbed development.

Conclusion: Since testicular descent is regulated by testis-derived hormones, cryptorchidism may reflect a functional defect of the testis.

INTRODUCTION
Cryptorchidism, i.e. undescended testis, is a common abnormality in newborn boys, and recent prospective studies have described rates of 2–8% among full term boys (reviewed in Ref. 1). Undescended testis is associated with adverse effects on male reproductive health, i.e. a 4–5-fold increased risk of testicular cancer and a risk of infertility (1–3). Here we review current opinions of the fetal and neonatal development of the testis, mechanism of testicular descent, and abnormalities of these processes in cryptorchidism.

NORMAL DEVELOPMENT OF TESTIS DURING PREGNANCY AND EARLY CHILDHOOD
In both genders the development of the indifferent gonad starts on the mesonephros during the 5th and 6th weeks after fertilization, when the germ cells migrating from the yolk sac arrive in the genital ridge, the mesenchyme just medial to the mesonephros. Cranially, at its border to the gonad, the mesonephros is connected with the later diaphragm by the cranial suspensory ligament (also called the cranial mesonephric ligament or the diaphragmatic ligament). Caudally to the mesonephros, the mesonephric fold, as the connecting gubernaculum, extends to the abdominal wall at the place of the later internal inguinal ring. During week 7–8 the tight connection between the mesonephros and the testis almost disappears and the testis assumes a rounded shape (4–6). In both genders, between the 6th and 9th weeks, the metanephros, the final kidney, enlarges and ascends from the sacral region to a lumbar site just below the suprarenal gland. This movement results in lateral displacement of the gonad caudally to the final kidney (4,7). The cranial suspensory ligament and the mesonephros regress, and from about the 13th week the testis is anchored to the internal inguinal ring by the gubernaculum testis (4,6,8).

The primordial germ cells migrate from the yolk sac into the genital ridges and differentiate into gonocytes. Activation of first the SRY gene and also of factors such as WT-1, SF-1, SOX9, Fgf9 and Dax1 leads to differentiation of cells of the coelomic epithelium of the genital ridge into Sertoli cells (5,9). The first sign of the differentiation of the sexually bipotential gonad into the testis is the aggregation of Sertoli cells around the germ cells to form the primary testicular cords at around 6–7 weeks (10). Testicular cords include also peritubular myoid cells (9). By the end of week 9 the interstitial cells arise, and differentiate into steroid secreting Leydig cells. Sertoli cells start to secrete anti-Müllerian hormone (AMH) (also called Müllerian inhibiting substance (MIS)) by the 8th week, which causes regression of the Müllerian ducts that would otherwise develop into female internal accessory reproductive organs (10).

The Leydig cells produce testosterone, that induces both the differentiation of the Wolffian ducts into male internal accessory reproductive organs between weeks 8 and 12, and masculinization of the external genitalia after being converted to dihydrotestosterone (DHT) (10). Furthermore, between the 15th and 25th week the central and peripheral nerve system, especially the nucleus of the genitofemoral nerve, possibly has to be masculinized by androgens (11). In addition to testosterone, the Leydig cells secrete Insulin-like hormone 3 (INSL3), which induces male-like development of the gubernaculum, at least in mice (reviewed in 12).

Normally, differentiation of fetal gonocytes into spermatogonia begins around gestational weeks 13–15 with down-regulation of stem cell markers (e.g. OCT-3/4, NANOG, TFA2PC, KIT) and appearance of additional germ cell specific proteins (e.g. cancer-testis antigens, such as MAGEA4).
in an increasing proportion of germ cells, while they move towards basal lamina (13,14). Differentiation of gonocytes is morphologically recognized in semithin sections as the appearance of A dark spermatogonia during the first months of life (15,16). The last remaining gonocytes disappear in infancy (13,17).

During the first three months after birth the number of Leydig and Sertoli cells increase concomitantly with the increase in the levels of testosterone and another testicular hormone; inhibin B (18–20). This hormonal surge is called the minipuberty, and it includes also the rise of gonadotropins during the first weeks of life with peak values at 2–3 months of age (21). After the age of three months the serum levels of gonadotropins and testosterone decrease within a few months to the very low levels observed later in childhood, whereas the level of inhibin B falls to low levels only after 15 months of age (20). The level of AMH increases during the first months of life, to reach a peak at the age of 6 months and to decrease slowly thereafter (22).

The postnatal increase in the number of Leydig cells is followed by their regression during the first year of life, which is accompanied by an increase in the number of infantile Leydig cells (23). The total number of germ cells also increases transiently to a maximum at 3 months of age (24). The minipubertal activation of the hypothalamic–pituitary–gonadal axis has been proposed to be essential for the final transformation of the gonocytes or pre-spermatogonia into A dark spermatogonia (15,16,25). However, according to animal experiments the transformation is possible without androgens (26).

DESCENT OF THE TESTIS

The descent of the testis proceeds in two phases named the transabdominal phase and the inguinoscrotal phase. In the first phase the testis remains anchored to the inguinal area by the swollen gubernaculum, which prevents the testis from ascending as the embryo enlarges. In the second phase the testis is guided by the gubernaculum from the inguinal area into the scrotum (11). The timing of these two phases varies between species, so that, e.g. in rodents the second phase occurs only after birth, whereas in man it is usually completed by the time of birth (27). The two phases differ also in their hormonal regulation (see Fig. 1).

Rodent models of testicular descent

In mice the transabdominal phase of testicular descent is dependent on the Insl3-mediated enlargement of the gubernaculum (12). In male mice homozygous for the targeted deletion of either Insl3 gene or the gene of its receptor Lgr8 (also called Great) the male-like swelling of the gubernaculum is disrupted and thus the gubernaculum remains female-like, i.e. long and thin, and the testes are located high in the abdomen. In male mice heterozygous for the Insl3 deletion a normal swelling of the gubernaculum occurred normally, but there was delayed gubernacular regression and delayed testicular descent, which suggests that the descent of the testis is dependent on the dosage of Insl3 (12).

Other testicular factors seem to have a minor role in the male-like development of the gubernaculum, since such development is also observed in female Insl3-transgenic mice (28). Accordingly, both in male mice with androgen

Figure 1  Schematic figure of the phases of testicular descent [reproduced with permission from Elsevier Toppari et al. (60)].
In summary, the transabdominal phase of testicular descent is normal in male mice lacking androgen action. However, in mice, maternal exposure to estrogens caused downregulation of Insl3 expression in fetal Leydig cells, intra-abdominally located testes and a female-like development of the gubernaculum (29). In addition, in rats, fetal exposure to phthalates reduced the production of both testosterone and Insl3 mRNA and inhibited the transabdominal descent of the testis, possibly via altered maturation of the fetal Leydig cells (30,31).

The fetal expression of Insl3 does not seem to require gonadotropins, since mice lacking an active pituitary-gonadal axis (hypogonadal mice) express Insl3 (12). Accordingly, these mice and LH receptor knockout mice (LuRKO) show normal transabdominal phase of testicular descent although the inguinoscrotal phase is impaired (27). Testosterone treatment induces the inguinoscrotal descent in LH receptor knockout mice (32).

During the inguinoscrotal descent of the testis the gubernaculum grows and migrates towards the scrotum and also the tip of the processus vaginalis actively elongates to provide the intra-abdominal testis a peritoneal diverticulum to leave the abdomen. Androgens may control the migration and growth of the gubernaculum during this phase via the sensory branch of the genitofemoral nerve and its neurotransmitter calcitonin gene-related peptide (Cgrp) (11,26).

Maternal epidermal growth factor partially reversed the adverse effects of the antiandrogen flutamide on the inguinoscrotal descent of the testis in mice, but the mechanism remains unknown (33). Targeted disruption of the Hoxa10 gene in turn caused uni- or bilateral cryptorchidism and abnormal development of the gubernaculum, processus vaginalis, inguinal canal and scrotal sac in male mice (27).

**Descent of the testis in man**

The transabdominal phase of testicular descent in humans has been described to be completed at the 15th week of gestation (11). In male fetuses the cranial suspensory ligament regresses, but in female fetuses it is replaced by the suspensory ligament of the ovary (8). Both genders have the gubernaculum, which becomes surrounded by peritoneum, except where it is attached to the abdominal wall. The testis glides over the genital ducts, becomes embedded caudally in the gubernaculum, together with the epididymis, and enters the internal inguinal ring. In females, the gubernaculum is long and thin and will form the round ligament of the uterus, whereas in male fetuses it temporally increases, but after the testis has reached the scrotum it regresses to the scrotal ligament (8).

Before the testis and epididymis descend through the inguinal canal the absolute and relative mass of the gubernaculum increases because of an increase in its water content (8,34). The diameter of the gubernaculum reaches its maximum during the seventh month, which induces the widening of the inguinal canal. The intra-abdominal pressure and the shrinkage of the gubernaculum may force the testis through the inguinal canal. After that, the gubernaculum, the testis and the epididymis are covered by peritoneum pouch of the prolonged processus vaginalis (8). The caudal end of the gubernaculum enters the scrotum, but is neither firmly attached to any structure nor does it extend to the bottom of the scrotum. After the testis has reached the bottom of the scrotum the distal part of the processus vaginalis is called the tunica vaginalis testis and the connection to the peritoneum involutes. The gubernaculum shrinks, becomes more fibrous and persists as the scrotal ligament (6,8,34).

No gubernaculum has been observed distal to the external inguinal ring before 23rd gestational week. The descent of the testis through the inguinal canal has been described to occur rapidly and to be completed by the end of seventh month of gestation (8,34). The inguinoscrotal phase of testicular descent is proposed to be completed by the end of 35th week (11).

Human cryptorchidism is usually due to abnormalities in the inguinoscrotal phase of testicular descent, whereas the transabdominal phase is more seldom disrupted (35,36), and only about 5% of operated undescended testes are intra-abdominally placed (6).

**CRYPTORCHIDISM**

**Aetiology of cryptorchidism**

Several etiologies for cryptorchidism have been proposed: Abnormal action of the hypothalamic-pituitary axis, abnormal testicular differentiation, deficient androgen production/action, deficient production/action of AMH, and deficient action of INSL3. In addition, cryptorchidism occurs in many syndromes and in caudal developmental field defects (reviewed in Ref. 1). Furthermore, familial occurrence of cryptorchidism has been described (see ref. in 37). Cryptorchidism has been proposed to be associated with an increased GGN repeat length of the androgen receptor gene, which may cause decreased function of the androgen receptor (38,39). Furthermore, it has been suggested that bilateral cryptorchidism may be associated with an increased length of the CAG repeat in the androgen receptor gene (40), although previous studies concerning cryptorchidism found no such association (38,39). However, mutations for instance in androgen receptor gene or in the gene of 5-alpha-reductase seem to be rare in isolated cryptorchidism (41,42).

Also mutations in the HOXA10 gene seem to be rare among cryptorchid patients (see ref in 37). Insl3 and Lgr8 genes have a key role in the descent of the testis in mice, and mutations of these genes have been identified in some cryptorchid cases. However, only
heterozygous mutations of these genes have been described in human cryptorchidism (12,43). Furthermore, in a recent study cryptorchidism was associated with an increased incidence of a polymorphic allele of SF-1, that has a reduced transcription activity (44). In humans, SF-1 may affect the in vitro expression of both INSL3 and LGR8 (12). Thus, INSL3 and LGR8 genes may have a critical role in controlling the descent of the testis also in humans.

As mentioned above, genitofemoral nerve and CGRP have been associated with cryptorchidism in rodents. However, the human gubernaculum contains only a small amount of muscle cells and therefore it is unlikely that active contraction of the gubernaculum has an important role in the descent of the testis in man (45). Furthermore, mutation screening in patients with idiopathic cryptorchidism showed no pathogenic sequence changes in the CGRP pathway (46). On the other hand, CGRP caused fusion of the processus vaginalis in human inguinal hernia sacs in vitro, and thus the genitofemoral nerve and CGRP may control the obliteration of the processus vaginalis after the testis has descended (26). Furthermore, the frequency of cryptorchidism is increased to about 20% in boys with spina bifida in general, and to about 35% in cases of high lumbar lesions, suggesting that genitofemoral nerve may be important also in humans (26,47).

Intra-abdominal testes can also be due to the persistent Müllerian duct syndrome (PMDS), which is caused by genetic abnormality of AMH or its receptor. In PMDS the testes are very mobile and usually located in an ovarian position, but the testis may also be located in an inguinal hernia together with a Fallopian tube, the uterus and eventually the contralateral testis. The gubernaculum has been reported to be feminized in PMDS (48).

Human patients with complete or incomplete androgen insensitivity syndrome show normal transabdominal descent of the testis, but the second phase of testicular descent is disrupted (48). Some patients with androgen insensitivity syndrome had, however, their testicles descended into the labia, which may be explained by the effect of intra-abdominal pressure or the partial effect of androgens or development of a hernia (49).

Environmental factors may also play an etiological role in cryptorchidism (reviewed in Ref. 1). Prenatal phthalate exposure in male offspring, for example, was associated with a short anogenital distance (50). Moreover, in a series of boys 2–36 months of age the length of the anogenital index correlated negatively to the frequency of cryptorchidism.

In conclusion, although several possible etiologies for cryptorchidism have been described, in most cases they remain unknown. In some cases the cause of cryptorchidism is probably entirely due to environmental factors. However, in most cases the cause is likely to be a combination of genetic predisposition and environmental factors.

**Histological and hormonal findings in cryptorchidism**

Cryptorchidism may be associated with a reduced number of germ cells, defective or delayed maturation of germ cells, and a reduced number of Leydig cells (3,6,15,16,25,51). Changes in testis histology in cryptorchid testes are variable depending on the age of the individual at the time of biopsy/orchiopexy and the position and duration of cryptorchidism. In reported cryptorchid fetuses germ cells are present, but the number of germ cells may be reduced already at this early stage (6).

The process of testis differentiation and cell proliferation are compromised in the non-descended testes and, to some degree, also in the contralateral, normally descended testis (6,16) leading to a rapid loss of germ cells. The occasional transition into primary spermatocytes, which may occur already between 3 and 4 years of age, is also compromised in undescended testis (16). In cryptorchid boys the number of germ cells decreases dramatically within the first two years of life, especially after 6 months of age (Fig. 2; Ref. 51,3,6). After 2 years of life a normal number of spermatogonia is found only in about 10% of cryptorchid testis (6,51). Furthermore, from about 1.5 years of age an increasing proportion of patients have less than 1% of the normal number of germ cells (that is essentially no germ cells Ref. 6) at surgery for cryptorchidism (5,51).

The presence of a dark spermatagonia in patients who undergo orchiopexy before 2 years of age may be predictive of fertility in adulthood (15). In bilateral and unilateral cryptorchidism the finding of no germ cells at the time of surgery for cryptorchidism in childhood is associated with 78–100% and 33% risk, respectively, of oligozoospermia in adulthood (sperm count < 5 x 10⁶/mL) (3,6).

Undescended testes frequently contain distorted tubules, immature Sertoli cells or microcalcifications, indicating testicular dysgenesis (reviewed in Ref. 52). Adult men with a history of cryptorchidism may have a very heterogeneous testicular histology from Sertoli cell only pattern to normal (53). The prevalence of carcinoma in situ (CIS) has been estimated to be 2–3% of men with a history of cryptorchidism (1).

The adverse effects of cryptorchidism on germ cells may be due to the abnormal position of the testis, since an undescended testis has an abnormally high environmental
temperature (54), and animal studies showed that heat affects germ cells and Sertoli cells (reviewed in Ref. 55). Accordingly, in human studies, intra-abdominal testes exhibit more apoptosis than inguinal testes at the time of surgery (56). However, testicular biopsies taken before the age of one year showed more apoptosis than biopsies taken at an older age, and most germ-cell degeneration in cryptorchidism occurs before the age of 6 months (56). This is in line with the dramatic decrease in the number of germ cells within the first 2 years of life (Fig. 2; Ref. 51). On the other hand, already at birth the number of germ cells may be reduced in cryptorchidism (6). Therefore, besides acquired germ cell deficiency due to the abnormal position, deficiency of germ cells may be congenital, which supports the hypothesis that cryptorchidism represents testicular dysgenesis of fetal origin (52).

Reports on hormone levels associated with cryptorchidism in childhood have been conflicting (reviewed in Ref. 1). However, some recent studies suggested that cryptorchidism is associated with increased interstitial gonadotropin levels and reduced levels of inhibin B (57,58). Furthermore, cases with severe cryptorchidism had measurable serum androgen bioactivity at the age of three months (59). These results support the concept that cryptorchidism is associated with a primary testicular disorder. It is, however, not known whether this disorder is a cause or consequence of cryptorchidism (57). Comparison of hormone levels of cryptorchid and healthy boys at birth might enlighten this question.

CONCLUSIONS

Testicular descent is thought to proceed in two phases and the descent is usually completed at the time of birth in humans. Animal studies suggest that the first transabdominal phase is dependent on the Leydig cell hormone INSL3. The second inguinoscrotal phase is regulated by androgens. Although several aetiologies for cryptorchidism have been described, causative mutations have been found only in a few patients, and in most cases the aetiology remains unclear. Probably, the aetiology of cryptorchidism is multifactorial.

Cryptorchidism is associated with impaired germ cell development and altered interstitial sex hormone levels. These observations suggest that there may be primary developmental disorders in cryptorchid testes. The mechanism and origin (fetal, postnatal or combined) of these changes remain to be elucidated.

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