Testicular factors involved in testis descent

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Testiculaire factoren betrokken bij testisindaling

Proefschrift

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ABBREVIATIONS

ABC	avidin-biotin complex
AHC	adrenal hypoplasia congenita
AIS	androgen insensitivity syndrome
AMH	anti-Müllerian hormone
AR	androgen receptor
BrdU	5'-bromodeoxyuridine
BSA	bovine serum albumin
CAIS	complete androgen insensitivity syndrome
CD	campomelic dysplasia
cDNA	complementary DNA
CSL	cranial suspensory ligament
CGRP	calcitonin gene-related peptide
DAB	3,3'-diaminobenzidine tetrachloride
Dax1	DSS-AHC critical region on the X chromosome, gene1
DSS	dosage-sensitive sex reversal
DES	diethylstilbestrol
DHT	5α-dihydrotestosterone
DHTP	5α-dihydrotestosterone propionate
DNA	deoxyribonucleic acid
E #	embryonic day # or # days post coitum
ECM	extracellular matrix
EGF	epidermal growth factor
ER	estrogen receptor
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GAG	glycosaminoglycans
GAPD	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
HA	hyaluronic acid
HMG	high mobility group
Hox	homeobox
hpg	hypogonadal

3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
IGF	insulin-like growth fator
Insl3	insulin-like factor 3
kb	kilo base
kDa	kilo Dalton
Ley I-L	Leydig cell insulin-like factor
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
P450c17	17α-hydroxylase
P450scc	cholesterol side-chain cleavage enzyme
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMDS	persistent Müllerian duct syndrome
R1881	methyltrienolone
RLF	relaxin-like factor
mRNA	messenger ribonucleic acid
SEM	standard error of the mean
SF-1	steroidogenic factor 1
SMBA	spinal and bulbar muscular atrophy
Sox	Sry related HMG-box
Sry	sex determining region Y
α-SM actin	α-smooth muscle actin
StAR	steroidogenic acute regulatoy protein
TDT	terminal deoxynucleotidyl transferase
Tfm	testicular feminization
TGFβ	transforming growth factor β
TUNEL	TdT-mediated dUTP nick end labeling
Wt1	Wilm's tumor suppressor gene

General introduction

TESTIS DESCENT IS the male-specific developmental process by which the gonads descend from their original position in the abdomen to the final position in the scrotum. Scrotal positioning of the testes, as it occurs in many mammals, is essential for spermatogenesis. Since the time of John Hunter in 1762, many scientists have studied testis descent, which turned out to be a complex process, involving an interplay of different structures and factors. Due to this complexity, the understanding of the structural and molecular mechanisms underlying the process of testis descent is still far from complete.

MAMMALIAN SEX DIFFERENTIATION

Sex determination

The testes originate in the abdominal cavity, starting as indifferent gonads that are morphologically indistinguishable from the female gonads. Although the gonads of both sexes are phenotypically identical during early fetal development, the genetic sex is established at the time of fertilization, when the egg is fertilized by a sperm cell carrying either an X or an Y chromosome. The actual number of genes controlling all aspects of mammalian sex determination and differentiation is unknown, but key genes have become restricted to one pair of chromosomes, the sex chromosomes, usually referred to as the X and Y chromosomes. The determination of sex in mammals is strictly chromosomal, with the male being the heterogametic sex (XY) and the female the homogametic sex (XX). Following the discovery at the beginning of the last century that sex in the fruit fly *Drosophila* is determined by the ratio between the number of X chromosomes and autosomes, with the Y chromosome playing no role in sex determination, it was assumed that the same was probably true for mammalian species, since sex determination seems such an important and basic mechanism. It was not until 1959 that the pivotal role of the mammalian Y chromosome in determining the male sex became apparent (Ford *et al.*, 1959; Jacobs and Strong, 1959). The development of the indifferent gonads towards testis differentiation is triggered by the action of at least one gene on the Y chromosome. In the absence of the Y chromosome, the indifferent gonad develops as an ovary. Once the gonads start to differentiate, their male- or female-specific endocrine function is responsible for the sexual development of the rest of the embryo.

A different kind of sex chromosome organization and action is found in birds (Stevens, 1997). The sex chromosomes are designated Z and W, with the female (ZW) being the heterogametic sex and the male (ZZ) the homogametic sex. The Z chromosome is a large chromosome, and it contains almost all the known sex-linked genes, whereas the W chromosome is generally much smaller. Sex determination is dependent on the ratio of autosomes to Z chromosomes.

Besides chromosomal sex determination, other mechanisms exist, including environmental sex determination and pathogenesis. Environmental sex determination is characterized by sex determination in response to environmental cues, such as pH and most notably temperature (Johnston *et al.*, 1995). In many species of fishes and reptiles, males and females are genetically identical, with the temperature of the eggs during a certain

period of development being the sex-determining switch.

Gonad formation

Morphology
The development of the various organs of the urogenital system, including the gonads and kidneys, is closely linked due to their common origin in the intermediate mesoderm (Corliss, 1976; Wartenberg, 1990). The intermediate mesoderm runs along the length of the embryo on both sides of the midline of the dorsal abdominal wall forming the urogenital ridge. This ridge gives rise to three excretory organs: the pronephros and mesonephros, which are two transient kidney-like organs, and the metanephros which is the true kidney. The pronephros develops first, at the cranial end of the intermediate mesoderm, and its duct runs towards the cloaca, at the posterior end of the embryo. In most mammals, the pronephros soon begins to degenerate as the mesonephros develops just caudal to it and takes over its duct, which is the so-called mesonephric duct. The indifferent gonad first develops as a thickening of the coelomic epithelium on the ventromedial surface of the mesonephros. As development proceeds, the gonadal region is progressively more demarcated from the mesonephros, forming a separate genital ridge, but remains connected to the mesonephros. At a later stage of development, tissue buds off from the mesonephric duct near the caudal end, which forms the duct of the metanephros. With the establishment of the metanephros, the mesonephros begins to degenerate.

Genes involved in gonadal formation
Until recently, only two genes were known to be involved in the process of gonad formation, the *Wt1* and *Ftz-f1* genes. Due to the availability of mutant mouse models, which show gonadal dysgenesis, the number of genes related to gonad development has rapidly increased. Since the gonads, adrenals and kidneys are all derived from the intermediate mesoderm, mutations in these genes often involve both gonadal dysfunction and renal and/or adrenal dysfunction. Several genes involved in the formation of the indifferent gonad are indicated in Figure 1.1. A more detailed description is given in two recent reviews by Swain and Lovell-Badge (1999) and Roberts *et al.* (1999) and references therein.

Steroidogenic factor 1 (SF1), also called adrenal 4-binding protein (Ad4BP), is an orphan nuclear receptor protein for which no activating ligand has been identified yet (Parker and Schimmer, 1997). SF1 is the product of the *fushi tarazu factor 1 (Ftz-f1)* or *Sf1* gene in the mouse. SF1 was originally isolated as a transcription factor for a variety of different steroidogenic enzyme genes in the adrenal glands and gonads (Lala *et al.*, 1992; Morohashi *et al.*, 1992). In the adult mouse, *Sf1* mRNA is expressed in the steroidogenic cells of the adrenal gland and the gonads, in the pituitary gland and in a part of the hypothalamus (Ikeda *et al.*, 1993; Ingraham *et al.*, 1994; Shinoda *et al.*, 1995). In the developing mouse embryo, *Sf1* transcripts are detectable from approximately embryonic day 9.0 (E9) in the genital ridge of both sexes (Ikeda *et al.*, 1994). As the indifferent gonad differentiates into a histologically recognizable testis at E10.5-E11.5, *Sf1* remains highly expressed in fetal Sertoli cells and interstitial or Leydig cells. Although *Sf1* transcripts are readily detectable in the ovary at E12.5, the expression level is markedly descreased to re-appear during late gestation
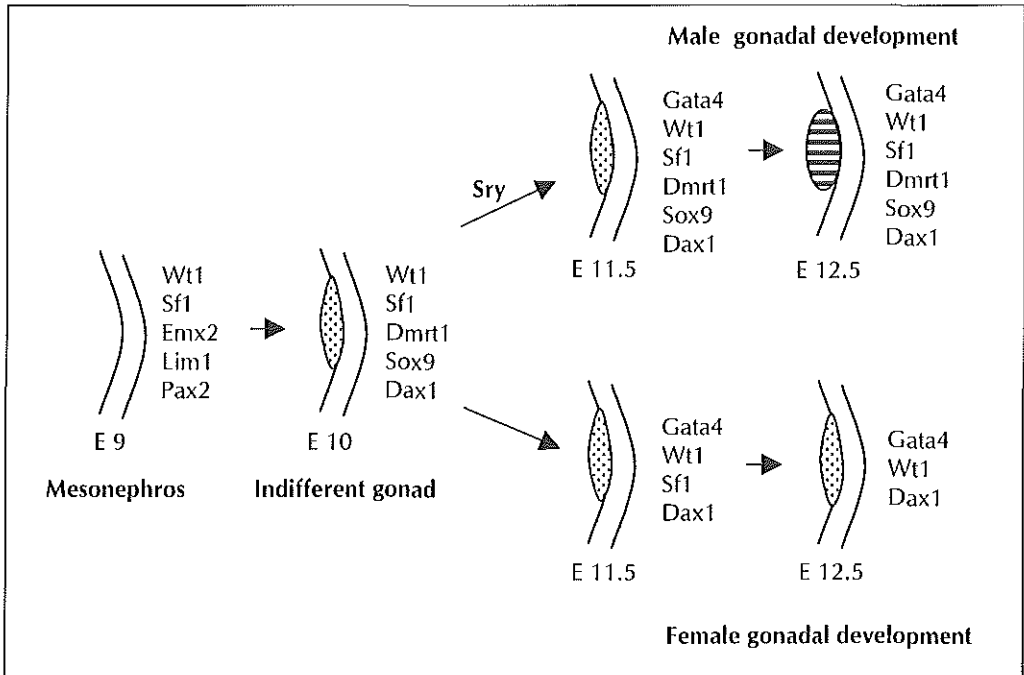
Figure 1.1 – Gene products involved in mouse sex determination and differentiation
The scheme illustrates the common sex-specific regulatory factors involved in gonad development at the different stages of mouse embryonic development (E = gestational day). There are no observations on expression of *Emx2, Lim1* and *Pax2* after E9. Expression of *Gata4* at the early stages is not known.

(E18.5). To assess directly the role of SF1 *in vivo*, mice with a homozygous targeted disruption of the *Sf1* gene were generated. All *Sf1* gene knockout mice lacked adrenal glands and gonads, which is in agreement with *Sf1* mRNA expression in these tissues (Luo *et al.*, 1994). The earliest stages of genital ridge development do occur, but development stops between E11 and E11.5 and the gonads degenerate due to apoptosis. It remains to be determined whether SF1 has a critical role in sex determination, beyond its function in gonadal development and steroidogenesis. The human *SF1* gene has also been cloned and characterized (Wong *et al.*, 1996). To date, no mutations have been identified in this gene.

Wilm's tumor is a childhood tumor of the kidney, and mutations in at least two different genes predispose to Wilm's tumor. One of these genes is isolated, the Wilms' tumor suppressor gene, *WT1*, which encodes a protein involved in early gonad and kidney development (Little *et al.*, 1999). *WT1* is a very complex gene, encoding a protein that has many characteristics of a transcription factor, acting either as an activator or a repressor. During mouse embryonic development, *Wt1* is expressed in both male and female indifferent gonads and remains highly expressed during development of the gonad (Pelletier *et al.*, 1991c; Armstrong *et al.*, 1993). In the developing testis, *Wt1* expression is confined to Sertoli cells and absent in Leydig cells. *Wt1* transcripts are also found in the supporting cell lineage of the fetal ovary, the granulosa cells. Homozygous deletion of the *Wt1* gene

in mice resulted in an arrest of gonadal and kidney development, whereas heterozygous littermates were unaffected (Kreidberg *et al.*, 1993). In contrast to mice, humans with a heterozygous mutation in the *WT1* gene exhibit cryptorchidism (undescended testis) and hypospadias (misplaced penile urethral opening) and are predisposed to develop a Wilms' tumor, suggesting that these malformations are the result of a gene dosage effect. A heterozygous mutation in the DNA binding domain of the *WT1* gene leads to a more severe phenotype; the Denys-Drash syndrome which includes a Wilms' tumor associated with severe defects in urogenital and gonadal development (Pelletier *et al.*, 1991a; Pelletier *et al.*, 1991b). The genital anomalies are only observed in XY individuals, demonstrating ambiguous or completely female genitalia. However, defects in gonadal development are found in both XY and XX individuals, suggesting no sex-specific action of WT1.

The genes *Lim1*, *Emx2* and *Pax2* are homeobox genes, containing a conserved DNA sequence which encodes a DNA-binding domain. These genes are expressed during early urogenital development in the mesonephros, metanephros and associated ducts (Fujii *et al.*, 1994; Torres *et al.*, 1995; Miyamoto *et al.*, 1997). Mice which are homozygous for a targeted deletion of either the *Emx2* or the *Lim1* gene completely lack kidneys, gonads and urogenital ducts (Shawlot and Behringer, 1995; Miyamoto *et al.*, 1997). Pax2 deficient mice also lack kidneys, ureters and genital ducts, but the gonads are formed (Torres *et al.*, 1995). *Emx2* and *Pax2* mutant mice demonstrate normal adrenal development, indicating that the gonadal defect observed in these mice cannot be due to a defect in the SF1 positive cells of the gonad. The expression of *WT1* in the early kidneys of *Emx2* mutant mice was not affected, placing EMX2 downstream of WT1 in the cascade of gonad development. In contrast, *Lim1* and *Pax2* expression was reduced or completely lost in *Emx2* mutant mice. *Emx2*, *Lim1* and *Pax2* gene products are all involved in early gonad development but the precise roles and possible functional interactions between these products have to be studied yet.

Gonadal sex determination and differentiation

Morphology

The gonad arises as a ridge-like thickening, the genital ridge, on the ventral border of the mesonephros around E9.5 in mice (Corliss, 1976; Byskov and Hoyer, 1994). The mesonephros is thought to contribute to two somatic cell lineages of the genital ridge, the supporting cell precursors and the steroidogenic cell precursors. During the indifferent stage of gonadal development, the genital ridges are invaded by the primordial germ cells following their migration from the yolk sac, along the newly formed gut, dorsal mesentery and mesonephros (Ginsburg *et al.*, 1990). The next step is the inclusion of the primordial germ cells into testicular cords, formed by alignment of the supporting precursor Sertoli cells. Testicular cord formation is the first morphological sign of testis formation, visible in the mouse around E12.5. Ovarian differentiation takes place later and will be first visible around E14.5 in mice. The fetal testis cords are covered by a basal lamina and a layer of peritubular myoid cells. Between the cords, the interstitial cells start to differentiate into Leydig cells and become steroidogenically active at day E13 in the mouse (Greco and Payne, 1994).

Genes involved in differentiation of the gonads

Following development of an indifferent gonad, many genes are required for the complete process of testis differentiation. Several of these genes are characterized, of which the most important ones are shown in Figure 1.1 (Roberts *et al.*, 1999; Swain and Lovell-Badge, 1999).

Genetic analysis of sex-reversed individuals, XX males and XY females, resulted in the isolation of the testis-determining gene on the human Y chromosome, termed the *SRY* gene (Sinclair *et al.*, 1990; Hawkins, 1995). In the mouse, the Y chromosomal *Sry* gene is expressed for a short period in the male embryo, specifically by cells of the genital ridge between E10.5 and E12.5, starting just before morphological differentiation of the testis (Koopman *et al.*, 1990). SRY is thought to act solely in the supporting cell lineage, triggering them to differentiate into Sertoli cells (Palmer and Burgoyne, 1991). Once Sertoli cells start to differentiate, they are thought to trigger the other cell lineages in the gonad to follow the male differentiation pathway. The SRY protein possesses a HMG (high mobility group) type of DNA binding domain, and has proporties consistent with its action as a factor which can control expression of downstream genes. With one exception, all mutations found in the human SRY in association with human sex reversal are clustered in the HMG domain, indicating that the switch in cell fate brought about by SRY is likely to occur at the level of transcription (Hawkins, 1995). Direct evidence that SRY is sufficient to induce testis differentiation and subsequent male development was shown in XX mouse embryos transgenic for *Sry* (Koopman *et al.*, 1991). While *Sry* is important in initiating testis development, many other genes are likely required at different stages of gonadal development.

A recently identified gene, *DMRT1*, might be one of the genes involved in testis development (Raymond *et al.*, 1998). This gene encodes a protein which contains a so-called DM domain, a novel DNA-binding motif. This DM domain was first identified in both the sex determining gene *Doublesex* of the fruitfly *Drosophila* and the sex determing gene *Mab-3* of the nematode worm *Caenorhabditis elegans*. *DMRT1* is mapped to a human locus on chromosome 9 which is linked to human XY sex reversal. Characterization of the expression patterns of *Dmrt1* in mice demonstrated exclusive expression in the genital ridges of both sexes at E9.5 (Raymond *et al.*, 1999). During gonadal sex differentiation, *Dmrt1* expression is maintained only in the XY gonad, becoming restricted to the testicular cords. Also in the chicken, *Dmrt1* is expressed in the genital ridges and Wolffian ducts prior to sex differentiation and is expressed higher in the male (ZZ) than in the female (ZW) embryos. Moreover, in the embryonic alligator urogenital system and gonads *Dmrt1* expression has been shown to be upregulated at the male-determining temperature (Smith *et al.*, 1999). Since both birds and reptiles lack *Sry* but do show male-specific *Dmrt1* expression during the testis determining period, *Dmrt1* might be an ancient sex determining gene, expressed at early testis development irrespective of the type of primary sex determining switch.

This might also hold true for another gene, *SOX9* (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). *SOX9* has been identified by genetic analysis of sex reversed individuals (Foster *et al.*, 1994; Wagner *et al.*, 1994). Inactivating mutations on one *SOX9* allele can cause both campomelic dysplasia (CD), a human skeletal malformation syndrome, and XY sex

reversal. The genital morphologies found in XY CD patients range from hypospadias to female genitalia with streak-like gonadal rudiments. The *SOX9* gene belongs to the family of *SOX* (*SRY*-related HMG box) genes and encodes a putative transcription factor structurally related to *SRY* (Denny *et al.*, 1992). Expression studies in mice demonstrate that *Sox9* is present in the genital ridges of both sexes from about E10.5 (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). At E11.5, *Sox9* is highly expressed in the genital ridges from male embryos but not in those of female embryos, being consistent with a role in testis differentiation. By E13.5, *Sox9* transcripts are present within the fetal Sertoli cells and this expression persists throughout life. In mammals, it is conceivable that SRY is directly involved in regulation of *SOX9* transcription. Recently, *SOX9* duplication has been associated with autosomal XX sex reversal, which suggests that an extra dosage of SOX9 is sufficient to initiate testis differentiation in the absence of *SRY* (Huang *et al.*, 1999).

Similar as *SRY* and *SOX9*, the *DAX1* gene was identified on basis of genetic analysis of human XY females. Dosage-sensitive sex reversal (DSS) is a locus on the human X chromosome which causes sex-reversal in an XY individual when duplicated (Arn *et al.*, 1994). From this region, *DAX1* (DSS-AHC-critical region on the X chromosome, gene1) was isolated, which encodes a protein belonging to the nuclear hormone receptor superfamily (Muscatelli *et al.*, 1994; Zanaria *et al.*, 1994). Functional deficiency of DAX1 is responsible for adrenal hypoplasia congenita (AHC), characterized by the absence of the adrenal cortex and hypogonadotropic hypogonadism. Since XY individuals with *DAX1* mutations develop as males, it has been proposed that this gene is required for ovarian development but not for testis formation. In the mouse, *Dax1* is expressed in the genital ridge of both sexes at E10.5 and peaks around E12 (Swain *et al.*, 1996). The level of *Dax1* decreases dramatically as the testicular cords begin to appear, but persists in the devloping ovary. Transgenic mouse experiments have shown that overexpression of *Dax1* alone was able to result in XY female sex reversal (Swain *et al.*, 1998). However, loss of DAX1 function in female mice did not affect ovarian development and fertility (Yu *et al.*, 1998). Thus, *Dax1* is not an ovary-determining gene but can act as an anti-testis gene during development, by directly antagonizing *Sry*. Since the tissue distribution of *Dax1* mRNA expression is similar to that of *Sf1* and mutations in both genes cause phenotypes that include adrenal insufficiency and hypogonadotropic hypogonadism, DAX1 and SF1 might act in the same pathways (Ikeda *et al.*, 1996).

In addition to the gene products that are known to have definite roles in gonadal development and differentiation, like SF1, DAX1 and SOX9, there are other factors that might be regulators of gonadal gene expression, for example GATA4. This protein is a member of the GATA family of transcription factors and is present in developing gonads. In the mouse, GATA4 protein is detected in the indifferent gonads of both sexes at E11.5 and subsequently in both fetal Sertoli and Leydig cells of the testis andin the developing ovary (Viger *et al.*, 1998). The expression of *GATA4* is downregulated in the ovary from E13.5 onwards. Since mice deficient for GATA4 die *in utero* before gonadal development takes place (Molkentin *et al.*, 1997), conditional inactivation of the *GATA4* gene at a later stage of development will be necessary to study its role in gonadal development.

The above described sex-determining genes, *SRY*, *DMRT1*, *SOX9* and *DAX1*, all dem-

onstrate dosage or threshold effects. This is of interest in view of the fact that gene dosage effects are also of importance in sex determination in species such as *Drosophila* and *Caenorhabditis elegans*. Gene dosage appears to be less important for genes encoding general transcription factors involved in gonadal formation, such as *SF1*, *WT1* and *PAX2*.

With the identification of several genes within the last ten years, the elucidation of the molecular mechanisms involved in gonadal differentiation has started, but many aspects of the early embryology and cell biology of the gonads still needs to be worked out. Up till now, it is difficult to fit all gene products in a model, in part due to missing components but also because the factors do not seem to act in a simple linear pathway but take part in an interactive network (Swain and Lovell-Badge, 1999).

HORMONE PRODUCTION BY THE FETAL TESTIS AND DIFFERENTIATION OF THE GENITAL DUCTS

Morphology

As the mesonephroi degenerate, with the establishment of the metanephroi or permanent kidneys, their ducts become attached to the developing gonads (Corliss, 1976). These mesonephric, or Wolffian, ducts give rise to the male reproductive tract. At their lower ends, the Wolffian ducts reach the lateral wall of the cloaca and form the primitive urogenital sinus, of which the caudal part gives rise to the definitive urogenital sinus. Cranially and laterally on the mesonephric ducts, a second pair of ducts arises as invagination of the coelomic epithelium, which grow in a caudal direction. This pair of ducts, para-mesonephric or Müllerian, will develop into the female genital tract. In their upper portions, the Müllerian ducts are located externally to the Wolffian ducts; at the lower level of the gonads, the Müllerian ducts cross over ventrally and run internally along the Wolffian ducts. At their lower ends, the two Müllerian ducts fuse and form the uterovaginal duct, finally making contact with the posterior wall of the urogenital sinus. During the indifferent stage of gonadal development, all embryos have both the mesonephric and the paramesonephric ducts (Figure 1.2). In the male, under the influence of hormonal signals from the developing testes, the Wolffian ducts are maintained and develop into epididymides, vasa deferens, seminal vesicles and ejaculatory ducts, while the Müllerian ducts regress. In the female, as sex differentiation occurs, the gonads differentiate into ovaries and the Müllerian ducts develop into oviducts, uterus, cervix and upper vagina, while the Wolffian ducts regress. It was established by Jost (1953) that the phenotypic male sex characteristics are induced by two hormones secreted by the fetal testis: anti-Müllerian hormone (*AMH*) and testosterone. In the absence of these two hormones, the default pathway of sex differentiation is phenotypically female.

AMH

AMH and the AMH receptor
AMH is the first hormonal factor to be produced by the testis upon initiation of testis
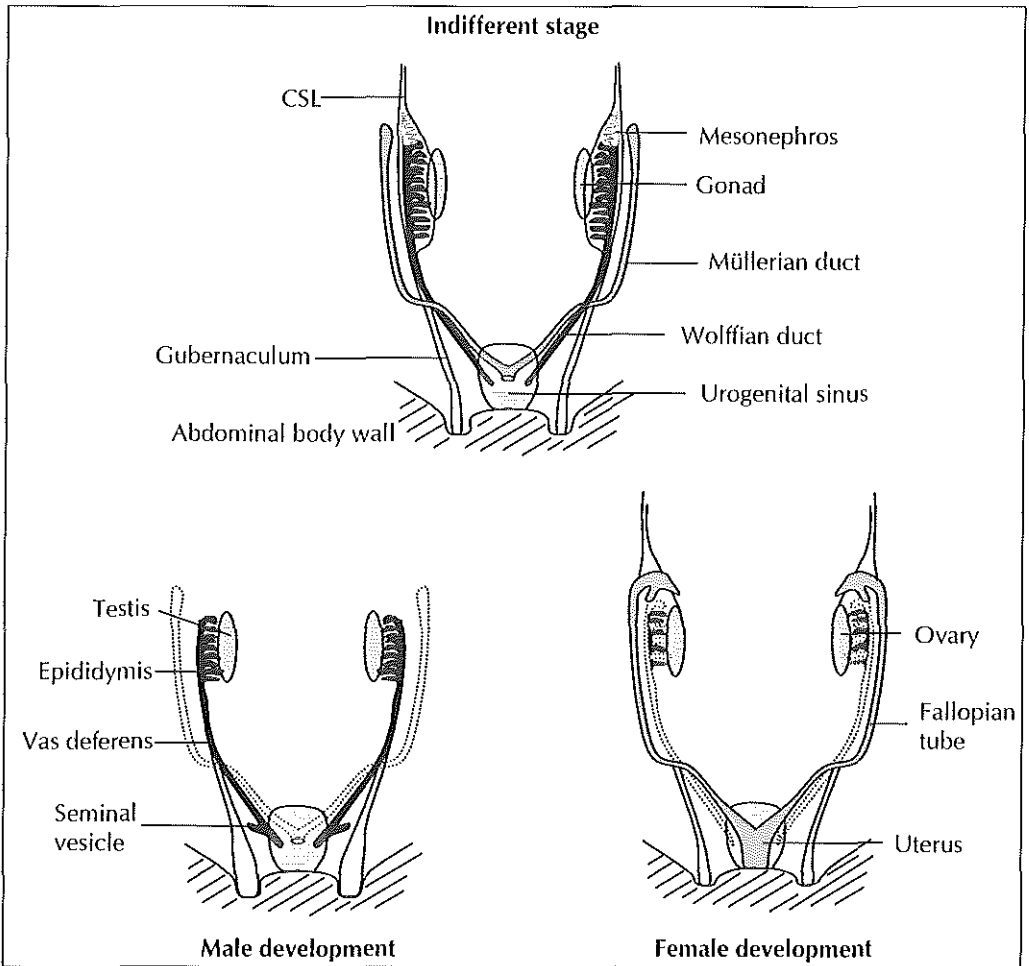
Figure 1.2 – Schematic representation of sex differentiation of male and female internal genitalia
During the indifferent stage, the gonads and two pairs of ducts are formed; the Wolffian and the
Müllerian ducts. In the male, the gonads develop into testes, the Wolffian ducts stabilize and further
differentiate, whereas the Müllerian ducts regress. In the female embryo, the gonads develop into
ovaries, the Müllerian ducts differentiate, whereas the Wolffian ducts regress. The prostate is not
shown. CSL, cranial suspensory ligament.

differentiation, which induces regression of the Müllerian ducts in the male embryo (Josso
et al., 1993; Lee and Donahoe, 1993). *AMH*, also known as Müllerian inhibiting substance
(MIS), is a glycoprotein secreted by fetal Sertoli cells. This factor is a member of the trans-
forming growth factor β (TGFβ) gene family of growth and differentiation factors, and
is produced as a large precursor that requires proteolytic processing to produce a biologi-
cally active molecule (Cate et al., 1986; Pepinsky et al., 1988). The action of *AMH* is medi-
ated through a membrane-bound receptor complex containing a type II serine/threonine
kinase receptor. The type II receptor for AMH (AMHRII) has been cloned and character-

ized (Baarends *et al.*, 1994; di Clemente *et al.*, 1994; Grootegoed *et al.*, 1994). Similar to what has been shown for TGFβ and other family members, the AMH receptor complex probably also contains a type I receptor. Alk2, one of the type I receptors that have been cloned, is considered a candidate AMH type I receptor (He *et al.*, 1993).

Expression of AMH and AMHRII

Amh gene expression is first detectable in the mouse testis at E11.5 (Münsterberg and Lovell-Badge, 1991) and in the rat testis at E13 (Josso *et al.*, 1977) when the testicular cords become visible, and this expression is continued throughout fetal development. Postnatally, *AMH* expression in Sertoli cells strongly decreases, being low in the adult testis (Münsterberg and Lovell-Badge, 1991; Baarends *et al.*, 1995a). In female rodents, *AMH* mRNA is first detectable in the granulosa cells of the ovary within a few days after birth and persists throughout life (Münsterberg and Lovell-Badge, 1991; Baarends *et al.*, 1995b). *AMHRII* transcripts can be found in the mesenchymal cells adjacent to the Müllerian ducts of male and female embryos at E15 (Baarends *et al.*, 1994; di Clemente *et al.*, 1994). In the male embryo, *AMHRII* expression in the genital duct system decreases from E15 onwards, being absent at E19, which correlates with the degeneration of the Müllerian ducts. The expression around the female ducts remains high. Surprisingly, *AMHRII* mRNA is also expressed in the fetal and adult gonads, in the Sertoli cells of the testis and the granulosa cells of the ovaries (Baarends *et al.*, 1994; di Clemente *et al.*, 1994; Baarends *et al.*, 1995b).

AMH action

The role of AMH *in vivo* was studied by means of different transgenic mouse models. Female transgenic mice that ectopically express human *AMH* (*hAMH*) during development undergo Müllerian duct regression, and lack a uterus and oviducts (Behringer *et al.*, 1990). Combined with the observation that male mice which are homozygous for a targeted deletion of either the *AMH* or the *AMHRII* gene develop a female genital tract including oviducts and uterus, in addition to the development of the male genital tract, the essential role of AMH in Müllerian duct regression has been established (Behringer *et al.*, 1994; Mishina *et al.*, 1996). Recently, female transgenic mice overexpressing *hAMH* on an AMHRII-deficient background were generated (Mishina *et al.*, 1999). Although these female mice had high levels of circulating hAMH, they had normal reproductive tracts and were fertile. These findings demonstrate that the reproductive tract lesions found in transgenic females mice which ectopically express *hAMH* are mediated by the AMH type II receptor and that AMH does not exert any effect through another type of receptor system (Behringer *et al.*, 1994; Mishina *et al.*, 1999).

Besides its pivotal role in male sex differentiation, AMH has also been suggested to play a role in gonadal development and function. The first indications for such a role of AMH came from observations in bovine freemartins. A freemartin is a XX female, which is exposed *in utero* to hormones coming from the male twin through fusion of the fetal blood vessels. In addition to the absence of the Müllerian ducts, freemartins also exhibit masculinized ovaries with testicular cords and Leydig cell development. It was suggested that this effect could be due to AMH produced by the male twin (Jost *et al.*, 1972). Indeed, this destructive effect of AMH on ovary development could be reproduced *in vitro* by

exposure of fetal rat ovaries to AMH (Vigier *et al.*, 1987). Moreover, female transgenic mice highly overexpressing *hAMH* demonstrated degeneration of the ovaries associated with the formation of testicular cord-like structures, albeit inconsistently and only after birth (Behringer *et al.*, 1990). The ability of AMH to direct the ovary into a more testis-like structure suggested a role in male gonadal development. Subsequent studies demonstrated that AMH is not essential in testis determination but is involved in testis differentiation and function. Leydig cell hyperplasia was incidentally found in adult AMH- and AMHRII-deficient male mice (Behringer *et al.*, 1994; Mishina *et al.*, 1996). Coupled to the observation that transgenic male mice overexpressing *hAMH* are sometimes incompletely masculized, caused by a deficiency in androgen production by the Leydig cells (Lyet *et al.*, 1995), it has become apparent that AMH is affecting Leydig cell differentiation and function. *In vitro* experiments demonstrated that AMH affects Leydig cell function directly in a paracrine fashion via its receptor (Racine *et al.*, 1998).

Although the effect of AMH on the prenatal ovary is quite destructive, both *AMH* and *AMHRII* are expressed in the granulosa cells of the postnatal ovary during stages of folliculogenesis (Baarends *et al.*, 1995b). Since no gross abnormalities were found in female AMH- and AMHRII-deficient mice, which are fertile and have normal litter sizes, it was initially assumed that AMH played a redundant role in ovarian function (Behringer *et al.*, 1994; Mishina *et al.*, 1996). However, careful examination of the follicle population in ovaries at different ages revealed that ovaries of AMH-deficient mice show increased recruitment of the primordial follicles into the growing pool of follicles, compared to ovaries of wild-type mice, indicating a role of AMH in the long-term control of the primordial follicle pool and normal ovarian function during reproductive life (Durlinger *et al.*, 1999).

Development of both the Wolffian and the Müllerian ducts as observed in male mice deficient for AMH or AMHRII can also occur in men and is denoted as the persistent Müllerian duct syndrome (PMDS). This rare syndrome is characterized by the presence of Müllerian duct derivatives, i.e. Fallopian tubes and uterus in otherwise normal virilized XY individuals (Josso *et al.*, 1997). Following the characterization of both the human *AMH* (Cate *et al.*, 1986) and *AMHRII* genes (Imbeaud *et al.*, 1995; Visser *et al.*, 1995), mutations in either one of these genes have been implicated as etiological factors of PMDS (Josso *et al.*, 1997).

AMH gene transcription is regulated by several transcription factors described above, which are involved either in gonad formation or in testis development. SF1 has been directly implicated in regulation of *AMH* gene expression, although SF1 alone was not sufficient to induce *AMH* expression (Shen *et al.*, 1994; Giuili *et al.*, 1997). *In vitro* studies proposed the involvement of three additional transcription factors, WT1, SOX9 and GATA4 (De Santa Barbara *et al.*, 1998; Nachtigal *et al.*, 1998; Tremblay and Viger, 1999). In any case, transcriptional control should enable fetal testicular expression, while ovarian expression is postponed to the period of reproductive life.

Testosterone

Testosterone synthesis and action during sex differentiation
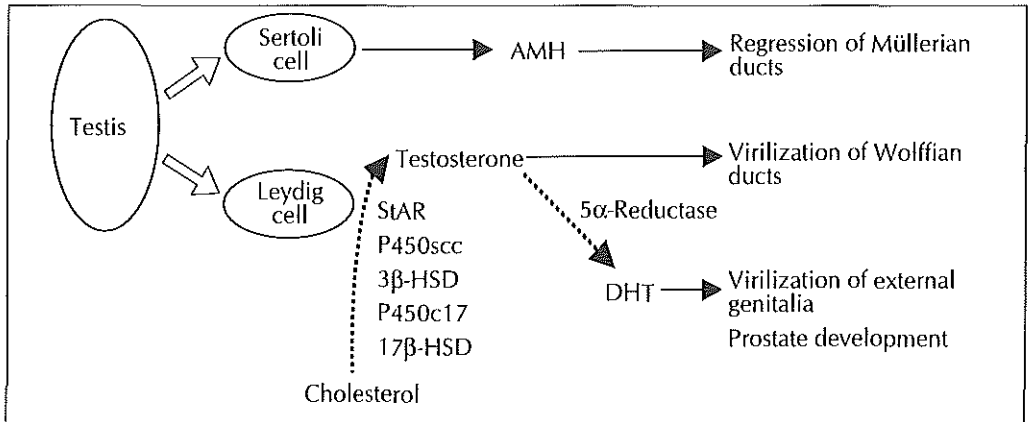In addition to production and secretion of AMH by fetal Sertoli cells, the fetal Leydig

Figure 1.3 – Involvement of testicular hormones in male sex differentiation
AMH, anti-Müllerian hormone; StAR, steroidogenic acute regulatory protein; P450scc, P450 side chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c17, 17α-hydroxylase; 17β-HSD, 17β-hydroxysteroid dehydrogenase. Enzymatic conversions are denoted by a dotted line.

cells of the developing testis start to produce testosterone (Figure 1.3). Testosterone is a steroid hormone, responsible for the stabilization and differentiation of the Wolffian ducts into epididymides, vasa deferens and seminal vesicles (Wilson *et al.*, 1981b). Testosterone is synthesized from cholesterol in a series of steps requiring several enzymes, including the steroidogenic enzymes cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase/c17,20-lyase (P450c17) and 17α-hydroxysteroid dehydrogenase (17β-HSD) (Miller, 1988). A specific protein, steroidogenic acute regulatory (StAR) protein, is involved in the transfer of cholesterol from the outer to the inner mitochondral membrane where P450scc is located (Stocco and Clark, 1996). The fetal mouse testis has the capacity for testosterone biosynthesis as early as E13 (Greco and Payne, 1994). There is a significant increase in the amount of fetal testosterone in the serum during fetal development, peaking at E17 in mouse and E18.5 in rats (Hadziselimovic, 1983a, Habert and Picon, 1984).

Fetal Leydig cell differentiation and function appears to be independent of luteinizing hormone (LH) (El-Gehani *et al.*, 1998; O'Shaughnessy *et al.*, 1998). Since SF1 binding sites are identified in the promoters of most genes encoding steroidogenic enzymes, SF1 is considered one of the key regulators of steroidogenesis (Parker and Schimmer, 1997). During testis development, testosterone production by testicular Leydig cell comes under control of LH.

The androgen receptor
Testosterone exerts its effect through the intracellular androgen receptor (AR). The gene encoding the AR was cloned in 1988 by several groups (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Trapman *et al.*, 1988; Tilley *et al.*, 1989). The *AR* gene consists of 8 exons, encoding a protein of approximately 910 amino acids. The AR is a ligand-dependent transcription factor, belonging to the superfamily of nuclear receptors which also includes receptors for

the other steroid hormones, thyroid hormone, retinoids, vitamin D and a large group of orphan receptors, including SF1 (Evans, 1988; Mangelsdorf et al., 1995). The AR contains several functional domains: an amino-terminal domain involved in transcription activation, a centrally located DNA-binding domain and a C-terminal part involved in ligand binding. Upon ligand binding, the AR dissociates from heat shock proteins and binds to specific DNA elements in the promoter regions of androgen-target genes, regulating transcription of these genes (Beato and Sanchez-Pacheco, 1996; Brinkmann et al., 1999). In addition to testosterone, the metabolite 5α-dihydrotestosterone (DHT), which is formed by the action of 5α-reductase, also serves a ligand for the AR. Testosterone and DHT are the most important androgens.

Expression of the androgen receptor during sex differentiation
The AR is expressed in many different tissues including the male and female reproductive organs, adrenal, skeletal and cardiac muscle, kidney, lung, liver, hypothalamus and pituitary gland (Takeda et al., 1990). The presence of the AR in the developing male urogenital structures has been demonstrated by many different research groups, including a few studies on the systematic analysis of the cellular distribution of AR expression in the developing urogenital system in the rat (Bentvelsen et al., 1995; Majdic et al., 1995) and the mouse (Cooke et al., 1991; Crocoll et al., 1998). In the mouse, the AR is detected in the mesenchyme around the Wolffian duct of both sexes at E12.5, before testosterone production has started (Cooke et al., 1991; Crocoll et al., 1998). The Wolffian duct develops in a craniocaudal fashion into epididymis, vas deferens and seminal vesicle. During this process, a shift of AR expression from the mesenchymal cells to the epithelial cells of the duct proper is observed, starting at about E15.5. A similar shift of AR expression is observed in the urogenital sinus, from which the prostate, among other structures, will develop. The mesenchyme around the differentiating urogenital sinus is already positive in both sexes from E12.5 onwards, whereas the epithelial part remains negative during embryogenesis. No AR positive cells are found in the mesenchyme of the Müllerian ducts. Findings in the mouse are in agreement with studies in the rat (Bentvelsen et al., 1995; Majdic et al., 1995).

During the early stages of genital development, AR expression is observed in both the male and the female developing urogenital tract. At the time the fetal testis starts to produce testosterone, AR expression in the male genital tract increases whereas expression in the female tract decreases. Initially, AR expression appears to be hormone independent but continued expression depends upon testicular hormone expression (Bentvelsen et al., 1994). Exposure of female fetuses to androgens prevents the decrease of AR expression. *Vice versa*, exposure of male fetuses to the anti-androgen flutamide leads to decreased AR expression (Bentvelsen et al., 1994; Bentvelsen et al., 1995). Up till now, the regulatory mechanisms of AR expression during embryonic development have remained largely unknown.

Defective androgen action
The essential role of androgens in male sex differentiation is best illustrated by defective androgen action, which can be due to either end organ insensitivity or ligand deficiency (Grumbach and Conte, 1998). These defects can result in partial or complete lack of masculinization in 46,XY individuals with normally developed testes.

Androgen insensitivity syndrome (AIS) in man is an X-linked genetic disorder resulting from malfunction in the AR (Quigley *et al.*, 1995; Brinkmann *et al.*, 1996). Mutations in the *AR* gene can result in a wide spectrum of phenotypes in affected individuals, ranging from patients with female external genitalia, the complete phenotype, to a male phenotype exhibiting infertility. All these individuals develop testes, with normal endocrine functioning of both Leydig and Sertoli cells. Due to normal AMH production and action, Müllerian duct derivatives are usually absent in these patients. However, Wolffian duct-derived structures are affected, even being absent in the complete phenotype. Over 350 mutations have been reported in the *AR* gene in individuals with AIS (Gottlieb *et al.*, 1999). Mutations identified in AIS patients are mostly single base mutations resulting in amino acid substitutions, although complete or gross deletions of the *AR* gene do occur (Brinkmann *et al.*, 1999). The single base mutations are mainly found at different sites in the sequences encoding the DNA- and ligand-binding domains of the AR, and cause both partial and complete forms of androgen insensitivity. Very few substitution mutations in exon 1, encoding the transcription activation domain of the AR, have been found.

Androgen insensitivity has also been noted in other mammalian species including the rat (Bardin *et al.*, 1970) and the mouse (Lyon and Hawkes, 1970). Androgen insensitivity in the mouse was shown to be X-linked, and the respective locus was designated *Tfm* for testicular feminization. Both animal models have served as useful models for studying androgen insensitivity, being unresponsive to physiological concentrations of androgens and hence showing development of the androgen insensitivity syndrome. However, the rat model still responds to pharmacological doses of androgens and can be considered as not being completely androgen insensitive, leaving the *Tfm* mouse as the most appropriate model (Sherins and Bardin, 1971; Naess *et al.*, 1976). Similar as in AIS patients with complete androgen insensitivity, *Tfm* mice completely lack Wolffian duct-derived tissues and have female external genitalia. This complete androgen insensitivity is caused by a frameshift mutation in exon 1 encoding the amino terminus of the AR, resulting in a premature translational termination of AR protein synthesis (He *et al.*, 1991).

Two other human pathologies which are also linked to androgen receptor defects are spinal and bulbar muscular atrophy (SBMA) and prostate cancer. SBMA, also called Kennedy's disease, is characterized by progressive muscle weakness and atrophy which can be associated with signs of androgen insensitivity and infertility (Arbizu *et al.*, 1983). The disease is linked to an expanded polymorphic glutamine stretch, located in the NH_2-terminal part of the AR (La Spada *et al.*, 1991). Mutations in the *AR* gene, but also *AR* gene deletions or amplification have been identified in human prostate cancer, indicating the involvement of the AR in the progression of prostate cancer, although its precise role is not fully understood (Jenster, 1999).

Genetic defects in any of the steroidogenic enzymes involved in testosterone biosynthesis can also result in incomplete development of the Wolffian ducts and male genitalia, which has been shown for the enzymes P450c17, 3β-HSD and 17β-HSD (Grumbach and Conte, 1998; Boehmer *et al.*, 1999). A more severe phenotype is seen in patients with mutations in the gene encoding StAR protein, but this phenotype also includes defects in male sex differentiation (Stocco and Clark, 1996).

Dihydrotestosterone synthesis and action during sex differentiation

In contrast to the internal genitalia, the external genitalia of both sexes are derived from common primordia, the genital tubercle, folds and swellings (Wilson *et al.*, 1981a; Grumbach and Conte, 1998). In the female, the genital folds remain as labia minora, the genital swellings become the labia majora and the genital tubercle develops into the clitoris. In the male, fusion and elongation of the urethral folds causes formation of the penile urethra and penile shaft, and the genital swellings will fuse and give rise to the scrotum.

The differentiation of the male penile urethra and external genitalia, but also the prostate, depends on the local conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase (Wilson *et al.*, 1981b). Two isoforms of 5α-reductase have been identified, which have distinct biochemical properties and distinct tissue-specific expression patterns (Wilson *et al.*, 1993). 5α-Reductase isotype II is involved in male urogenital tract development. During differentiation, the Wolffian ducts lack 5α-reductase activity, whereas in the urogenital sinus and tubercle the capacity for DHT formation is already maximal before testosterone production by the testis even has started (Siiteri and Wilson, 1974). Experimental data and studies in 46,XY individuals with 5α-reductase isoenzyme type II deficiency substantiated the concept of preferential target tissues for testosterone and DHT (Imperato-McGinley *et al.*, 1974; Andersson *et al.*, 1991; Wilson *et al.*, 1993). Affected individuals have normal testosterone production but a profound impairment of DHT formation; virilization of the Wolffian ducts is normal, but in the most severe cases the urogenital sinus and external genitalia are predominantly female. Male mice without the type II isoenzyme also fail to virilize properly, although the effect of this loss on the phenotype is less severe than that of human males (Mahendroo and Russell, 1999).

Testosterone and DHT bind to the same androgen receptor, since both *Tfm* mice and AIS patients lack virilization of both internal and external genitalia. DHT is bound to the AR with higher affinity, has a slower dissociation rate from the receptor and is a more potent activator of androgen-responsive genes *in vivo* (Wilbert *et al.*, 1983; Deslypere *et al.*, 1992). It is still unexplained how binding to the same receptor is translated into different physiological effects. It has been proposed that the weaker interaction of testosterone with the AR can be compensated for by a higher hormone concentration (Veyssiere *et al.*, 1982; Grino *et al.*, 1990). In this way, testosterone acts in the vicinity of the testis at a locally high concentration to virilize the Wolffian ducts. Virilization of structures that are more distantly located from the testis, such as the urogenital sinus and external genitalia, may require strengthening of the testosterone signal. Early experiments by Jost (1953) with rabbit embryos are consistent with this hypothesis. If one testis was removed prior to sex differentiation, the Wolffian duct was normal at the sham-operated side, whereas the Wolffian duct on the castrated side was less developed or even absent. In contrast, the presence of one testis appeared to be sufficient to masculize the urogenital sinus and external genitalia.

In addition to exposure of target tissues to different concentrations of testosterone, it can be postulated that the recruitment of transcription intermediary factors might be ligand specific, leading to regulation of different genes, although no such factors have been identified yet (Randall, 1994).

Additional actions of androgens

Testicular androgens are not only essential for the development of the male genital tract, but also for maintenance of male sex characteristics, including spermatogenesis. In view of the absence of AR expression in spermatogenic cells, the Sertoli cells probably are the main target cells for androgens in the control of spermatogenesis (Grootegoed *et al.*, 1977; Bremner *et al.*, 1994). The testicular peritubular myoid cells and Leydig cells also express AR. In the fetal rat testis, AR positive cells are found in the interstitial cell population after testosterone secretion has started (Majdic *et al.*, 1995). As testis differentiation proceeds, the number of AR positive interstitial cells increases, probably being the peritubular myoid and progenitor Leydig cells. Fetal Sertoli cells are AR negative. The role of androgens in the development of the testis itself has remained unclear.

Although female fetuses do express the AR, no virilization of the Wolffian ducts occurs due to lack of the ligand, testosterone. The absence of fetal ovarian steroid hormone production is the result of lack of expression of three of the steroidogenic enzymes (P450scc, P450c17 and P450arom), despite a low level of SF1 expression (Greco and Payne, 1994). Recent genetic studies in mice demonstrated that steroidogenesis in the female fetus is actively suppressed in the fetal ovary by a member of the *Wnt* gene family of growth factors, *Wnt4* (Vainio *et al.*, 1999). *Wnt4* knockout males appear normal, whereas *Wnt4* knockout females are masculinized; the Wolffian duct continues to develop, resembling that of a male.

In the adult female ovary, androgens are produced by the theca cells, being converted into estrogens in the granulosa cells (Hillier and Tetsuka, 1997). Besides this role as substrate in estrogen synthesis, the role of androgens in female reproductive function is less well defined. Delayed puberty and/or reduced pubic hair have been associated with 46,XX individuals heterozygous for an AR mutation (Pinsky *et al.*, 1985; Sai *et al.*, 1990). Although females can be carriers of an inactivating AR mutation, complete deficiency of AR in females has not been observed, because this would require spermatogenesis in AIS males. It would be very interesting to generate female mice which are completely AR-deficient to study the function of the AR in females.

Factors involved in reproductive tract development

Reproductive tract development in the male is regulated by androgens. As noted above, AR expression is first detected in mesenchymal cells of the Wolffian ducts and urogenital sinus, followed by expression in the epithelium at a later stage of development. Since androgen-dependent processes do already occur in the AR negative epithelium, a key role of mesenchyme as a paracrine mediator of androgenic effects in the epithelium has been proposed (Cunha *et al.*, 1992; Cunha *et al.*, 1983). Strong evidence for the importance of the mesenchymal rather than epithelial AR in urogenital tract development has been well-established through analysis of androgenic effects in *Tfm*/wild-type tissue recombinants (Cunha and Chung, 1981). A variety of growth factors have been postulated to be candidate paracrine mediators of mesenchymal-epithelial interactions, including insulin like-growth factors, nerve growth factor, platelet-derived growth factor, epidermal growth factor, transforming growth factor β and fibroblast growth factors (Cunha *et al.*, 1992). Most of these fac-

tors, however, were studied in adult tissues. Two growth factors implicated as mesenchymal paracrine regulators of epithelial growth in the developing prostate and seminal vesicles are keratinocyte growth factor (KGF) and fibroblast growth factor (FGF10) (Thomson *et al.*, 1997; Thomson and Cunha, 1999). Although the signalling pathways of these two factors may crosstalk with AR signalling, the *FGF10* and *KGF* genes are not directly regulated by androgens.

A growth factor involved in androgen-dependent Wolffian duct differentiation is epidermal growth factor (EGF). EGF and the EGF receptor are both expressed in the developing male genital tract (Gupta *et al.*, 1991). In an organ culture bioassay system, it was shown that EGF, like androgens, induced Wolffian duct stabilization whereas anti-EGF antibodies prevented Wolffian duct stabilization even in the presence of androgen. The mechanism by which EGF stimulates Wolffian duct differentiation is not known, but it might potentiate the effect of testosterone (Gupta, 1999).

Female reproductive tract development and function is regulated by estrogens and depends also upon specific mesenchymal-epithelial interactions (Cunha *et al.*, 1983). Several gene transcripts are associated with sites of epithelial-mesenchymal interactions, among them mRNAs encoded by members of the *Wnt* gene family. Three members of this family (*Wnt4*, *Wnt5a* and *Wnt7a*) are expressed in the developing and adult female reproductive tract (Miller *et al.*, 1998). Mouse knockout models have been generated for both *Wnt4* and *Wnt7a*. *Wnt4*$^{-/-}$ female mice showed complete lack of Müllerian duct development (Vainio *et al.*, 1999). In *Wnt7a*$^{-/-}$ female mice, the Müllerian ducts do develop but proper development of these ducts into the female reproductive tract is affected (Miller and Sassoon, 1998).

Several mammalian *Hox* genes have also been linked to reproductive tract development and function. *Hox* genes are characterized by a conserved DNA sequence of about 180 bp, the homeobox, which encodes a DNA-binding homeodomain (Krumlauf, 1994). The products of these genes act as regulatory transcription factors, which bind to specific DNA sequences and are critical in development of the basic body plan of many types of animal species, including *Drosophila* and mammals. The mammalian *Hox* genes, comprising 39 genes, are organized in four clusters, *Hox-a, -b, -c* and *-d*. The most 5' genes of the clusters, the *AbdominalB*-like (*AbdB*) subclass of *Hox* genes, show overlapping domains of expression in both developing limbs and the posterior digestive and/or urogenital structures (Dollé *et al.*, 1991). *Hox* genes which are expressed in the developing urogenital system include *Hoxa-9, Hoxa-10, Hoxa-11, Hoxa-13, Hoxd-10, Hoxd-11, Hoxd-12* and *Hoxd13* (Dollé *et al.*, 1991; Hsieh-Li *et al.*, 1995; Ma *et al.*, 1998). Targeted deletion of *Hoxa-10, Hoxa-11, Hoxa-13* and *Hoxd-13* genes, respectively, demonstrated the distinct role of each of these genes in urogenital tract development of both sexes (Hsieh-Li *et al.*, 1995; Rijli *et al.*, 1995; Satokata *et al.*, 1995; Podlasek *et al.*, 1997; Warot *et al.*, 1997). Since *Hoxa-9, Hoxa-10, Hoxa-11* are also expressed in the adult mouse uterus and are regulated by ovarian hormones, an additional role of *Hox* genes in the functioning of the female reproductive tract has been suggested (Ma *et al.*, 1998).

GONADAL POSITION

The initial position of the developing gonads in male and female mammals is identical. As soon as the indifferent gonads start to develop into testes, the testes start to migrate down the abdomen, through the inguinal canal, into the scrotum. This complex process is called testis descent and involves an interplay of different structures and factors. As discussed below, disturbance of this process may cause an arrest of the migration of the testis somewhere along the path of descent, leading to an undescended or cryptorchid testis. Complete testis descent is essential to obtain full functional development of the testis, including spermatogenesis. Studies of testis descent are based on observations obtained from different mammalian species, including rats, mice, pigs, dogs, cows and human. Unless indicated, the processes described herein are generally applicable to different mammalian species.

Morphology

Before changes in gonadal position between the sexes are being discussed, the original position of the gonads and genital mesenteries will be described (Lemeh, 1960; Youssef and Raslan, 1971; Corliss, 1976; Wartenberg, 1990). The urogenital system as a whole arises in the dorsal abdominal body wall, covered on its ventral aspect by a layer of peritoneum. When the mesonephros begins to grow, it bulges into the abdominal cavity. During this process, the mesonephros remains covered by peritoneum which, at both ends, forms into a mesenteric sheet or fold. One fold runs between the cranial part of the mesonephros and the area of the developing diaphragm, and within this fold the diaphragmic ligament or cranial suspensory ligament develops. The other fold extends from the caudal pole of the mesonephros to the inguinal area, which contains the inguinal ligament.

The indifferent gonad develops on the ventromedial aspect of the mesonephros, positioned within the peritoneal covering of the mesonephros. As development proceeds, the gonad increases in size and becomes progressively more elevated from the mesonephros, and two distinct ligaments develop between gonad and mesonephros; the cranial and caudal gonadal ligaments. With the establishment of the metanephros or permanent kidney, the mesonephros degenerates and its duct becomes attached to the developing gonad, whereas its covering becomes that of the gonad. At the cranial end, the gonadal ligament fuses with the ligament of the cranial mesonephric fold. In this way, the gonad itself becomes connected to the diaphragm via the cranial suspensory ligament (Corliss, 1976; van der Schoot, 1993). In contrast, the caudal gonadal ligament connects the gonad to the mesonephric or Wolffian duct and does not fuse with the inguinal ligament (Ludwig, 1993). The inguinal ligament associates closely with the mesenchymal sheet of the Wolffian duct at the prospective boundary between the epididymis and vas deferens. The region, where the caudal end of the inguinal ligament attaches to the abdominal body wall, is the region in which the processus vaginalis and inguinal canal will develop and is therefore called inguinal region. The inguinal ligament contributes to the so-called gubernaculum. During the process that follows, the structures derived from the mesonephric folds, the cranial suspensory ligament

and gubernaculum, appear to play an important role.

Testis descent

The process of testis descent in mammals is generally subdivided into two phases (Rajfer and Walsh, 1977; Habenicht and Neumann, 1983; Hutson and Donahoe, 1986; Wensing and Colenbrander, 1986). In the initial concept by Gier and Marion (1969), the first phase was further subdivided into two stages: nephric displacement and transabdominal migration.

Nephric displacement
During the indifferent stage of gonad development, the metanephros develops at the posterior end of the urogenital ridge and starts migrating anteriorly, dorsal from the mesonephros. While the metanephros migrates, it increases rapidly in size and occupies the place of the developing gonad, which is being pushed caudally and further laterally. The final position of the indifferent gonad, after this migration, is near the caudal pole of the kidney. This lateral or nephric displacement has been indicated as the initial phase of testis descent, occurring at the time that the development of the testicular cords has already started. However, as it occurs in both male and female fetuses, it seems preferable to be considered as gonadal migration, being no part of testis descent. Herein, the biphasic model of testis descent will be further followed, excluding the nephric displacement as a phase of testis descent.

First phase of testis descent: transabdominal migration
During the first phase of testis descent, the testis moves from a position lateral to the kidney to the abdominal bottom. Some authors, however, have expressed doubt whether the testis actually migrates during this phase (Youssef and Raslan, 1971; Rajfer and Walsh, 1977; Heyns, 1987; Shono *et al.*, 1994b). Although the upper pole of the developing testis corresponds at first with the last thoracic segment, it is never far from the abdominal bottom. A relative growth theory has been proposed, stating that the rapid increase in the distance between the testis and the kidney is not caused by any downward movement of the testis but is accounted for by rapid growth of the lumbar part of the vertebral column and adjacent structures. The kidney is carried upwards together with the ovary, whereas the testis is fixed at the abdominal bottom by the gubernaculum.

The first or transabdominal phase takes place between E15.5 and E17.5 in the mouse (Hadziselimovic *et al.*, 1980) and between E16 and E20 in the rat (Wensing, 1986). In the human fetus, this transabdominal phase occurs between week 8 and week 17 of pregnancy (Hadziselimovic, 1983a; Heyns and Hutson, 1995).

The involvement of the gubernaculum in the first phase of testis descent is very evident, as this structure is developing dramatically before and during transabdominal testis descent, whereas the gubernaculum in the female remains thin. A logical theory appeared to be that the gubernaculum, developing between the testis in the inguinal region, is pulling the testis to the abdominal bottom. However, this theory was disputed mainly because the human gubernaculum does not contain muscle fibers for performing contraction. It has also been suggested that outgrowth of an extra-abdominal part of the gubernaculum
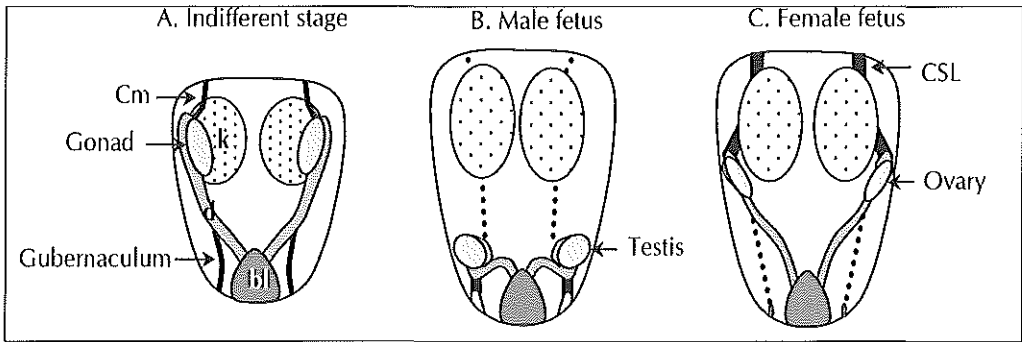
A. Indifferent stage   **B. Male fetus**   **C. Female fetus**

Cm
Gonad
Gubernaculum

CSL
Ovary
Testis

Figure 1.4 – Sex dimorphic development of the cranial suspensory ligaments (CSLs) and gubernacula

(A) During the indifferent stage, the gonads of both sexes are located on the ventromedial aspect of the kidneys. Gonads and ducts are attached to the abdominal body wall via the cranial mesenteries and gubernacula. *(B)* In the male fetus, gubernacula develop whereas development of CSLs is lacking and the testes are anchored at the abdominal bottom. *(C)* In the female fetus, CSLs develop whereas the gubernacula remain small and the ovaries are located at the lower pole of the kidneys. CSL, cranial suspensory ligament; bl, bladder; Cm, cranial mesentery; d, duct; k, kidney.

would exert traction on the intra-abdominal part of the gubernaculum with attached duct and testis, pulling it distally (Wensing, 1968). This process was compared with the reaction of a balloon, which upon inflation moves out of a narrow constriction.

Involvement of the cranial suspensory ligament (CSL) in the determination of the gonadal position was indicated, based on the observation that the CSL develops in the female rat fetus, whereas outgrowth of this ligament is lacking in male rats (van der Schoot and Elger, 1992). The low abdominal position of the testis might be a direct result of lack of CSL development. This hypothesis was modified by Hutson *et al.* (1997), who suggested that the final position of the gonad is determined by the differential development of the two mesonephric ligaments, both the CSL and the gubernaculum, as shown in Figure 1.4. Failure of CSL development in combination with gubernaculum outgrowth causes the testis to establish a position at the inguinal region, low in the abdomen. In contrast, a persisting CSL attaches the ovary to the lumbar region without being anchored to the inguinal region due to the absence of gubernaculum outgrowth, leading to a high abdominal position of the ovary. This process which leads to different abdominal positions of ovaries and testes is generally called the first phase of testis descent.

Second phase of testis descent

During the second and final stage of testis descent, the inguino-scrotal phase, the testis moves from the abdominal body wall into the scrotum, passing through the inguinal canal. In the human fetus, the testis passes through the inguinal canal at about 28 weeks and reaches the scrotum at 35 to 40 weeks (Hadziselimovic, 1983a; Heyns, 1987). In rodents, this phase occurs after birth (Hadziselimovic *et al.*, 1980; Wensing, 1986).

A multitude of theories has been proposed to explain the mechanism by which the testis migrates from the abdomen into the scrotum, reviewed by Heyns and Hutson (1995),

and references therein. In general, these theories include that the testes are pulled or pushed from the abdomen into the scrotum, or that they reach their destination by a combination of growth and involution processes. Increase in abdominal pressure has been proposed as a primary force by which the testis is expelled from the abdomen, through the inguinal canal (Gier and Marion, 1969; Frey *et al.*, 1983). Others have suggested that contraction of the abdominal muscles during respiration or crying provides the primary force, and even gravity has been regarded as an important factor. Hadziselimovic and Herzog (1993) proposed that testis descent is secondary to epididymal descent, with the testis "incidentally" accompanying the epididymis, and might be caused by epididymal and mesonephric differentiation.

In many theories, the gubernaculum plays an active role in the process of testis descent by pulling the testis into the scrotum. Atrophy, degeneration or shrinking of the gubernaculum has been proposed to bring about testis descent, whereas others suggested that swelling of the gubernaculum is of more importance, causing dilation of the inguinal canal (Rajfer and Walsh, 1977; Backhouse, 1981). More recently, the genitofemoral nerve and a neurotransmitter, calcitonin gene-related peptide (CGRP), released from the nerve terminals of the genitofemoral nerve have been indicated as important factors in inguino-scrotal descent (Hutson *et al.*, 1997). CGRP is released from the genitofemoral nerve in the scrotum and might provide directional chemotactic guidance for the gubernaculum, followed by the testis.

These are all mechanical events, which might possibly be involved in testis descent, some appearing logical while other suggested mechanisms seem very unlikely. Central to the controversies surrounding the mechanism of testis descent is the precise role played by the gubernaculum, although most investigators do agree that the gubernaculum is intimately involved in the mechanism of testis descent.

The gubernaculum in more detail

The term gubernaculum was first used by Hunter (1762), who gave this name to the structure which directs the testis into the scrotum. From that date onwards till the present time, the extensions, attachments, parts, structure and the role of the gubernaculum are debated (Heyns and Hutson, 1995).

Morphology
The mechanism of testis descent differs between large mammals including man, monkey, horse, pig and dog, and small mammals including rat, mouse, and rabbit (Wensing, 1986; Heyns and Hutson, 1995). Comparison of experimental data obtained from rodents, which are more often used as experimental model due to their size and rapid reproduction cycle, with data obtained from larger mammals or from clinical observations in man is quite possible, but should be done with care (Wensing, 1986; van der Schoot, 1996c). The various structures caudal from the testis of the rat and the pig, respectively, are schematically shown in Figure 1.5; this scheme is mainly based on the description by Wensing and Colenbrander (1986).
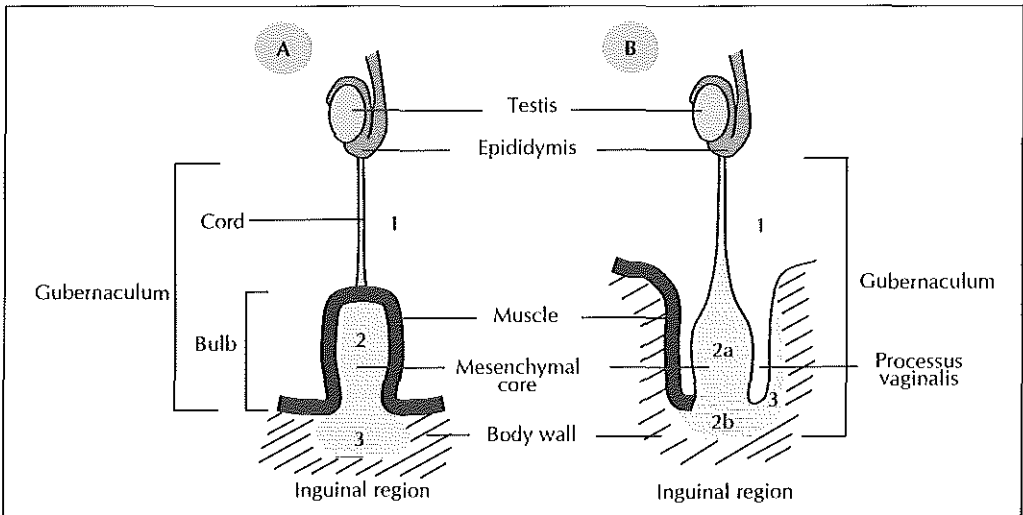
Figure 1.5 – Schematic representation of the gubernaculum of the rat (A) and the pig (B)
The homologous parts of the gubernaculum are indicated by similar numbers. A-1, gubernacular cord; A-2, mesenchymal core of the gubernacular bulb; A-3, extra-abdominal part of the gubernaculum. B-1, intra-abdominal part of the gubernaculum proper; B-2a, extra-abdominal part of the gubernaculum proper; B-2b, infravaginal part of the gubernaculum; B-3, vaginal part of the gubernaculum. Based on the description by Wensing and Colenbrander (1986).

– *Rat (also representing mouse and rabbit)* – The gubernaculum runs between the testis and the inguinal area, and can be subdivided into two parts. The cranial part (1) is the gubernacular cord, the original caudal mesonephric mesentery. The caudal part, consisting mainly of mesenchyme, is subdivided into an intra-abdominal (2) and extra-abdominal segment (3). The intra-abdominal segment has been named gubernacular cone, gubernacular bulb or conus inguinalis, and consists of a loose mesenchymal core covered by a muscular layer that is continuous with the developing layers of the abdominal wall musculature. Within the present thesis, the above-described gubernacular cord (1) and gubernacular bulb (2) will be considered the gubernaculum.

Initially, the whole complex is present in both male and female fetuses. During the phase of transabdominal testis descent, differences between male and female gubernaculum development become apparent. In the male, the gubernacular cord shortens and thickens whereas the bulb enlarges substantially, a process which is often referred to as gubernacular swelling. In the female, the gubernacular cord lengthens and the bulb remains the same size. During the second phase of testis descent, the mesenchyme of the gubernaculum regresses and the bulb starts to invert or prolapse. In this way, a sac will be formed with the muscular outerlayer of the cone as wall. This sac forms the processus vaginalis and its muscular wall is the cremaster muscle, which has a sac-like shape. Since the processus vaginalis will not develop into a narrow inguinal canal, as seen in larger mammals, the testis can freely pass from scrotum to the abdominal

cavity. It remains speculative what exactly happens with the gubernacular bulb during the second phase, whether it first regresses and then inverts, or only regresses or only inverts (Heyns and Hutson, 1995; van der Schoot, 1996c).

- *Pig (also representing dog, horse, monkey, and men)* – The gubernaculum runs between the testis and the inguinal area and can be subdivided into three parts. The gubernaculum proper, consisting of an intra-abdominal part (1) and an extra-abdominal part (2a). The second part is the caudal end of the gubernaculum, which is not invaded by the processes vaginalis, called the infravaginal part (2b). The third part of the gubernaculum is called the vaginal part (3), which forms the wall of the processus vaginalis.
  During transabdominal testis descent, the extra-abdominal part of the gubernaculum expanses, whereas the intra-abdominal part becomes shorter, bringing the testis towards the inguinal region and causing dilation of the inguinal canal. During the second phase of testis descent, the gubernaculum proper regresses, creating space for further descent. Other explanations are equally possible, but the dramatic increase in the size of the gubernaculum appears to be essential.

The cremaster muscle of larger mammals and men is strip-like, in contrast to the sac-like muscle of rodents and rabbits. Furthermore, the processus vaginalis in large mammals can already be identified before the process of testis descent starts, first noted as a dimple at the caudal attachment of the inguinal ligament, present in both sexes (Lemeh, 1960). In the male, the processus vaginalis will gradually become deeper and larger in diameter, being shaped by the developing gubernaculum, forming the inguinal canal. After completion of testis descent, the inguinal canal becomes very narrow and the testis cannot enter the abdominal cavity anymore. The inlet of the inguinal canal consists of the internal inguinal ring and its outlet of the external inguinal ring.

In spite of the quite pronounced anatomical differences in the process of testis descent between mammalian species, different parts of the gubernaculum are comparable, indicated in parts A and B of Figure 1.5 by the same numbers.

The gubernaculum is present in both sexes. However, the female gubernaculum remains underdeveloped in most mammalian species (van der Schoot, 1996b). Yet, it can be identified in adult females as a slender ligament, which is named the uterine teres ligament. An exception is the human uterine teres ligament, which is a well-developed structure, running from the uterus towards the inguinal canal, and is also called the round ligament (Attah and Hutson, 1991; van der Schoot, 1996b).

Gubernaculum outgrowth and regression
The term gubernaculum outgrowth or swelling reaction is used to define the time period when rapid expansion of the gubernacular mesenchyme takes place. Analysis of male human fetuses demonstrated that the wet mass of the gubernaculum relative to the fetal mass rapidly increased prior to descent (Heyns, 1987). In addition, an increase in the wet/dry mass ratio of the gubernaculum was seen, denoting an increase in its water content prior to descent. Data obtained from pig fetuses demonstrated that this rapid increase in volume was due to rapid cell proliferation, accompanied by the disposition of a significant

amount of extracellular matrix (ECM) (Heyns *et al.*, 1986; Fentener van Vlissingen *et al.*, 1989; Heyns *et al.*, 1990). Components of the ECM synthesized by gubernaculum cells during the outgrowth phase were collagen, sulphated-glycosaminoglycans (s-GAG) and the glycosaminoglycan hyaluronic acid (HA, also called hyaluronan or hyaluronate). GAG serve a hydrophylic function resulting in water accumulation within the gubernaculum and swelling.

During the second phase of testis descent, the gubernaculum regresses and becomes fibrous. During this phase, cell proliferation ceased whereas DNA concentration increased, indicating an increase in cell density (Fentener van Vlissingen *et al.*, 1989). The total amount of s-GAG per gubernaculum increased at a slower rate during this phase then in the gubernaculum outgrowth phase. The total amount of HA steadily decreased during this phase, suggesting that HA metabolism might be crucial for gubernaculum outgrowth and regression. In the study of Heyns *et al.* (1990), the HA fraction in the gubernaculum did not change during testis descent, although relative loss of water from the gubernaculum after descent was observed. It was noted that the percentage of HA in the gubernaculum was two times higher then in other tissues, indicating that this fraction might be responsible for the increased water content of the gubernaculum during descent.

Little is known about possible involvement of ECM modulation in the outgrowth and regression of the rodent gubernaculum. Development of the muscular layer of the rat gubernacular bulb has been analyzed by Radhakrishnan *et al.* (1979). The gubernacular bulb primarly consists of indifferent loose, round mesenchymal cells. As the male bulb starts to grow, the mesenchymal cells in the periphery begin to take on an elongated fibrillar configuration, converting first into myoblasts and then to rhabdomyoblasts. When gubernaculum outgrowth is maximal, around birth, the periphery of the gubernacular bulb thickens and differentiates from rhabdomyoblasts into cords of striated muscle. In the female gubernacular bulb, cells in the periphery differentiate into fibrillar cells and rhabdomyoblasts but then undergo degeneration and are replaced by fat.

Androgen receptor in the gubernaculum
The androgen receptor (AR) has been shown to be present in the gubernacular bulb of the rat and the gubernaculum of the pig (George and Peterson, 1988; Oprins *et al.*, 1988; Heyns and Pape, 1991). A high level of AR expression was seen in the undifferentiated mesenchymal cells that form the core of the rat gubernacular bulb (Husmann and McPhaul, 1991a). The level of expression was rapidly decreasing in the bulb towards the end of gubernaculum outgrowth, around birth (Husmann and McPhaul, 1991a; Bentvelsen and George, 1993). Based on these findings, the mesenchymal cells of the fetal gubernaculum were proposed to be a primary target of androgen action. It remains to be determined whether the loss of AR is due to a decrease in expression level, loss of mesenchymal cells or differentiation of these cells in myoblasts.

The cranial suspensory ligament (CSL) in more detail

Presence of the CSL primordium at an early stage of sex differentiation in both sexes and the disappearance of this structure in the male fetus has been observed by many investiga-

tors. However, an actual role for this ligament in testis descent has not been studied by many laboratories and is, at the moment, still considered controversial. As the CSL has been recently reviewed by Van der Schoot and Emmen (1996), this section is directed towards more recent studies on this structure.

Morphology
The CSL, also called cephalic ligament or diaphragmatic ligament, is a muscular cord-like structure, which borders the cranial mesonephric mesentery. In adult females of most mammalian species, it is an easy distinguishable structure, which runs from the ovary and genital duct towards the diaphragm. The most obvious function of the ligament is to keep ovary and uterus in place, especially during pregnancy, but an additional role in the sympathic innervation of the ovary has been suggested (Crouch and Lackey, 1969; Mohsin and Pennefather, 1979; Lawrence and Burden, 1980). The CSL consists of smooth muscle, which allows stretching of the ligament during pregnancy. Contractions of the CSL may also participate in ovulatory events, possibly by assisting ovum trapping by the fimbriae of the Fallopian tube. However, such a role is considered very speculative (Melton and Saldivar, 1970; Mohsin and Pennefather, 1979).

The CSL is absent in most males, which has been related to a low abdominal position of the testis. A typical exception is the male sheath-tail bat (Jolly and Blackshaw, 1988). This animal exhibits testicular migration, with the testis located only in the scrotum during the breeding season. Straps of muscle run from the testis cranially towards the diaphragm and caudally towards the scrotum. The muscular structure running cranially may be analogous to the CSL and necessary to pull the testis into the abdomen.

During early development, cranial mesonephric ligaments are present in both male and female fetuses (van der Schoot *et al.*, 1995). In the female fetus, these ligaments further develop into the CSLs, whereas in the males the ligaments do not persist to the same extent and disappear. This sex dimorphic development of the CSL occurring during genital development correlates with the sex-specific position of the gonads.

The role of the CSL in determining gonad position
Persistence of CSLs in the male correlates with disturbed testis descent, as shown by flutamide exposure of male rodent fetuses and observations on androgen insensitive mice and men (Hutson, 1986; van der Schoot and Elger, 1992; Barthold *et al.*, 1994; Shono *et al.*, 1994a; Cain *et al.*, 1995). It is questioned, however, whether persistence of the CSLs can be the primary etiological cause for undescended testes or that it is just a reflex response to the lack of developing gubernacula (Cain *et al.*, 1995; Lee and Hutson, 1999). A case study of a dog with bilateral undescended testes demonstrated that persistence of the CSLs could have been the major determining factor in causing this disturbance (Kersten *et al.*, 1996). Caudally from either testis, a structure was running between the caudal pole of the kidney and the area of the internal inguinal ring. This structure was identified as the processus vaginalis sac, being inverted and elongated. The sac normally was developed, but instead of growing downwards it had grown upwards, possibly due to the developed CSL. Similar observations were obtained from the study of freemartinism (van der Schoot *et al.*, 1995). These female animals demonstrate male-like development of the gubernaculum and proc-

essus vaginalis, due to the presence of either AMH and/or another testicular factor from the male co-twin. In contrast, their CSLs are normally developed and inversion of the gubernacula, extending into the abdominal cavity, is observed. In both studies, the inversion of the gubernaculum is seen as a reflex response.

As it appears to be difficult to discriminate the separate roles of both the CSL and gubernaculum in the process of testis descent, a model has been proposed in which both ligaments act in concert in determining the final position of the testis, as shown in Figure 1.4 (Hutson *et al.*, 1997).

CSL in human
The presence of the CSL during early development of the human fetus is shown in many textbooks and articles (Lemeh, 1960; Hadziselimovic, 1983a; England, 1990; van der Schoot and Emmen, 1996; Cortes, 1998). There is no clear evidence, however, for the existence of the CSL at later stages of human genital development or in the adult female. Due to the upright body position of the human, the pelvic configuration is different from that of other mammals. This may have caused a relatively low abdominal position of the ovaries and uterus, as compared to other mammalian species, and may also have led to a less obvious CSL. The well-developed uterine teres ligament, the female gubernaculum, possibly has taken over the function of the CSL.

Cortes (1998) suggested that the involution of the CSL is important for the descent of the testis to a level beyond the internal inguinal ring. This is based on clinical observations on 13 boys (17 intra-abdominal testes) who underwent laparoscopy. The CSL was present in all cases, ranging from large and fan-shaped to small. Generally, the testes which were located high in the abdomen had a larger CSL. Cortes *et al.* (1996) also related a rare condition called splenogonadal fusion to a persisting CSL. Patients with splenogonadal fusion often have uni- or bilateral undescended testes. In this condition, splenic tissue is found to be connected with gonadal tissue with often a cord running from the normally positioned spleen to the gonad. In the early part of their development, possibly as the splenic anlage and the developing CSLs are crowded close together, they may become fused to each other (Cortes *et al.*, 1996). As a consequence, CSL regression will be disturbed.

The term ovarian suspensory ligament is often applied to a ligament of the adult human female genital tract which contains the uterine and ovarian blood supply (van der Schoot, 1993). This ligament does not originate from the cranial mesonephric ligament. It might be confusing that the term ovarian suspensory ligament is also generally applied to the CSL, both in adult females of other mammalian species and in the human fetus.

Hormonal control of testis descent

Endocrine factors involved in the first phase of testis descent
Not only the morphological process of testis descent has remained unclear, also its regulatory mechanism is not fully understood. It has been well established that hormones from the testis are important for testis descent, but the exact hormones and their target structures are still debated.

The first phase of testis descent starts as soon as the testis secretes both AMH and

testosterone. Experimental studies in different animal models demonstrated involvement of androgens in the development of many specific structures of the male genital system, such as the epididymis, vas deferens, seminal vesicles and prostate, but not in gubernaculum development (Habenicht and Neumann, 1983; Wensing and Colenbrander, 1986; van der Schoot, 1992; Hutson *et al.*, 1997). However, in *Tfm* mice and rodents exposed to anti-androgens, the testes are located at the bottom of the abdomen and the gubernacular bulb is normally developed. Individuals with complete androgen insensitivity syndrome also demonstrate normal transabdominal testis descent (Hutson, 1986). These findings not only suggest that gubernaculum development is an androgen-independent process, but also that the first phase of testis descent occurs in the absence of androgen action. However, Habenicht and Neumann (1983) reported that cyproterone acetate, which exerts an anti-androgenic effect, did affect the position of the testes in rat fetuses to some degree. At the end of transabdominal descent, the testes were situated somewhat higher and displaced more laterally, despite normal development of the gubernacular bulb. Furthermore, lengthening of the gubernacular cord was seen.

As the role of androgens in the first phase of testis descent was by no means settled, the involvement of another factor or hormone was suggested. Gubernaculum development does depend on the presence of a fetal testis, since orchidectomy causes gubernaculum atrophy (Baumans *et al.*, 1982). Additional evidence for the necessity of a testis for gubernaculum developed is derived from analysis of genital abnormalities in freemartins (Colenbrander and Wensing, 1975; van der Schoot *et al.*, 1995). As described above, a freemartin is a female porcine or bovine fetus, prenatally exposed to fetal testicular factors from a male co-twin due to fusion of the blood circulation. Analysis of freemartins revealed outgrowth of the gubernaculum, without any sign of androgen-induced modifications in their genital system. Since both regression of the Müllerian ducts and stunted ovarian development was observed in freemartins, AMH has been identified as a possible effective male hormone in this respect (Jost *et al.*, 1972; Vigier *et al.*, 1987). Possible involvement of *AMH* in testis descent was further substantiated by clinical observations. In patients with persistent Müllerian duct syndrome (PMDS), the testes often fail to descend (Josso *et al.*, 1997). Furthermore, a portion of boys born with undescended testes have lower levels of AMH (Yamanaka *et al.*, 1991). These findings correlate abnormal testis descent with alterations in AMH production or function, suggesting a role for this hormone in the process of testis descent (Habenicht and Neumann, 1983; Hutson and Donahoe, 1986). However, in most cases of PMDS at least one testis is descended in the scrotum (Josso *et al.*, 1997). An alternative explanation for the observed disturbed testis descent in PMDS, is indirect impairment of testis descent due to tight attachment of the testis to the retained Müllerian duct derivatives.

Experimental data obtained from animal models do not support a role for AMH in testis descent. Rabbits and dogs with persistent Müllerian duct syndrome have normally descended testes (Tran *et al.*, 1986; Meyers-Wallen *et al.*, 1993) The generation of transgenic mice overexpressing the human *AMH* gene and mice with targeted disruption of the *AMH* gene provided excellent models to further study the role of AMH in testis descent (Behringer *et al.*, 1990; Behringer *et al.*, 1994). Male *AMH* knockout mice do have retained

Müllerian ducts but their testes normally descent. Female transgenic mice overexpressing *hAMH* demonstrate neither an abnormal ovarian position nor gubernaculum development (Lyet *et al.*, 1996). Exposure of these transgenic female fetuses to androgens induced a slight increase in gubernaculum outgrowth, which was, however, by no means similar to gubernaculum development in males. This is in agreement with earlier observations from *in vitro* studies with fetal porcine gubernaculum cells, demonstrating that the growth response induced in these cells by testicular tissue could neither be mimicked by AMH nor by androgens (Fentener van Vlissingen *et al.*, 1988; Visser and Heyns, 1995). Concerning gubernaculum development, involvement of a third unidentified testicular factor was proposed.

Studies undertaken to gain more insight in the regulation of transabdominal testis descent were primarily focussed on factors involved in gubernaculum development and little attention was paid to possible androgen target tissues. Van der Schoot and Elger (1992) noticed that the sex-dimorphic development of the cranial suspensory ligament occurring during the transabdominal phase of testis descent in rodents, shown in Figure 1.4, was under the control of androgens. Prenatal exposure of female rats to androgen prevents outgrowth of the CSL, whereas males prenatally exposed to anti-androgen show CSL development. The androgen-dependent regression of the CSLs is confirmed by other investigators (Barthold *et al.*, 1994; Shono *et al.*, 1994a; Cain *et al.*, 1995; Lee and Hutson, 1999). Persistence of CSLs in mice with the androgen insensitivity syndrome is consistent with the involvement of androgens in the prevention of CSL outgrowth (Hutson, 1986).

Endocrine factors involved in second phase of testis descent
The second, inguino-scrotal, phase of testis descent is considered to be androgen dependent, as this phase does not occur in rodents exposed to anti-androgen and in mice and human individuals with the androgen insensitivity syndrome. Proposed mechanisms of this androgen action are lengthening of the epididymis, vas deferens and spermatic vessels, increasing the size of the scrotum, increasing abdominal pressure by acting as anabolic steroid on the abdominal wall musculature and causing changes in the gubernaculum that lead to a scrotal position of the testis (Heyns and Hutson, 1995).

During the inguino-scrotal phase of testis descent, occurring prenatally in the pig and man and shortly after birth in rodents, gonadotropins become essential for maintenance of normal testicular androgen production (Wensing and Colenbrander, 1986; Pelliniemi *et al.*, 1996; O'Shaughnessy *et al.*, 1998). This is supported by both clinical and experimental data. Clinically, undescended testes are common in Kallman's syndrome, a genetic deficiency of luteinizing hormone-releasing hormone (LHRH) secretion, and in other endocrine disorders involving abnormal gonadotropin secretion or action. In addition, hypogonadal (*hpg*) mice that lack GnRH have undescended testes (Grocock *et al.*, 1988). Postnatal treatment of male *hpg* mice with testosterone results in normal testis descent, indicating failure of the inguino-scrotal phase of testis descent. In decapitated pig fetuses, with undetectable levels of LH and FSH, gubernaculum outgrowth took normally place, whereas regression of the gubernaculum appeared to be retarded to some extent (Colenbrander *et al.*, 1979). All these studies confirm that gonadotropin-stimulated androgen secretion is important for inguino-scrotal descent, with gubernaculum regression as one of the possible mechanisms by which

androgens regulate this phase.

In the dog, orchidectomy performed after completion of gubernaculum outgrowth prevents the subsequent regression phase and could be reversed by administration of exogenous testosterone (Baumans et al., 1983). Failure of gubernaculum regression was also reported after exposure of porcine fetuses to anti-androgens (McMahon et al., 1995). Incomplete regression of the gubernaculum, with a remnant still being present, has been observed in a pig with androgen insensitivity syndrome and in AIS patients (Wensing et al., 1975; Radhakrishnan and Donahoe, 1981). All these studies confirm that androgens are important in gubernaculum regression. However, conflicting results were obtained from experimental studies in rodents (Heyns and Hutson, 1995). This might be due to the fact that it is unclear what happens with the rodent gubernaculum during this phase (see previous section). An alternative hypothesis was proposed, stating that androgens do not act directly on the gubernaculum itself but control development and the activity of the genitofemoral nerve which, in turn, controls gubernaculum migration during the inguino-scrotal phase (Hutson et al., 1997). Despite the multitude of data in favour of this theory, data against such a role have been reported and the extrapolation of the experimental data obtained from rodents to humans appears to be problematic (Barthold et al., 1994; Husmann and Levy, 1995). The precise mechanism by which androgens induce gubernaculum regression remains to be elucidated.

Measurement of the serum AMH concentration in the human fetus revealed that AMH is produced by the fetal testis during the inguino-scrotal phase of testis descent (Schwindt et al., 1997). In comparison, AMH mRNA expression could also be detected in the post-natal rat testis during this phase of testis descent, albeit at a very low level (Baarends et al., 1995a). However, a putative role for AMH during the second phase of testis descent has not been suggested so far. The putative third testicular hormone, proposed to regulate gubernaculum outgrowth during the first phase of testis descent, might also be involved in the inguino-scrotal phase.

A biphasic model for hormonal control of testis descent

Hutson and Donahoe (1986) proposed a biphasic model for testis descent, in which the two phases of testis descent are not only separated in time but also are under separate hormonal control. According to this model, the transabdominal phase of testis descent is under the putative control of AMH, whereas the inguino-scrotal phase is controlled by androgens. However, the control of the process of testis descent appears not to be as sharply defined as proposed in this biphasic model of testis descent (Hadziselimovic, 1995; Husmann and Levy, 1995). Some of the conflicting data are already summarized in the previous sections, including the lack of direct evidence for a role of AMH in testis descent and the controversial data on the involvement of androgen action in gubernaculum regression in rodent models.

Based on both experimental data and clinical observations, androgens appear not to be involved in the first phase of testis descent. However, Habenicht and Neumann (1983) observed that a slight positional shift of rat testes is present at the time of birth after prenatal exposure to anti-androgen. Van der Schoot and Elger (1992) reported that the

sex-dimorphic development of the CSL occurring during the first phase of testis descent is androgen-dependent. Furthermore, treatment of female rodent fetuses with androgen causes a more caudal position of the ovary and a slight increase in gubernaculum outgrowth (Lyet *et al.*, 1996). Clearly, androgens alone cannot cause complete transabdominal descent, but these data indicate at least some role of androgens in the first phase of testis descent.

Typically, experiments in which the anti-androgen flutamide was used to study time-specific effects of this compound on testis descent, demonstrated that the critical period of androgen action occurs during the transabdominal phase (Husmann and McPhaul, 1991b; Spencer *et al.*, 1991). Exposure of male rat fetuses to flutamide during different time intervals showed maximal disturbance of testis descent when this compound was administered during the early phase of sex differentiation, which is at the beginning of the transabdominal phase of testis descent. When flutamide administration was started at the end of the transabdominal phase, around birth, testis descent was not affected anymore. These results strongly support the view that androgens act during the early phase of testis descent, although the exact site of this action is unclear. The androgen receptor, a prerequisite for androgen action, was found to be highly expressed in the developing gubernaculum, whereas the expression level dramatically decreases from fetal to postnatal development (Husmann and McPhaul, 1991a; Bentvelsen and George, 1993). However, many data support an androgen-independent regulation of gubernaculum outgrowth (Wensing and Colenbrander, 1986; Heyns and Hutson, 1995). In contrast, regression of the CSLs is an androgen-dependent process, but it is debated whether CSL regression can be the primary effector of transabdominal descent (van der Schoot and Elger, 1992; Cain *et al.*, 1995; Hutson *et al.*, 1997; Lee and Hutson, 1999).

A third testicular hormone in testis descent; insulin-like factor 3 as possible candidate
It has been suggested by several investigators that gubernaculum outgrowth is dependent neither on androgen nor on AMH, but requires a third, yet unidentified testicular factor (Fentener van Vlissingen *et al.*, 1988; Wensing, 1988; van der Schoot *et al.*, 1995; Visser and Heyns, 1995). Porcine fetal gubernaculum cells in culture were specifically responsive to a low molecular mass extract from the fetal testis, which was distinct from known polypeptide growth factors and known hormones. Fentener van Vlissingen *et al.* (1988) proposed the term descendin for the active factor (< 3.5 kDa), whereas Visser and Heyns (1995) named the candidate novel hormone (< 30 kDa) gubernaculotropin.

Development of the fetal mammalian testis starts with formation of the testicular cords, containing the fetal Sertoli cells (Corliss, 1976; Byskov and Hoyer, 1994). Later in the course of testis formation, Leydig cells appear in the interstitial region, by differentiation of precursor cells. Yet, during the outgrowth period of the gubernaculum, a functional population of Leydig cells is present in the fetal testis (Hullinger and Wensing, 1985). Thus, both these cells and the Sertoli cells could be responsible for production of the putative third testicular factor. Decapitation of fetal pigs resulted in a dedifferentiation of the Leydig cell population, demonstrating that fetal Leydig cell development becomes dependent on fetal gonadotropin (Colenbrander *et al.*, 1979). As gubernaculum outgrowth remained unaf-
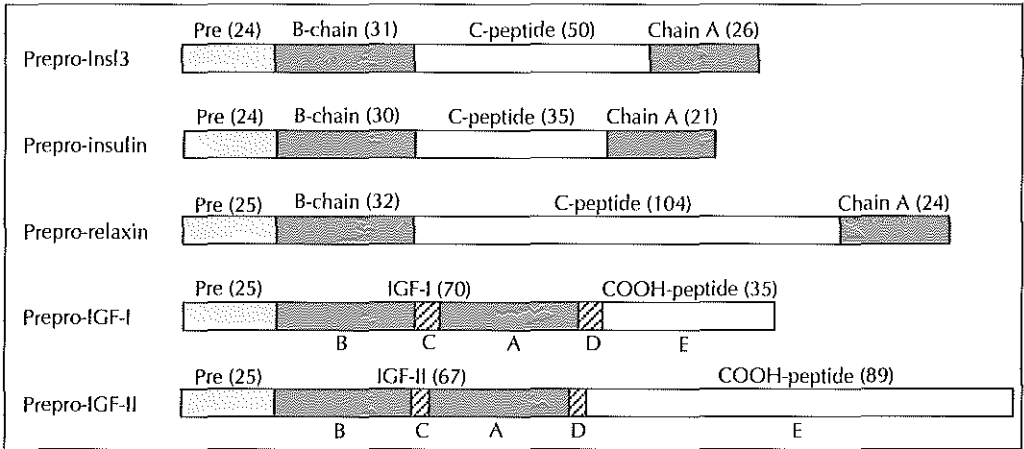
Figure 1.6 – Diagram illustrating the primary structures of prepro-Insl3, prepro-insulin, prepro-relaxin, prepro-IGF-I and prepro-IGF-II
The number of amino acid residues in the different parts of the polypeptide chains are shown between parentheses (modified from Adham et al. (2000)).

fected in these decapitated animals, it was proposed that the swelling reaction of the gubernaculum does not correlate with Leydig cell function (Colenbrander et al., 1979). Similar results were obtained in the rabbit, in which decapitation was performed at the onset of sex differentiation, without a noticeable effect on gubernaculum development (van der Schoot, 1996a).

Unexpectedly, a Leydig cell factor has been recently linked to the process of testis descent, being a good candidate for the previously proposed third testicular hormone (Chapter 3). This factor, insulin-like factor 3 (Insl3) was identified by cDNA cloning as a product from Leydig cells from the porcine testis. Insl3 is also called Leydig cell insulin-like factor (Ley I-L) or relaxin-like factor (RLF) (Adham et al., 1993). The gene encoding Insl3 has been characterized in human, porcine and mouse (Burkhardt et al., 1994; Koskimies et al., 1997; Zimmermann et al., 1997). Insl3 is a member of the insulin-like hormone superfamily, which includes insulin, relaxin, and insulin-like growth factors I and II (IGF-I and IGF-II) (Figure 1.6). Insl3 is probably produced as a prepropeptide, consisting of a signal peptide, B-chain, a connecting C-peptide and an A-chain. It is expected that bioactive Insl3 is formed after enzymatic removal of the C-peptide, similar as for insulin and relaxin. In contrast, prepro-IGF-I and prepro-IGF-II contain a small C-peptide, which is maintained in the active proteins. Insl3 might exert its action through binding to a high affinity receptor on the membrane of target cells (Bullesbach et al., 1999). Analysis of Insl3 expression in mice revealed that Insl3 is specifically expressed in pre- and postnatal Leydig cells of the mouse testis and in postnatal theca cells of the ovary (Zimmermann et al., 1997). Fetal Leydig cells express Insl3 in a gonadotropin-independent way, whereas LH is essential for Insl3 expression in adult Leydig cells (Balvers et al., 1998). Since functional SF1 binding sites are identified in the promoter of the Insl3 gene, SF1 might be essential in the regulation of

Insl3 during pre- and postnatal development of the testis (Zimmermann *et al.*, 1998). The sex dimorphic pattern of *Insl3* expression during development suggested a possible role for Insl3 in sex differentiation, gonadal function and/or germ cell development. The generation of mice in which the *Insl3* gene has been inactivated, demonstrates involvement of this gene product in male sex differentiation (Zimmermann *et al.*, 1999). Similar observations were reported by Nef and Parada (1999). Male mice lacking Insl3 are infertile and have undescended testes, whereas Insl3-deficient females are unaffected (Chapter 3).

Cryptorchidism

In the human, and in many other mammalian species, spermatogenesis is dependent on the relatively cool environment of the scrotum (Frankenhuis and Wensing, 1979; Hadziselimovic, 1983b; Setchell, 1998). If the testis does not descend normally into the scrotum, it is described as cryptorchid. The fertility of bilateral cryptorchid individuals is generally very poor. This is not true for all mammalian species, as there are mammals with incompletely descended testes and mammals without testis descent, among them the elephant, the hyrax and the sea cow. Mammals that are naturally cryptorchid belong to evolutionary older species (Williams and Hutson, 1991).

It is still an enigma why the testis in many mammalian species needs a lower temperature for normal function (Hadziselimovic, 1983b; Setchell, 1998). The scrotum might be a sexual signal to the female of capability to produce offspring. It has been suggested that the lower temperature might have the effect of reducing the higher mutation rate during male gametogenesis (Short, 1997). However, this higher mutation rate may also be important as a source of genetic diversity. Furthermore, a lower temperature of the testis probably leads to a lower metabolic rate, but the advantage of this is not clear.

Definition of cryptorchidism
Absence of the testis in the scrotum may occur due to a retractile, ectopic or incompletely descended testis (Kaplan, 1993; Rozanski and Bloom, 1995). Both the ectopic and the incompletely descended testis are considered cryptorchid (undescended) testes. The incompletely descended testis is located somewhere along the normal path of testis descent, whereas the ectopic testis is found outside the usual anatomical path of descent. A retractile testes has a range of movement from the scrotum to the inguinal canal, but can be manually moved to the base of the scrotum where it will remain for some time. The retractile testis is often confused with cryptorchidism.

Incidence of cryptorchidism
Cryptorchidism is the most common disorder of sex differentiation in man. At birth, 3.7 - 5 % of all boys have undescended testes. However, due to subsequent spontaneous descent, the incidence is 0.97 - 1.78 % by the age of 3 months (Scorer, 1964; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Berkowitz *et al.*, 1993). In 10 to 15% of boys with persistent cryptorchidism, the defect is bilateral (Scorer, 1964; Cendron *et al.*, 1993). Approximately 20% of the undescended testes are impalpable, of which 40% is located in the inguinal canal, 40% is intra-abdominal and 10% is absent (Levitt *et al.*, 1978; Hazebroek and Molenaar, 1992).

Cryptorchidism is closely associated with infertility and subfertility and is an important risk factor for testicular malignancy (Chilvers *et al.*, 1986). Estimations of the prevalence of infertility in the normal population greatly vary and are difficult to compare with studies on infertility in patients with undescended testes. A conclusion that can be drawn from the available data is that fertility in men treated for bilateral cryptorchidism is significantly less than in those who had undergone treatment for an unilateral undescended testis (Elder, 1988). The risk of testicular cancer in individuals with a history of cryptorchidism is 2% to 3%, approximately 5 to 7 times higher than in the general population (Swerdlow *et al.*, 1997; Cortes, 1998).

Management of undescended testes
There are two kinds of therapy available for placing the cryptorchid testis into the scrotum; hormonal and surgical. Hormonal treatment of cryptorchidism is based on the knowledge that testis descent is at least partially under gonadotropic regulation. Patients can be treated with either luteinizing hormone-releasing hormone (LHRH), administered via a nasal spray, or with human chorionic gonadotropin (hCG), which possesses mainly LH activity and is administered intramuscularly. However, the use of hormonal therapy for the treatment of cryptorchidism is controversial since success rates of the clinical studies markedly vary, from 0-55% with hCG and from 9-78% with LHRH (de Muinck Keizer-Schrama, 1988; Pyorala *et al.*, 1995). The efficacy of the treatment of cryptorchidism with hCG or LHRH appears to be highly dependent on the testis position before treatment; the lower the position, the better the result. Especially the inclusion of retractile testis in the treatment groups can lead to an overestimation of the effectiveness of the hormonal treatment (Rajfer *et al.*, 1986; de Muinck Keizer-Schrama, 1988; Pyorala *et al.*, 1995). Retractile testes are not considered truly undescended testes, as they most often take a permanent scrotal position around puberty and do not require treatment. It is commonly agreed upon that surgical intervention is the primary treatment for abdominal testis.

The surgical placement of the undescended testis into the scrotum, orchiopexy, has evolved as the most common method for treatment of cryptorchidism (Elder, 1988; Rozanski and Bloom, 1995). Orchiopexy is currently recommended in early infancy, at the age of 1 to 2 years (Kogan *et al.*, 1990; Cortes, 1998). Early intervention might minimize loss of fertility, based on the observed progressive deterioration of testicular morphology due to the relatively high temperature of the non-scrotal testis (Gaudio *et al.*, 1984; Canavese *et al.*, 1993). In addition, early operation might reduce the risk of the subsequent occurrence of a testicular tumor in the undescended testis, especially in case of an intra-abdominal position, although the evidence is sparse (Chilvers *et al.*, 1986; Cortes, 1998).

Etiology of undescended testes in men
Since normal testis descent appears to be an interplay of different structures and factors, it follows that a variety of defects in the hypothalamic-pituitary-gonadal axis or anatomical abnormalities may result in cryptorchidism (Elder, 1987). Cryptorchidism is commonly found in patients with defects in gonadotropin, androgen and AMH production or action, including patients with Kallman's syndrome, androgen insensitivity syndrome and persistent Müllerian duct syndrome (Clarnette *et al.*, 1997). Alternatively, cryptorchidism might

also result from anatomical obstructions. The incidence of cryptorchidism is relatively high in boys with congenital abdominal wall defects (Koivusalo *et al.*, 1998). Patients with the complex prune belly syndrome characteristically have deficient abdominal wall musculature, defects in urinary tract development and bilateral undescended testes, often located in the abdomen (Wheatley *et al.*, 1996). Failure of testes descent in these patients has been related to inadequate abdominal pressure due to the absence of the abdominal muscles, but also to failure of gubernaculum development (Nunn and Douglas Stephens, 1961; Elder, 1987). As previously mentioned, cryptorchidism is also often observed in patients showing splenogonadal fusion, which has recently been linked to the lack of regression of CSLs due to fusion with the spleen during development (Cortes *et al.*, 1996).

Cryptorchidism is a common feature in a variety of syndromes and chromosomal abnormalities (Elder, 1987). In the majority of boys showing cryptorchidism, however, undescended testes is an isolated anomaly of which the pathogenesis is unknown (Rajfer and Walsh, 1977; Elder, 1987). Since isolated cryptorchidism can occur in several members of the same family, and epidemiological studies have shown a consistent inheritance of isolated cryptorchidism, genetic factors might be involved. Recent genetic studies revealed that mutations in the gene encoding the androgen receptor are not associated with isolated cryptorchidism (Wiener *et al.*, 1998). However, alterations in the *HOXA-10* gene might be an etiological factor of disturbed testis descent (Kolon *et al.*, 1999). The analysis of the *HOXA-10* gene in relation to cryptorchidism in man was based on the finding that male Hoxa-10-deficient mice, created through targeted inactivation of the *Hoxa-10* gene, exhibit uni- and bilateral cryptorchidism (Rijli *et al.*, 1995; Satokata *et al.*, 1995). The initial analysis of this gene in patients with isolated cryptorchidism and in control individuals suggests that genetic alterations of the *HOXA-10* gene might be present in some patients (Kolon *et al.*, 1999).

Animal models in understanding cryptorchidism
Careful analysis of urogenital tract anomalies in patients with defects in gonadotropin, androgen or AMH action, has been very valuable in the identification of possible factors and structures involved in normal and abnormal testis descent (Clarnette *et al.*, 1997). In addition, the usefulness of experiments with laboratory animals in understanding testis descent is well-established, although the animal model must be thoughtfully considered if extrapolation to humans is to follow (Heyns and Hutson, 1995). Exposure of rodents to androgens, estrogens or anti-androgens, combined with naturally existing *Tfm* mice, androgen insensitive rats and *hpg* mice, have been very commonly used as *in vivo* models. Naturally occurring mutations in the *AMH* or *AMH receptor* gene in mice have not been described. However, mice have been generated with a targeted inactivating mutation in the *AMH* gene or in the *AMHRII* gene, for studying the role of AMH in sex differentiation.

Rodent models, which are currently used in the study of testis descent, include:
1. Androgen-insensitive mice and rats, the so-called testicular feminized (*Tfm*) mice and rats with a functional defective androgen receptor (AR), due to inactivation of the AR gene (Yarbrough *et al.*, 1990; He *et al.*, 1991).
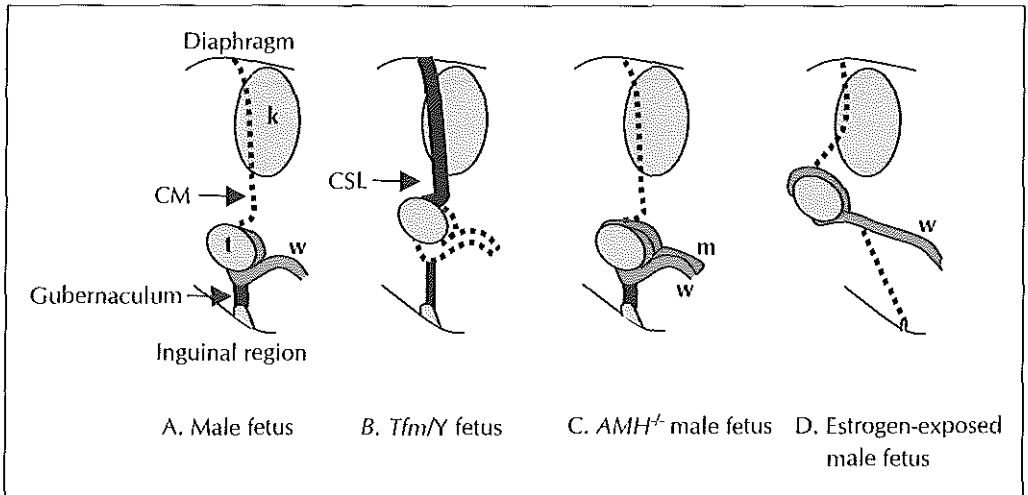2. AMH- and AMHRII-deficient mice. Mice with a targeted deletion in the *AMH* gene or

Figure 1.7 – A schematic representation of the transabdominal phase of testis descent in different mouse models

(A) In the male fetus, the gubernaculum is developed, whereas CSL development is lacking. *(B)* In the *Tfm*/Y fetus, both the CSL and the gubernaculum are developed. Outgrowth of the gubernacular cone is unaffected whereas the cord is less shortened compared with a normal male fetus. The Wolffian duct is not stabilized. *(C)* In the AMH-deficient male mice *(AMH$^{-/-}$)* both the Müllerian and the Wolffian duct systems are stabilized. CSL development is lacking, whereas the gubernaculum demonstrates normal development. *(D)* In the estrogen-exposed male fetus, CSL development did not occur and gubernaculum development is affected; the gubernacular bulb does not swell and the cord is lengthened. CM, cranial mesentery; CSL, cranial suspensory ligament; k, kidney; m, Müllerian duct; t, testis; w, Wolffian duct.

the *AMHRII* gene, which are either AMH deficient or resistant (Behringer *et al.*, 1994; Mishina *et al.*, 1996). Both mouse models have the same phenotype.

3. *Tfm*/Y AMH-deficient mice. Double mutant male mice in which the action of both androgens and AMH is eliminated (Behringer *et al.*, 1994).

4. Hypogonadal (*Hpg*) mice. Mice carrying a large deletion in the *GnRH* gene (Mason *et al.*, 1986).

5. Mutant trans-scrotal (TS) rats. Inbred rat-strain, which shows uni- or bilateral ectopic testis in more than 70% of males (Ikadai *et al.*, 1988).

Within the context of the present thesis, *Tfm* and AMH-deficient mice are the most relevant models, and the phenotypes are schematically shown in Figure 1.7.

Estrogens and cryptorchidism

Diethylstilbestrol (DES), a synthetic estrogen, was used for estrogen therapy to prevent early termination of pregnancy, till it became clear that *in utero* exposure to DES caused urogenital tract abnormalities (Stillman, 1982). An increased incidence of cryptorchidism is one of the genital tract abnormalities observed in exposed men.

As cryptorchidism can also be induced in laboratory animals by prenatal exposure to

estrogenic compounds, rodent models have been used to study the possible mechanism by which estrogens cause cryptorchidism (Greene et al., 1939; Raynaud, 1958; Hadziselimovic et al., 1980; Habenicht and Neumann, 1983; Newbold and McLachlan, 1996). The position of the testes in exposed male mice ranges from firmly attached to the lower pole of the kidney to high in the scrotal sac. The first phase of testis descent is clearly affected by estrogens, with the gubernaculum of exposed animals being poorly developed. It was proposed that estrogens suppress fetal pituitary function and consequently Leydig cell function (Hadziselimovic et al., 1980). However, fetal Leydig cell function during the first phase of testis descent is gonadotropin-independent (El-Gehani et al., 1998; O'Shaughnessy et al., 1998). Although the androgen level might be suppressed in estrogen-exposed animals, this will probably not be the cause of defective gubernaculum development as this process has been shown to be androgen-independent. Since male rodents exposed to DES demonstrate retained Müllerian duct derivatives, an effect of estrogens on AMH action has been suggested (Newbold and McLachlan, 1996). As AMH has also been proposed as an active hormone during the first phase of testis descent, estrogens might interfere with testis descent due to inhibition of AMH action (Shono et al., 1996). However, a prominent role of AMH in testis descent is questioned (Fentener van Vlissingen et al., 1988; Lyet et al., 1996).

Estrogen-treatment is an experimental model for inhibition of gubernaculum outgrowth (Figure 1.7), although the mechanism by which estrogens exert their action has remained unclear. The effect of estrogens on the male genital tract involves a toxic effect of unscheduled and unwarranted exposure, with no apparent role for estrogen action in the development of the male reproductive tract (Couse and Korach, 1999).

SCOPE OF THIS THESIS

Both the cranial suspensory ligament (CSL) and the gubernaculum appear to be important structures involved in determining gonadal position. During the process of testis descent, two hormones are secreted by the developing testis: AMH and testosterone. Failure of action of these hormones has been related to disturbed testis descent. Despite all the research that has been focussed on elucidating the mechanisms of action of these hormones during testis descent, many aspects have remained unclear, indicating the complexity of the process of testis descent. This thesis is focussed on the hormonal control of CSL and gubernaculum development during the first phase of testis descent.

Chapter 2 describes the androgen-dependent suppression of CSL development. The development of the CSL was studied during sex differentiation in both male and female rat fetuses, and in animals which had been exposed to androgens. As the androgen receptor (AR) is a prerequisite for androgen action, the presence of the AR in the CSL was investigated.

Chapter 3 describes the generation of mice with a targeted deletion of the *insulin-like factor 3 (Insl3)* gene, in order to study the *in vivo* function of Insl3. The analysis of the phenotype demonstrates that Insl3 is involved in testis descent. Based on this observation,

and the identification and characterization of the *INSL3* gene in the human (Burkhardt *et al.*, 1994), involvement of INSL3 in the process of testis descent in humans is suggested. Therefore, the *INSL3* gene was analyzed in patients with bilateral undescended testes and compared with control individuals (Chapter 4).

It has been suggested that fetal exposure to environmental endocrine disrupters, in particular environmental estrogens, might be a causal factor in the increased incidence of cryptorchidism that has been observed in recent years. Prenatal exposure to estrogens, including diethylstilbestol (DES), inhibits the first phase of testis descent. Chapter 5 describes the influence of prenatal DES exposure on gubernaculum development, and on the expression of *Insl3* mRNA in the fetal mouse testis. To further investigate the role of Insl3, AMH and androgens in gubernaculum development, an organ culture system has been established which is described in Chapter 6.

In Chapter 7, the obtained data are integrated in a new model for the first phase of testis descent, and directions for future research are discussed.

Androgen action during male sex
differentiation includes suppression
of cranial suspensory ligament
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Androgen action during male sex differentiation includes suppression of cranial suspensory ligament development

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Summary

The cranial suspensory ligament is located in the border of the cranial (mesonephric) mesentery in adult female mammals, which runs between the cranial pole of the internal genitalia and the dorsal abdominal wall. Absence of the cranial suspensory ligament in male mammals depends upon exposure of its primordium to fetal testicular androgens and is a prerequisite for testicular descent. Female rats were exposed to 5α-dihydrotestosterone propionate at different stages of genital development, and cranial suspensory ligament development was studied in neonatal and in adult animals. Androgens suppressed cranial suspensory ligament development when exposure started during the early stages of genital development, until E19. Androgen receptor expression was immunohistochemically detected in the cranial mesentery of both sexes from E16 onwards. A decrease of androgen receptor expression in female fetuses from E18 onwards coincided with the appearance of a differentiated cranial suspensory ligament, as evidenced by the expression of two cell differentiation markers: α-smooth muscle actin and desmin. α-Smooth muscle actin was located in the outer border of the cranial mesentery of both sexes at E17, and expression increased only in female fetuses. At E19, desmin expression was also detectable in the α-smooth muscle actin positive cells. Proliferation and apoptosis indices of cells in the cranial mesentery, as analyzed by 5′-bromodeoxyuridine incorporation and by detection of DNA strand breaks (TUNEL-method) respectively, did not show any difference between the sexes, neither at E17 nor at E18. Since primordial cells of the cranial suspensory ligament highly express the androgen receptor during the period of gestation when androgens can suppress cranial suspensory development, altered morphogenesis of these cells may be a direct consequence of androgen action.

INTRODUCTION

The male-specific process of testis descent, as it occurs in many mammalian species, has been indicated as a partially androgen-dependent process, and different research groups have been searching for the target structures of this androgen action (Hutson *et al.*, 1994; Heyns and Hutson, 1995; Husmann and Levy, 1995). Almost exclusive attention has been paid to structures caudal to the testis, with the gubernaculum as main target structure. However, in contrast to other specific structures of the male genital system such as the epididymis, vas deferens, seminal vesicles, and prostate, it is still a matter of debate whether androgens are involved in male-specific gubernacular development (Heyns and Pape, 1991; van der Schoot and Elger, 1993; Husmann and Levy, 1995). An under-exposed phenomenon in the discussion of possible targets is the absence of the cranial suspensory ligament (CSL) in adult males of most mammalian species (van der Schoot and Emmen, 1996). The CSL is a muscular cord-like structure, which borders the cranial part of the mesonephric mesentery, attaching the ovary and genital duct to the craniolateral surface of the dorsal abdominal wall, near the ventral aspect of the last rib. Besides keeping the uterus in position during pregnancy, the CSL is supposed to play a role in the autonomic innervation of the ovary (Mohsin and Pennefather, 1979). During early genital development, the primordium of the CSL is present in both sexes of e.g. the rat, human and pig (van der Schoot and Emmen, 1996). In rats, the sexual dimorphism in CSL development occurs during the last days of fetal life and correlates with the noticeable sex-specific position of the gonads (van der Schoot and Elger, 1992). Lack of outgrowth of the CSL probably is a prerequisite for testis descent, although this does not imply that absence of the CSL results in testis descent. The complex process of testis descent involves an interplay of different structures and factors (Hutson *et al.*, 1997).

The prevention of outgrowth of the fetal CSL in male rodents is an androgen dependent process: prenatal exposure of females to androgens prevents development of the CSL, whereas males prenatally exposed to anti-androgens show CSL development in a female-like fashion (van der Schoot and Elger, 1992; Barthold *et al.*, 1994; Shono *et al.*, 1994; Cain *et al.*, 1995). These exposed male rats may demonstrate cryptorchidism in adulthood (van der Schoot and Elger, 1992). Furthermore, androgen insensitive mice and rats provide strong evidence for the essential role of androgens in CSL development; these animals have a non-functional androgen receptor and show persistence of the CSLs, combined with cryptorchidism (Hutson, 1986; Barthold *et al.*,1994). Studies on testis descent in experimental animals may contribute to our understanding of the pathogenesis of testis descent in the human, which is of much interest in view of the high incidence of cryptorchidism in newborn boys (Frey and Rajfer, 1982).

The aims of the present study were: (1) to examine whether androgen action on CSL development is limited to a critical period of genital development; (2) to gain further support for androgen action by demonstrating the local and/or time-specific presence of the androgen receptor in the primordium of this structure; (3) to understand in more detail the cellular and molecular mechanism underlying the specific suppressive androgen action.

MATERIALS AND METHODS

Animals and tissue preparation

Wistar rats (R-Amsterdam strain) were obtained from TNO (Rijswijk, The Netherlands). Animals were housed in the departmental animal quarters on a fixed lighting regime (lights on at 5 am and off at 7 pm), and were provided with tap water and standard laboratory pelleted food *ad libitum* (Hope Farms Standard Laboratory Diet, The Netherlands). Rats were mated overnight, and the day when copulatory plugs were found in the morning was considered day 0 (E0) of pregnancy.

Neonatal females were injected subcutaneously on postnatal days 1 (= day of birth), 3 and 5 with 2 mg 5α-dihydrotestosterone propionate (DHTP; Steraloids, USA) in 0.1 ml olive oil (n=10), or with oil only (n=10). They were killed at day 7 or at 3 months of age. Furthermore, pregnant female rats were daily injected subcutaneously with 10 mg DHTP in 0.2 ml oil, or oil only, from E11, E15, E17, or E19 up to and including E21. They were killed on the expected day of parturition (=E22). Fetuses were removed from the uterus, placed on ice, decapitated and fixed in 10% neutral buffered formalin. After preliminary fixation for 4-6 h, the abdomen was opened and intestines were removed. Specimens were embedded in paraffin, sectioned parasagittally at 7 μm, mounted on slides (1:10) and stained with hematoxylin and eosin. Other fetuses were kept alive and reared by foster mothers. The animals were killed at the age of 3 months by cervical dislocation and the internal genitalia were visualized through macroscopical dissection and photographed *in situ*. In each treatment group, at least 5 animals were studied at the day of birth and 5 animals in adulthood.

5'-Bromodeoxyuridine (BrdU) is a thymidine analogue which can cross the placenta and is incorporated into DNA during the S-phase of the cell cycle (Packard *et al.*, 1973). Pregnant rats were injected intraperitoneally with a single pulse of BrdU (Boehringer Mannheim, Germany) dissolved in saline (100 mg/kg body weight) on E17 or E18. Two h after injection, fetuses were removed from the uterus, fixed in 10% neutral buffered formalin and further processed for immunohistochemical detection of incorporated BrdU. Maternal intestine was included as control tissue.

For immunohistochemistry, fetuses were removed from the uterus by caesarean section on E16 through E22 and further prepared as described above. Tissue sections were cut at 5 μm and sections were selected for immunostaining. At each time point, one unilateral part of the urogenital tract was studied of at least 4 male and 4 female fetuses, which had been obtained from two different mothers.

Scanning electron microscopy

E16, E17, E18, E20, and E22 rat fetuses were processed for scanning electron microscopy (SEM) as follows. After preliminary fixation with Bouin's fixative for 6 h, the abdomen was opened and intestines were removed. After 48 h of fixation, tissue was dehydrated through a graded ethanol series to hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, PA, USA), mounted onto specimen stubs, gold coated, and examined in

a JOAL JSM 25-CF scanning electron microscope at 15 kV.

Immunohistochemistry

Sections were mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma, St Louis, MO, USA). Negative controls were always included. After deparaffinization, sections were treated with 3% H_2O_2/methanol solution to block endogenous peroxidase activity and transferred to phosphate-buffered saline (PBS).

Immunohistochemical detection of the androgen receptor (AR) was performed on paraffin embedded sections as described by Janssen *et al.* (1994) (Janssen *et al.*, 1994). Sections were microwaved for 3x5 min at 700 W in 0.01 M citric acid monohydrate buffer, pH 6.0 (Merck, Germany). The sections were then preincubated with normal goat serum, followed by incubation at 4°C overnight with primary polyclonal antibodies SP197 (raised against a synthetic peptide corresponding to the first 20 amino acid residues of the N-terminal domain of the human and rat AR), diluted 1:7000 in 5% BSA/PBS (Bentvelsen *et al.*, 1995). After rinsing in PBS, the sections were treated with biotinylated goat anti-rabbit antibody (dilution 1:400; Dako, Glastrup, Denmark) followed by treatment with streptavidin-biotin-peroxidase complex (ABC; diluted 1:200; Dako) for 30 min at room temperature. Peroxidase activity was developed with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Fluka, Basel, Switzerland) for 7 min. Negative controls were included: replacement of SP197 antibody by preimmune serum (1:7000) or peptide-blocked SP197 (1:7000), prepared by incubation of the antibodies with an excess (1 mg/ml) of the free synthetic peptide that was used to raise the antibodies. Hematoxylin was used for counterstaining.

Detection of apoptosis was performed by the TUNEL-method (Gavrieli *et al.*, 1992). Briefly, tissue sections were incubated with proteinase K (7 μg/ml) for 15 min, before treatment with 3% H_2O_2 in PBS to block endogenous peroxidase activity. Sections were pretreated with terminal deoxynucleotidyl transferase (TDT) buffer (30 nM Trizma base, 140 nM sodium cacodylate, 4 mM cobalt chloride, 0.1 mM DTT) for 5 min and then incubated with biotinylated deoxyuridine triphosphate (dUTP; 10 μM Biotin-16-dUTP; Boehringer Mannheim) and TDT (0.3 U/μl; Promega, Madison, WI, USA) in TDT buffer for 2 h at 37°C. The reaction was terminated by transferring the slides into TB buffer (salt sodium citrate buffer) for 15 min. After washing for 10 min in 2% BSA in PBS, slides were incubated with ABC-complex (diluted 1:200) and a solution of 0.07% DAB.

For BrdU immunostaining, monoclonal anti-BrdU was used as primary antibody (diluted 1:25; Sigma). The sections were digested with pronase 0.1% (Boehringer Mannheim) for 30 min at 37°C, treated with 2 N HCl for 30 min at 37°C, and neutralized with 0.1 M borate buffer (pH 8.5). After pre-incubation with 10% normal goat serum (Dako) to reduce non-specific binding, sections were subsequently incubated with anti-BrdU for 1 h at 37°C, peroxidase-conjugated goat-anti-mouse (dilution 1:100; Sigma) for 30 min at room temperature and 0.07% DAB for 7 min.

To study smooth muscle cell differentiation, monoclonal anti-α-smooth muscle actin (diluted 1:200; Biogenex anti-αsm-1 clone 1A4, San Ramon, USA) and monoclonal anti-desmin (diluted 1:25; Sanbio mon-3001, Uden, The Netherlands) were used. Sections were treated as for BrdU staining except that pretreatment with pronase and HCL was not per-

formed and the second antibodies were biotin-conjugated goat-anti-mouse (Dako) diluted 1:50.

Evaluation of immunohistochemical results

Cells with nuclear (AR) or cytoplasmic (α-sm actin and desmin) immunostaining were interpreted as being positive. The intensity of positive staining was expressed in terms of three relative intensities: strong (+++), moderate (++) and low (+). Prostate and intestine of adult rat were used as a positive control for AR and actin/desmin staining respectively and were graded as +++.

Proliferation and degeneration indices of the cranial mesentery were determined by counting minimal 300 cells and maximal 1500 cells per specimen (n=4) and subsequent division of the number of positive staining nuclei by the total number of counted cells. The cranial mesentery was considered the area between the efferent tubules of the gonad and cranial point of attachment to the abdominal wall. The cranial mesentery was not divided in different cellular compartments. Statistical significance was evaluated using two-factor analysis of variance.

RESULTS

Normal development of CSL

At E16, the gonads of both male and female fetuses were found to be positioned on the ventrolateral aspect of the developing kidneys (Figure 2.1A,B). Differences in gonadal size and shape could be recognized: the ovary was more elongated and smaller compared to the testis. In both sexes, the gonads and adjacent ducts were connected with the abdominal body wall with two strands: one strand running from the gonad towards the diaphragm, which is the cranial (mesonephric) mesentery containing the CSL primordium, and a second strand connecting the duct with the inguinal abdominal wall, the gubernacular cord.

At E17, the testis showed a lower abdominal position than the ovary (Figure 2.1C,D). The ovaries of female rats were positioned lateral from the caudal pole of the kidneys, whereas the testes of male rats of the same age were situated well below the kidneys. The cranial mesentery also showed sexual dimorphism, appearing like a thickened cord in the female fetuses in contrast to the elongated tissue strand in male fetuses. Furthermore, a difference in gubernacular cord appearance between the sexes could be noticed; a thicker cord in the male compared to the female fetus.

From E18 onwards, the ovaries remained positioned lateral to the kidneys, and connected to the middle area of the last rib via a thick mesentery. In contrast, the testes gained a position well below the kidneys, and were connected to the abdominal wall at the level of the caudomedial tip of the kidney, by a flattened mesenteric fold that sometimes was hardly distinguishable. At light microscopical level, the cranial mesentery of female rats was bordered by a differentiated ligament, the CSL, which appeared as condensed mesenchyme
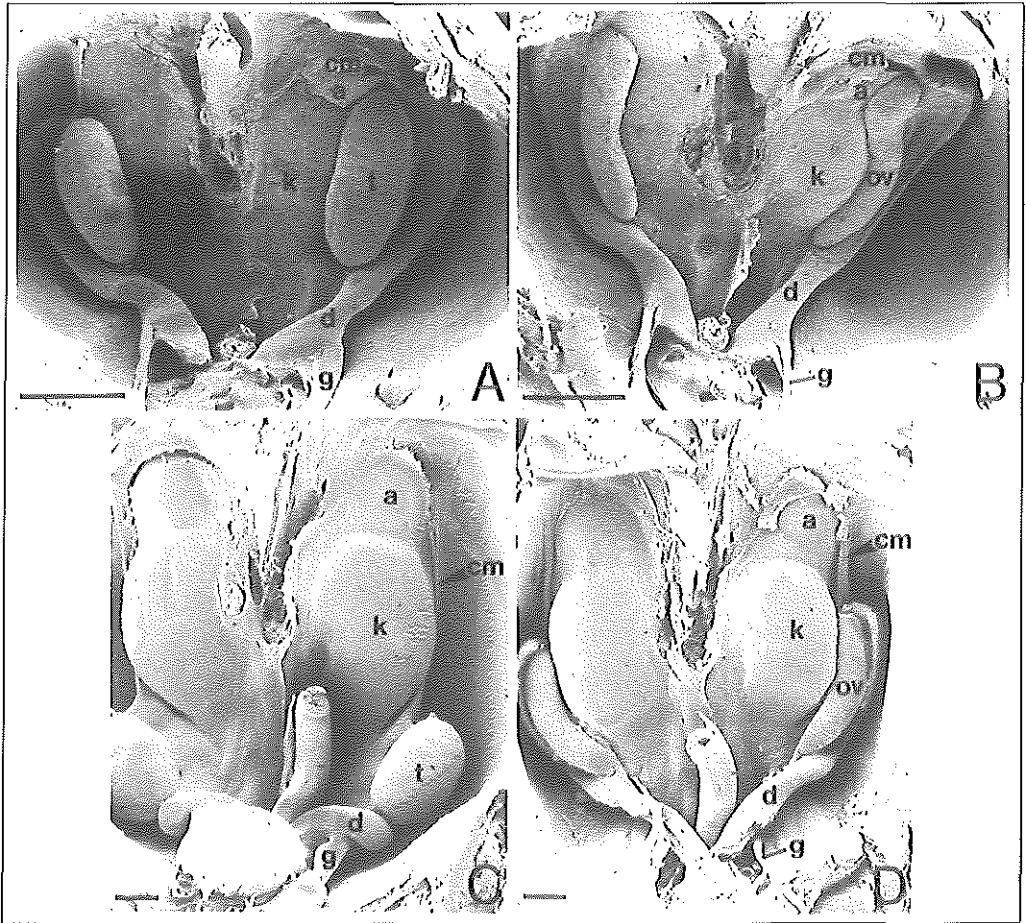
Figure 2.1 – Scanning electron microscopy images of the urogenital system of male and female rat fetuses. *(A, C)* Male, and *(B, D)* female rat fetuses at E16 (top) and E17 (bottom)
Except for the gonadal form and size, the genital system of both sexes at E16 is very similar; e.g. position of the gonads, appearance of cranial mesentery (cm), gubernacular cord (g), and duct (d). At E17, the testis (t) has gained a lower position compared to the ovary (ov). There is a marked difference in the shape of cranial mesentery and gubernacular cord between the sexes (gubernacular cord of the female broke during preparation). a, adrenal; k, kidney. Scalebar = 400 μm.

(Figure 2.2A) and later was identified as smooth muscle by immunohistochemistry. The male counterpart was a small mesenteric fold without specifically differentiated cells in its free border (Figure 2.2B).

Androgen exposure and CSL development

Rat fetuses were exposed to DHTP at different stages of development to determine the time period of inhibitory androgen action upon the CSL primordia. DHTP was used rather than testosterone propionate (TP), since DHTP virilizes the genital system
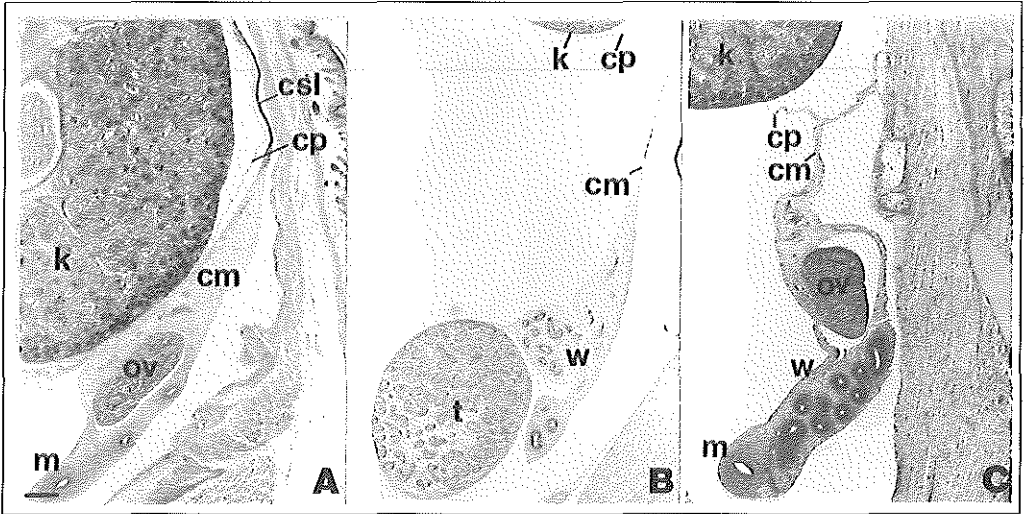
Figure 2.2 – Sagittal histological section of the urogenital system of male and female rat fetuses at E22 (day of birth). (A) control female, (B) control male and (C) female rat fetus exposed to DHTP from E15 to E21
In the female fetus (A), the ovary (ov) is lying at the caudal pole of the kidney (k) and is connected to the cranial part of the dorsal abdominal wall via the cranial mesentery (cm) which is bordered by the CSL (csl). The testis (t), surrounded by Wolffian duct derivatives (w), of the male fetus (B) has gained a position below the kidney and is connected to the abdominal wall via a thin fold of the cranial mesentery, without a specific structure. In the DHTP-exposed female (C), the ovary is located caudal from the kidney and surrounded by both Müllerian duct (m) and Wolffian duct derivatives. Only a thin mesenteric fold was observed between the genital system, the abdominal wall and the kidney capsule (cp). Scalebar = 200 µm.

Table 2.1
The presence of cranial suspensory ligament (CSL) in female rats after prenatal exposure to DHTP

Treatment group (gestational day)	at birth	in adulthood
11 - 21	0/8*	0/16
15 - 21	0/8	0/20
17 - 21	0/10	0/10
19 - 21	10/10	18/18
control	10/10	26/26

The fetuses were exposed to DHTP (10 mg/day), starting on different gestational days up to and including E21. Examination was performed at day of birth (E22) by histology and in adulthood by dissection.
* number of ovaries with CSL/ total number of ovaries.

of the female rat fetus to a similar degree as TP but avoids the frequent disruption of pregnancy observed after TP injection (Schultz and Wilson, 1974).

Prenatal exposure of male fetuses to DHTP had no visible effect on the cranial part of the genital system. All male animals, control and DHTP-treated, displayed no development of a CSL at birth and normally descended testes in adulthood (results not shown).

The CSL was absent in female rats from three out of four different treatment groups (Table 2.1). Histological analysis of the internal genitalia of E22 female fetuses exposed to DHTP before E19 showed no distinct ligament in the free border of the
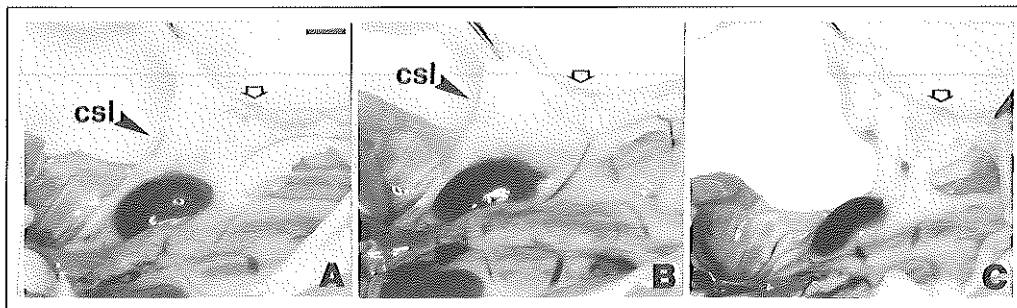
Figure 2.3 – Ventral macroscopical view on the left kidney and cranial part of the urogenital system of adult female rats. *(A)* **Control treatment,** *(B)* **postnatal exposure to DHTP and** *(C)* **prenatal exposure to DHTP**
By gently pulling at the left ovary (open arrow) presence of the CSL (csl, black arrow head) can be confirmed in the control (A) and postnatally treated (B) female rats, whereas in the prenatally treated (C) female rat only the cranial mesentery without a specific border structure is present. Scalebar = 2 cm.

cranial mesentery (Figure 2.2C). The ovaries were positioned below the caudal pole of the kidney. However, the ovaries were closer to the kidney than the testes and the cranial mesentery was united with the kidney capsule whereas in males the cranial mesentery was separated from the kidney capsule (Figure 2.2B,C). In female fetuses exposed to DHTP from E19 onwards, as well as control female fetuses, the ovaries were located lateral from the caudal pole of the kidneys and were connected via a well-developed ligament to the cranial part of the dorsal abdominal wall (Figure 2.2A). The degree of internal genital virilization varied between the different treatment groups, with the strongest degree of virilization in female fetuses exposed to DHTP from E11 onwards.

Autopsy of adult female rats from different treatment groups confirmed the microscopical observations at the time of birth (Table 2.1). Dissection of adult female rats, exposed to DHTP before E19, demonstrated absence of the CSL in all animals (Figure 2.3C). However, unlike the situation in normal males, the cranial mesentery had remained connected to the kidney capsule. In all exposed female rats, the morphological pattern of the ovarian-uterine vascular system was similar to that in control females, and no structure similar to a male-specific plexus pampiniformis was found. Virilization of exposed females from the different treatment groups was confirmed by presence of a penis with the urethra ending on its tip, enlargement of the anogenital distance, and absence of nipples.

Female rats neonatally exposed to DHTP showed unimpaired development of the CSL, visible microscopically on postnatal day 7 and macroscopically during adulthood when compared to control females (Figure 2.3A,B).

Developmental pattern of androgen receptor (AR) expression within the cranial part of the developing genital system

AR expression was examined in untreated fetuses of both sexes between E16 and E22. The AR was continuously expressed in the cranial part of the male urogenital system

Table 2.2
Expression of different markers in the cranial part of the genital system of male and female rats at different stages of development.

marker	E16	E17	E18	E19	E20	E22	day 7 postnatal
AR							
Male	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	nd
Female	+ +	+ + +	+ +/+	+ +/+	+	+/-	+/-
α-Sm actin							
Male	-	+	+	+	+	+	nd
Female	-	+	+ +/+	+ +	+ + +/+ +	+ + +	+ + +
Desmin							
Male	-	-	-	-	-	-	nd
Female	-	-	-	+	+	+ +	+ +

Intensity of positive immunostaining was graded as strong (+ + +), moderate (+ +), weak (+).
nd = not determined, - = no staining. Use of two symbols (+ + +/+ +, + +/+, +/-) indicates that intensity of positive staining varied among different specimen within one group.
At each time point, one unilateral part of the urogenital tract was studied of at least 4 male and 4 female fetuses.

during genital differentiation, from E16 up to and including E20 (Table 2.2). At E16, positive cells were found in the mesenchymal and epithelial cells of the mesonephric tubules, and in the cranial mesentery between the gonads and the diaphragm. This cranial part of the genital system had a more intense androgen receptor immunostaining than the caudal part, containing the Wolffian duct with AR positive mesenchymal cells surrounding the duct. At E17, the pattern of AR expression was similar as at E16 but the staining was more intense. AR was detected in virtually all cells of the cranial mesentery (Figure 2.4A). From E18 onwards, the AR was highly expressed throughout the male genital system and differential expression between the cranial part and the caudal part of the genital system was not detectable anymore.

In female fetuses, the level of expression of the androgen receptor was more dependent on the different stages of development than in male fetuses (Table 2.2). Highest expression was found at E17. A subsequent decrease in expression was observed, until a low level was reached at E20 and later days. The epithelium and surrounding mesenchyme of the efferent tubules showed moderate AR expression up to E22. As in male fetuses of E16 and E17, differential AR expression within the genital system could be observed; the cranial part of the female genital system stained more intensely than its caudal part, which contains the degenerating Wolffian duct, with a low level of AR in the surrounding mesenchymal cells. The AR expression pattern in the genital system of newborn female rats, prenatally exposed to DHTP, was similar to that in newborn males (results not shown).

AR expression was also studied in postnatal day 7 female rats, both in control and DHTP-treated rats. AR expression was hardly detectable in the genital system of control females (Table 2.2). In contrast, the cranial mesentery including the CSL contained a high
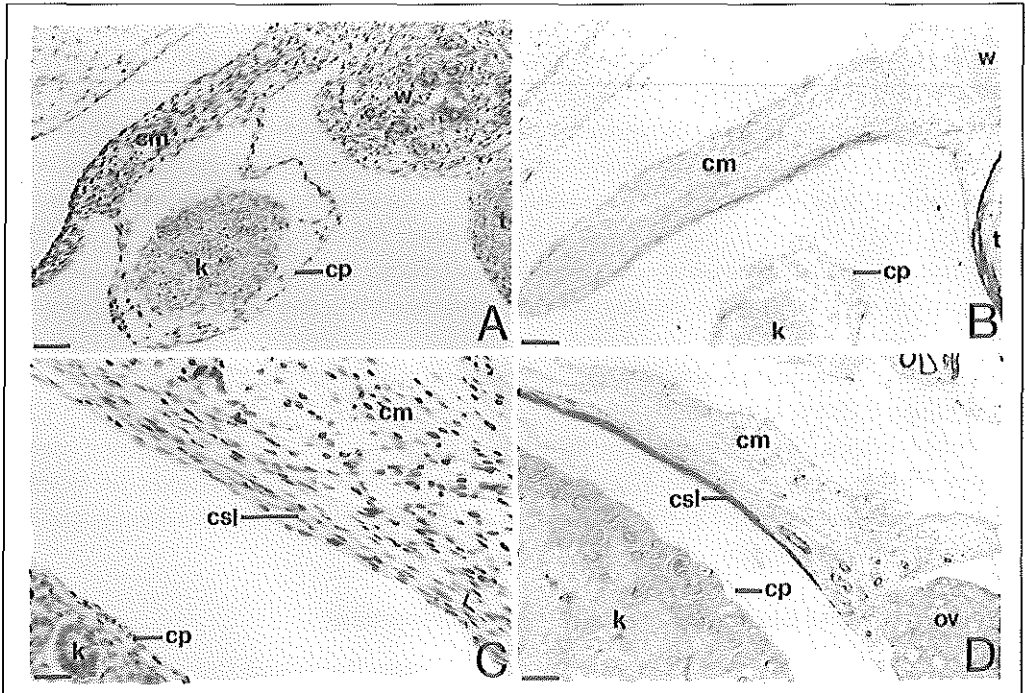
Figure 2.4 – Immunohistochemical localization of α-smooth muscle (α-SM) actin and androgen receptor (AR) in the cranial part of the urogenital system of male rat fetuses and female neonates. *(A, C)* AR immunostaining and *(B, D)* α-SM actin immunostaining in E17 male fetuses (top) and female neonates on postnatal day 7 (bottom). *(For a color version of this figure, see page 157.)*
In the E17 male fetus, AR (A) is expressed in virtually all cells of the cranial mesentery (cm), whereas α-SM actin-positive cells (B) are mainly found in the outer border of the mesentery, the primordial cells of the CSL. Caudally, the Wolffian duct (w) is surrounded by AR positive cells (A). The tunica albuginea demonstrates a strong anti-actin immunoreaction (B). Detail of CSL (csl) of a neonatal female after postnatal exposure to androgen (C), shows that many cells in differentiated CSL and surrounding mesenchyme have high expression of AR. In overview (D), the CSL on postnatal day 7 is present as the outer border of the cranial mesentery, containing strands of actin-positive cells. The ovary (ov) is located at the caudal tip of kidney (k). t, testis; cp, kidney capsule. Scalebar = (A, B) 50 μm, (C) 25 μm, (D) 200 μm.

level of AR expression in DHTP-exposed neonatal female rats (Figure 2.4C).

Smooth muscle differentiation in CSL during development

The immunolocalization of differentiating smooth muscle cells in the cranial part of the genital system was determined from E16 up to and including postnatal day 7, using the smooth muscle markers α-SM actin and desmin (Table 2.2).

In the E16 fetus, α-SM actin was not detectable in cells of the CSL primordia. From E17 onwards, α-SM actin was found to be located in the border of the cranial mesentery of both sexes, the primordial cells of the CSL (Figure 2.4B). Expression of α-SM actin continued to increase in the outer border of cranial mesentery in female rat fetuses. At

postnatal day 7, the α-SM actin-containing cells shape a distinct muscular ligament, the CSL, as anterior border of the cranial mesentery (Figure 2.4D). Differences in α-SM actin expression in the developing CSL between sexes were demonstrated from E19 onwards (Table 2.2). In male fetuses, the outer border of the cranial mesentery remained weakly positive for α-SM actin and no formation of a muscular outer layer could be observed at the end of fetal development. Extensive α-SM actin labeling was detected within the growing tunica albuginea (Figure 2.4B) and peritubular myoid cells of the testis at all the different stages, and in vascular cells of both sexes. From E17 onwards, the mesenchyme surrounding the mesonephric tubules and differentiating Müllerian and Wolffian ducts gave a positive reaction.

Desmin expression was absent from the CSL primordium at E16, E17, and E18 in both sexes (Table 2.2). At E19, desmin positive cells were first observed in the developing CSL of female fetuses, which were also positive for α-SM actin. At later developmental stages, desmin staining was increasing in the actin-containing cells. In male fetuses, no desmin positive cells were detected in the CSL primordia. In contrast, desmin was detected in the testis at all different stages.

Proliferation and apoptosis in CSL during development

Cells in S-phase of the mitotic cycle were labeled using BrdU/anti-BrdU immunohistochemistry. Many positive nuclei were found in all the different fetal tissues, including the cranial mesentery. Positive staining cells did not appear to be restricted to particular parts of the mesentery. Although there was large variability in cell proliferation rates within and between specimens, particular parts of the genital system showed consistent different proliferation rates. In E18 fetuses, proliferation rates of areas containing genital ducts were higher than those of the cranial mesentery.

The occurrence of DNA strand breaks, indicative of apoptotic cell death, was visualized *in situ* by the TUNEL-method. Although very few cells stained positive for dUTP, the nuclear staining could be found in the cranial mesentery. In female fetuses, the epithelium of the degenerating Wolffian duct and gonadal germ cells at E18 displayed a slightly higher incidence of positively stained nuclei compared to other structures of the genital system (not shown). Proliferation and apoptosis indices of the cranial mesentery determined at E17 and E18 are presented in Table 2.3 and were not different between the sexes.

Table 2.3
Percentages of cells undergoing proliferation and degeneration in the cranial mesentery at E17 and E18 in male and female rat fetuses

	E17	E18
Proliferation		
Male	12.5 ± 3.5	9.9 ± 1.7
Female	9.2 ± 3.3	11.1 ± 2.0
Degeneration		
Male	1.0 ± 0.9	0.5 ± 0.2
Female	1.5 ± 0.9	0.9 ± 0.4

Values are means ± SEM. No statistically significant differences were found.
Minimum 300 cells and maximum 1500 cells per specimen were counted.

Discussion

The results presented in this study address earlier observations that prevention of development of the CSL, during male sex differentiation in fetal rats, is an androgen dependent process (van der Schoot and Elger, 1992; Barthold *et al.*, 1994). Fetal treatment of female rats with androgens, but not postnatal treatment, did interfere with CSL development. Androgens prevented CSL differentiation in the anterior free border of the cranial mesentery in female fetuses, provided that the exposure had started at E17 or earlier. Thus, the critical period during which CSL development can be abolished by androgens ends around E17 or E18. Since the rat testis starts to produce testosterone on E15.5-E16.5 (Warren *et al.*, 1975), this androgen effect is an early phenomenon during male sex differentiation, with an onset well before fetal testosterone production reaches its maximum on E18.5 (Habert and Picon, 1984).

Scanning electron microscopical images demonstrated a possible correlation between the sex dimorphic development of the CSL and the development of the sex specific position of the gonads during the last days of rat fetal life. If suppression of CSL development in male rats is an important prerequisite for testis descent, the critical period for androgen action on the CSL primordia should correlate with the precise time period of androgen action on testis descent. Indeed, earlier studies demonstrated that inhibition of testis descent in male rats by anti-androgens was most effective when these compounds were applied during the early phases of genital differentiation (Husmann and McPhaul, 1991b; Spencer *et al.*, 1991). Exposure of male rat fetuses to the anti-androgen flutamide during different time intervals showed maximal disturbance of testis descent when this compound was administered at E16 and E17(Husmann and McPhaul, 1991b) or from E15.5 through E17 (Spencer *et al.*, 1991). Cain *et al.* (1995) measured CSL length in male fetuses exposed to flutamide, DHT or oil alone during E15-E17, the time period of maximal androgen action during testis descent. No difference in CSL length was observed at E18, but on E20 the flutamide-treated animals had significantly shorter CSL compared to both other treatment groups (Cain *et al.*, 1995). It should be noted that Cain *et al.* (Cain *et al.*, 1995) have used the term cranial ligament to point to the complete mesentery present cranial to the gonads, whereas we have applied this term to the ligament in the border of the mesentery. Recent reports on case studies on bilateral cryptorchidism in a dog and a pig also suggested a correlation between disturbed androgen action, persistence of CSL at both sides, and cryptorchidism (Kersten *et al.*, 1996; van der Schoot and Emmen, 1996).

In the present study, no difference was observed between the cranial mesentery of prenatally androgen-treated and control females, except for the anterior border of this mesentery; in control females the muscular CSL is present in this border, which is not identifiable in females exposed to androgens before E19. The absence of the CSL could explain the more caudal position of the ovaries compared to the normal ovarian position. In general, the ovaries, oviducts, and uterus are embedded in mesenteric folds, which attach the female genital system to the kidney capsule and posterior abdominal wall. This anatomical relationship was not abolished in the androgen-exposed female rats, in contrast to male rats, and could be of importance in determining the final position of the gonads. No ovarian

descent was observed in the DHTP exposed females, as was also observed in earlier studies (Elger *et al.*, 1970; van der Schoot and Elger, 1992). Possibly, exogenous androgen does not reach the developing genital system of the fetus in a sufficiently high concentration to cause complete virilization of that system (Schultz and Wilson, 1974; Bentvelsen *et al.*, 1995). Although complete stabilization and differentiation of the Wolffian ducts (Elger *et al.*, 1970; Schultz and Wilson, 1974), and marked virilization of external genitalia were observed after fetal exposure of females to androgen, the development of the ovarian vasculature into a male-like plexus pampiniformis and male-specific gubernacular development had not taken place in these androgen-treated females. The observed anatomical differences between male and androgen-exposed female rats cannot be explained by the absence of anti-Müllerian hormone (AMH) in the female fetuses. AMH-deficient (gene knock-out) male mice show normal testis descent, despite presence of Müllerian duct derivatives (Behringer *et al.*, 1994). Concerning gubernacular development, involvement of an unknown third testicular factor has been suggested (Fentener van Vlissingen *et al.*, 1988; van der Schoot *et al.*, 1995; Visser and Heyns, 1995). Lack of such a factor in androgen-exposed females cannot be ruled out as a possible explanation for the observed anatomical differences between androgen-exposed females and normal males.

Since local expression of the AR is a prerequisite for direct androgen action on the cranial part of the urogenital system, the distribution of nuclear AR within this part was analyzed. Immunolocalization of AR in the fetal urogenital system of the rat has been studied before (Bentvelsen *et al.*, 1995; Majdic *et al.*, 1995), but not in relation to the cranial part of this system. Madjic *et al.* (Majdic *et al.*, 1995) reported a heterogeneous distribution of AR among the mesenchymal cells of the mesonephric area in male rat fetuses on E16. The present study confirmed this observation, but also showed AR positive cells in the cranial mesentery. From E18 onwards, male fetuses showed intense nuclear AR staining in the cranial mesentery, whereas expression in female fetuses decreased. This sex-dependent difference in expression has also been observed in other structures of the fetal genital system, and it was suggested that the AR level in the fetal urogenital system is initially hormone independent, whereas continued AR expression is dependent upon testicular androgen production (Bentvelsen *et al.*, 1994). The present study demonstrated the presence of AR in the cranial mesentery, including primordial cells of the CSL, during the period when androgens are effective in suppressing CSL development (up to and including E17). Thus, the CSL primordium can be considered a direct target tissue for fetal testicular androgens. Furthermore, the present observations indicate that the primordium of the CSL has lost its androgen responsiveness by E17, although AR expression in the differentiated CSL continues after E17, but obviously without a morphogenic effect upon androgen exposure. Such a phenomenon was also observed in the nipple primordia of mice (Wasner *et al.*, 1983).

The cellular and molecular mechanisms of the suppressive androgen action on CSL development are of interest, in view of many other effects of androgens on growth and differentiation of fetal male tissues (Wilson *et al.*, 1981a; Cunha *et al.*, 1992). Another sex dimorphic process in which androgens suppress development, is differentiation of the nipples of the mammary glands in rats and mice. Nipple development is abolished in female

rats and mice after exposure to androgens during an early phase of sex differentiation (Goldman *et al.*, 1976; Kratochwil, 1977). A major difference between nipple and CSL development is, that the fetal nipple primordium consists of an epithelial bud surrounded by mesenchymal cells, whereas the CSL primordium only consists of mesenchymal cells. During destruction of the nipple primordium, androgens act on the AR positive mesenchyme, rather than that there is direct action of androgens on the epithelial cells, and mesenchymal-epithelial interactions eventually cause degeneration of the epithelial bud (Durnberger and Kratochwil, 1980). In the CSL primordium, suppression of development involves direct action of androgens on the mesenchymal cells.

The CSL of adult female rats is a ligament in the anterior border of the cranial mesentery, which contains smooth muscle cells (Mohsin and Pennefather, 1979). Since smooth muscle cells are characterized by expression of both α-SM actin and desmin (Lazarides and Hubbard, 1976), the expression patterns of these two markers in the developing CSL were analyzed. From E17 onwards, α-SM actin was found in the CSL primordia of both sexes, but this expression started to show a distinct sex-specific pattern betweenE18 and E19. From E19 onwards, the α-SM actin positive cells of the female CSL started to express desmin, which could not be demonstrated in the CSL of male fetuses. Such a sequence of first expression of smooth muscle markers is a common observation in smooth muscle differentiation, also demonstrated in prostate and seminal vesicle (Hayward *et al.*, 1996) Since desmin expression was first detected in prostate at postnatal day 5 and in seminal vesicle at postnatal day 10, smooth muscle differentiation in the CSL of female rats is an early event compared to muscle differentiation in prostate and seminal vesicle. The relative absence of smooth muscle cells in the CSL of male fetuses, as compared to the female, indicates that androgens suppress smooth muscle cell differentiation in the CSL. Mitotic activity and apoptosis of the cells in the cranial mesentery was studied, to investigate whether the difference in CSL development between the sexes was accompanied by a difference in cell kinetic characteristics of the cranial mesentery. However, no such a sex difference was detected. Gonadal germ cells in both sexes demonstrated cell degeneration and proliferation at E17 and E18, as reported by others (Hilscher *et al.*, 1974; Coucouvanis *et al.*, 1993). Although no sex difference in cell kinetics was detected, the cranial mesenteric tissue of the male fetus at E18 appeared more loosely structured compared to the female cranial mesentery with differentiating CSL. Furthermore, SEM images already showed a sex difference in cranial mesentery appearance at E17, when the sex difference in CSL differentiation could not yet be detected by immunohistochemistry. This could indicate changes in cell density, cell size, and/or extracellular matrix (ECM) between the male and female cranial mesentery. In general, the ECM plays an important role in morphogenesis (Hay, 1991). The ECM consists of different types of macromolecules including collagen, fibronectin and laminin, which can specifically bind to receptors at the cell surface. Cell growth and differentiation can by affected by cell-ECM interactions, also because the ECM can bind or activate peptide growth/differentiation factors such as TGFß, that in turn react with the cell. The ECM controls cell differentiation and morphogenesis, and can exert a direct effect on gene expression (Streuli *et al.*, 1991). Complex interaction between ECM, androgens and growth factors is involved in the control of prostate and mammary gland differentiation

(Streuli *et al.*, 1991; Kooistra *et al.*, 1995). A similar complex interaction may play a role in the control of the development of the cranial mesentery, containing the CSL primordium.

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Targeted disruption
of the *Insl3* gene causes
bilateral cryptorchidism

Targeted disruption of the *Insl3* gene causes bilateral cryptorchidism

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Summary

The sexual dimorphic position of the gonads in mammals is dependent on differential development of two ligaments, the cranial suspensory ligament (CSL) and the gubernaculum. During male embryogenesis, outgrowth of the gubernaculum and regression of the CSL result in transabdominal descent of the testes, whereas in the female, development of the CSL in conjunction with failure of the gubernaculum development holds the ovaries in a position lateral to the kidneys. Several lines of evidence suggest that regression of the CSL and induction of gubernaculum development are mediated by testosterone and a yet unidentified testicular factor, respectively. The *Insl3* gene (originally designated *Ley I-L*), a member of the insulin-like superfamily, is specifically expressed in Leydig cells of the fetal and postnatal testis, and in theca cells of the postnatal ovary. Here we show that male mice homozygous for a targeted deletion of the *Insl3* locus exhibit bilateral cryptorchidism with free moving testes and genital ducts. These malformations are due to failure of gubernaculum development during embryogenesis. In double mutant male mice for *Insl3* and *androgen receptor* genes, testes are positioned adjacent to the kidneys and steadied in the abdomen by the CSL. These findings demonstrate, that the Insl3 induces gubernaculum development in an androgen independent way, while androgen mediated regression of the CSL occurs independently from Insl3.

INTRODUCTION

Since the appearance of Jost's theory that male sexual differentiation in eutherian mammals is regulated by two fetal testicular hormones (Jost, 1953), several lines of evidence have demonstrated the role of anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance, from the Sertoli cells for the regression of Müllerian ducts and of androgen from the Leydig cells for the differentiation of Wolffian ducts into vas deferens, epididymis and accessory glands. In female fetuses, the absence of AMH and androgen lead to the development of the Müllerian ducts derivatives and the passive regression of the Wolffian ducts. However, the molecular mechanism underlying the sexual dimorphic position of the gonads in mammals was not included in Jost's theory and has received limited attention, although the different position of ovary and testis is of utmost importance for fertility. Over the last century, numerous theories have been proposed to explain the process of testis descent. Controversies between these theories are often concerned in the targeted structures and factors, which are involved in this process (van der Schoot and Emmen, 1996; Hutson *et al.*, 1997).

The genital mesentery of the internal genital tract is a retroperitoneal structure that connects the gonads and genital ducts to the abdominal wall. The differential development of two parts of the genital mesentery, the cranial suspensory ligament (CSL) and the caudal genital ligament also called gubernaculum, during male and female development has been determined and proposed to be responsible for a sexual dimorphic position of testis and ovary (van der Schoot and Emmen, 1996; Hutson *et al.*, 1997). In mammals, the process of testis descent has been divided into two functional phases (Hutson *et al.*, 1997). During first or transabdominal phase, occurring in the mouse development between E15.5 and E17.5, the development of the gubernaculum and regression of the CSL result in the transabdominal movement of the testis into the inguinal region. In the female embryo, development of the CSL and developmental impairment of the gubernaculum keep the ovary near the kidney (Figure 3.1). During the second or inguinoscrotal phase of testis descent, occurring in the mouse between postnatal weeks 2 and 3, the testis descends from the inguinal region to the scrotum and the gubernaculum is inverting or regressing.

To date, a substantial amount of literature data has accumulated to indicate the relevance of testicular factors in the differential growth of both ligaments during male embryogenesis. The prevention of outgrowth of the fetal CSL is an androgen-dependent process. Prenatal exposure of females to androgen prevents development of the CSL (Emmen *et al.*, 1998). The persistence of the CSL in the complete androgen-insensitive male mice that contain a mutation in the gene encoding the androgen receptor (*Ar*), and in male rats, which were exposed to anti-androgen during fetal life, further supports the role of androgen in suppression of the CSL development (Hutson, 1986; He *et al.*, 1991; van der Schoot and Elger, 1992). Development of a male-like gubernaculum in the bovine freemartin, a female fetus exposing to the blood of a male twin by chorioallantoic anastomosis, and in female rabbit fetus that has been grafted with a fetal testis, demonstrated the participation of fetal testicular factors in the gubernaculum development (Lillie, 1917; van der Schoot, 1993a; van der Schoot *et al.*, 1995). The fact that a proportion of human males with persist-
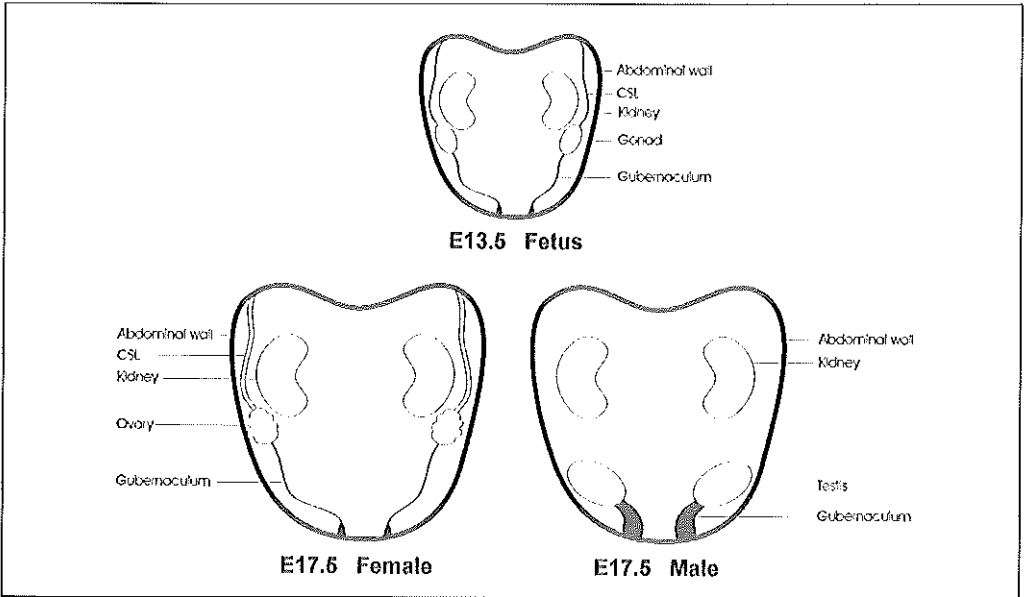
Figure 3.1 - Schematic representation of differential development of the cranial suspensory ligament (CSL) and the gubernaculum during sexual differentiation

At E13.5, position of the developed gonad is identical in male and female fetuses and attached to the abdominal wall by the CSL and the gubernaculum. Between E15.5 and E17.5, development of the gubernaculum and regression of the CSL in the male embryo result in the transabdominal descent of the testis into the inguinal region. In the female embryo, further development of the CSL and development impairment of the gubernaculum lead to sustain the ovary near the kidney.

ent Müllerian duct syndrome (PMDS), and human males with complete androgen insensitivity syndrome have undescended testes, suggested a potential role of both AMH and androgen in testicular descent (Cate *et al.*, 1990; Hutson *et al.*, 1997). However, recent data showed a normal testicular descent in the homozygous *AMH* and *AMH* type II receptor mutant mice and the outgrowth of the gubernaculum in male mice with testicular feminization rule out the direct action of AMH and androgen in induction of gubernaculum outgrowth during the transabdominal descent of the testis (Behringer *et al.*, 1994; Hutson, 1986; Mishina *et al.*, 1996). These data suggested a role of a third fetal testicular factor in gubernaculum development (Fentener van Vlissingen *et al.*, 1988; van der Schoot, 1993a; Visser and Heyns, 1995).

We have previously characterized a novel member of the insulin-like hormone superfamily, *Insl3*, which is specifically expressed in Leydig cells of the fetal and adult testis and in the theca cells of the postnatal ovary (Adham *et al.*, 1993; Roche *et al.*, 1996). The *Insl3* gene is expressed at high levels in the adult testis and at much lower levels in the adult ovary. Analyses of *Insl3* transcripts in testis and ovary throughout the pre- and postnatal life of the mouse revealed a sexual dimorphic pattern of *Insl3* expression during development. No *Insl3* transcripts were detected in female embryos of any stage, whereas in male embryos transcripts were first detected at E13.5. After birth, the level of *Insl3* transcription in testis

remains constant during the first 3 weeks, increases at the time at which the first wave of round spermatids undergoes spermiogenesis, and reaches the highest level in adult testis (Zimmermann *et al.*, 1997). These results lead us to suggest that the Insl3 factor plays an essential role in differentiation and maintenance of the male phenotype, and spermatogenesis (Adham *et al.*, 1993; Zimmermann *et al.*, 1997). In the female, expression of the *Insl3* is first detected in the ovary at day 6 after birth. This, taken together with the distinct expression pattern of *Insl3* during the estrous cycle and pregnancy, implies a functional role of Insl3 during follicular development (Zimmermann *et al.*, 1997).

To determine the role of Insl3 in sexual differentiation and gametogenesis, we have generated mice containing a targeted disruption of the *Insl3* gene. Morphological abnormalities were only observed in male *Insl3$^{-/-}$* mice, which exhibited bilateral cryptorchid testes located high in the abdomen. To investigate the role of Insl3 in the process of the testis descent, we have histologically analyzed gubernaculum development during transabdominal descent of the testis in the wild-type and the *Insl3* mutant males. To address the question whether the function of androgen and Insl3 in the development of CSL and gubernaculum is independent, we have generated double mutant male mice in which the action of both factors is eliminated. Finally, we have surgically descended the testis of the *Insl3$^{-/-}$* mice in the inguinal canal to determine the role of Insl3 for male germ cell development.

MATERIAL AND METHODS

Construction of the Targeting Vector

The *Insl3*-targeting vector was constructed by using the plasmid pPNT (provided by Dr. R. Mulligan). A 7.5 kb *SstI* fragment containing a 3' flanking region of the *Insl3* gene was isolated and ligated with the *XbaI/EcoRI* digested pPNT vector after filling the end with Klenow enzyme (clone *Insl3/1*) (Tybulewicz *et al.*, 1991; Zimmermann *et al.*, 1997). Finally, the 2.0 kb *SalI/XhoI* fragment (*Sa/I* site from polylinker of phage clone) containing a 5' flanking region of the *Insl3* gene was isolated and inserted in the *XhoI* digested clone *Insl3/1* by blunt end ligation. The resulting 16.5 kb targeting vector was linearized with *NotI* before electroporation.

ES Cell Culture, Generation of Chimeric Mice and Screening

The ES cell line MPI (provided by Dr. P. Gruss) was cultured as described (Wurst and Joyner, 1993). Confluent plates were washed in PBS buffer, trypsinized and the cells were suspended in the same buffer at $2x10^7$/ml. Aliquots of this cell suspension were mixed with 30 µg of linearized targeted vector and electroporated at 250 V and 500 µF using a Bio-Rad Gene Pulser apparatus. Cells were plated into nonselective medium in the presence of G418-resistant embryonic mouse fibroblasts. Thirty-six h later, selection was applied using medium containing G418 at 350 µg/ml and gancyclovir at 2 µM. After 10 days of selection, individual drug-resistant clones were picked into 24-well trays. Three days later,
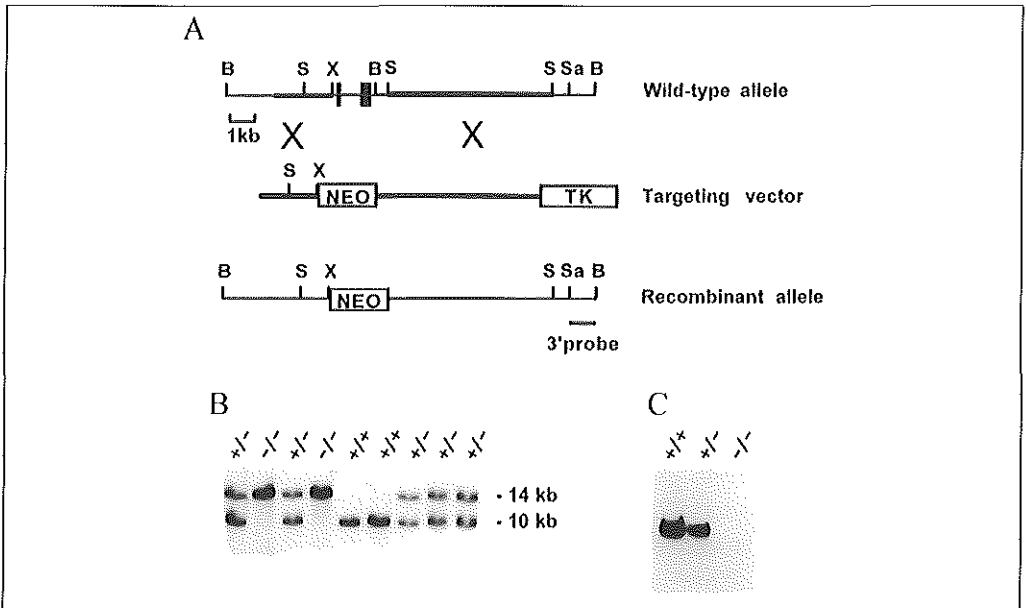
Figure 3.2 - Targeted disruption of *Insl3* gene, and RNA and DNA analysis of generated mice
(A) Structure of the wild-type allele, targeting vector, and targeted allele are shown together with the relevant restriction sites. A 1.8 kb *XhoI-SstI* fragment containing both exons of the gene was replaced by a *pgk-neo* selection cassette (NEO). TK, *Thymidine kinase* cassette; B, *BamHI*; S, *SstI*; Sa, *SalI*; X, *XhoI*. *(B)* Southern blotting of *BamHI* digested DNA from F_2 mice, hybridized with a 3′ external probe, revealing a 10 kb wild-type and a 14 kb mutated fragment. *(C)* Northern blotting of testicular RNA from *Insl3*$^{+/+}$, *Insl3*$^{+/-}$ and *Insl3*$^{-/-}$ adult mice, hybridized with the mouse *Insl3* cDNA probe, revealing a 0.9 kb mRNA prominent in *Insl3*$^{+/+}$, reduced in *Insl3*$^{+/-}$ and absent in *Insl3*$^{-/-}$ testes.

individual recombinant ES clones were replicated into 24-well trays for freezing and isolation of DNA.

Genomic DNA was extracted from ES cells, digested with *BamHI*, electrophoresed, and blotted onto Hybond N+ membranes (Amersham). The blots were hybridized with ^{32}P-labeled 1.3 kb *SalI/BamHI* fragment (Figure 3.2A) To confirm a correct homologous recombination event of the targeted *Insl3* gene and absence of additional random integration of targeted construct, a neomycin fragment was used to probe Southern blots. Hybridization was carried out at 65°C overnight in the following solution: 5x SSC/5x Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice at 65°C to final stringency at 0.2x SSC/0.1% SDS.

Chimeric mice from ES cells carrying the disrupted *Insl3* allele were generated by aggregating 10-15 compact ES cells with 2.5 day-old embryo of the CD1 mouse strain as described (Nagy and Rossant, 1993). Chimeric animals obtained were mated to CD1 or 129/Sv partner, and F_1 agouti offspring were genotyped by Southern blot analysis. Heterozygous animals were crossed to obtain homozygous mice, which were genotyped by Southern and PCR analyses. PCR was performed according to standard protocols to discriminate wild-type and mutant alleles in the DNA from the mouse tails and from the head of

embryos. Primer sequences were as follows: 1 (*Insl3* sense), 5'-CCGCACCTGGGAGAG-GACTTC; 2 (*Insl3* antisense), 5'-GTTATCCACGCTTGTCCAACC; 3 (*Pgk* antisense), 5'-TTCCATTGCTCAGCGGTG CTG. Thermal cycling was carried out for 30 cycles, denaturation at 94°C for 1 min., annealing at 58°C for 1 min., and extension at 72°C for 1 min. Animal studies were conducted in accordance with The Endocrine Society Guideline for the Care and Use of Experimental Animals.

RNA Analysis

Total RNA was extracted from testes of 12-week-old mice using the RNA now Kit (ITC Biotechnologies) according to the manufacturer's recommendation. The RNA was size fractionated by electrophoresis on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled *Insl3* cDNA fragment under the same conditions as used for Southern blot hybridization (Zimmermann *et al.*, 1997).

Generation of *Ar/Y Insl3$^{-/-}$* Mutant Mice

To generate *Ar/Y Insl3$^{-/-}$* double mutant mice, females *Ta Ar/$^{++}$*, which have *tabby* variegated coats owing to X chromosome inactivation, were mated with *Insl3$^{+/-}$* males. Females *Ta Ar/$^{++}$ Insl3$^{+/-}$* in the progeny were then crossed with *Insl3$^{+/-}$* males. *Ta Ar/Y Insl3$^{-/-}$* mice, which were phenotypic females with tabby coat, were identified by an *Insl3-* and a *Zfy*-specific PCR-based assay (Sah *et al.*, 1995).

Histological Analysis

Embryos (E15.5 and E17.5) were collected in PBS, fixed in Bouin's fixative, embedded in paraffin, sectioned at 6 μm and stained with haematoxylin-eosin. Testes from 5- and 15-day-old and 12-week-old mice were fixed with 5% glutaraldehyde in 0.2 M phosphate buffer, post fixed with 2% osmium tetroxide and embedded in epoxy (Epon) resin. Sections at 1 μm were stained with 1% toluidine blue/pyronine.

Scanning Electron Microscopy

After preserving material for genotyping, the abdominal cavity of the E17.5 was opened and the gastrointestinal tract and the urinary bladder were removed. After fixation by immersion in 1.5% glutaraldehyde in Locke's solution for 12 h, dehydration in a graded ethanol series, the embryos were critical point dried using ethanol as the transitional and CO_2 as the exchange fluid. The dried specimens were mounted with conducting silver and sputtered with gold-palladium to a layer of about 40 nm. Specimens were examined and photographed in a Zeiss DSM 960 scanning electron microscope.

Surgical Transplantation of the Cryptorchid Testis into the Inguinal Canal (Orchiopexy)

After anaesthesia of 3-week-old *Insl3$^{-/-}$* males, the abdominal cavity was opened by a 4 mm long transversal incision immediately below the umbilicus. The testicular artery was cut and

the testes were mobilized, brought down and steadied into the inguinal canal by suturing its capsule to peritoneum. These testes retained sufficient vascularity from collateral blood flow through the deferential artery.

RESULTS

Generation of Insl3-Deficient Mice

To elucidate the potential role of the *Insl3* gene, we deleted the gene in mice through homologous recombination. A replacement targeting vector was designed to delete the two exons encoding the Insl3 factor and replaced them with the neomycin phosphotransferase *(neo)* gene under the control of the phosphoglycerate kinase promoter. Introduction of a negative selection marker, the herpes simplex virus thymidine kinase *(tk)* gene, at the 3'end of the construct (Figure 3.2A) enabled us to use positive and negative selection (Mansour *et al.*, 1988).

MP1 ES cells were transfected with the targeting vector and selected for homologous recombination events (Voss *et al.*, 1997). Drug-resistant clones were picked, and DNA was isolated and screened by Southern blot analysis using an external probe (data not shown). Three recombinant clones had undergone homologous recombination. One clone produced germ line transmitting chimeras after aggreggation with morula derived from CD1 females. These chimeras were bred with CD1 and 129/Sv females to establish the *Insl3* deleted allele on a CD1 X 129/Sv hybrid and a 129/Sv inbred genetic background. Southern blot analysis on DNA isolated from tail biopsies was used to determine the genotype of the offspring. Hybridization with the 3'external probe (Figure 3.2A) visualized a 10 kb *BamHI* fragment in the case of a wild-type allele and a 14 kb fragment for a targeted allele (Figure 3.2B). Both male and female mice heterozygous for the *Insl3* mutation appeared normal and fertile. Heterozygous animals were mated, and approximately 25% (76 of 302) of the offspring were homozygous for the null allele. Northern blot analysis of RNA derived from testes of these mice revealed that the *Insl3*$^{-/-}$ mice failed to produce detectable *Insl3* mRNA (Figure 3.2C). These results confirm that the introduced mutation results in a complete loss of *Insl3* mRNA in testis of *Insl3*$^{-/-}$ mice. The phenotypes associated with the homozygous mutation that are described below were on a mixed (CD1 X 129/Sv) genetic background, but were not different from that on an inbred (129/Sv) genetic background.

Insl3 Homozygous Mutant Male Mice Are Sterile and Have Bilateral Cryptorchidism

The pattern of the *Insl3* expression in ovaries at various stages of the estrous cycle and during pregnancy showed a correlation with follicular development (Zimmermann *et al.*, 1997). However, homozygous mutant females underwent normal estrous cycles, as indicated by the cytology of vaginal smears, and after mating with wild-type or heterozygous male mice, they became pregnant and produced litters of normal size (9.1 ± 0.6 (n=18)
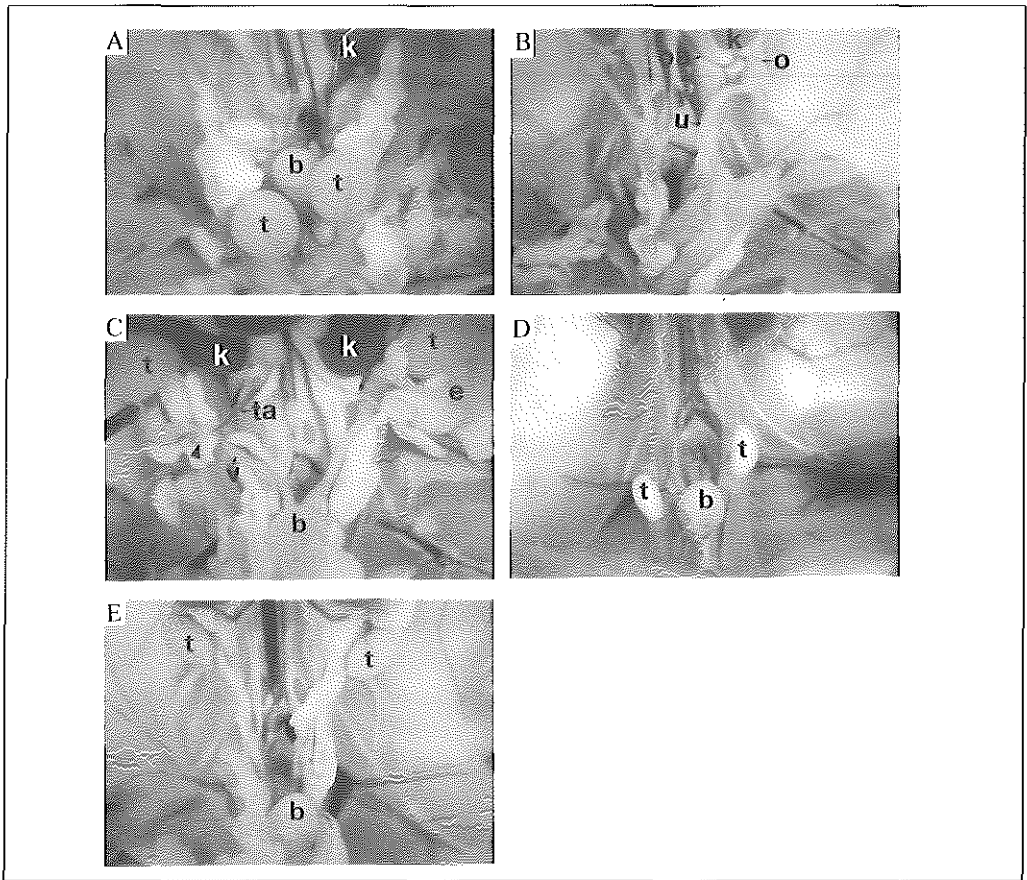
Figure 3.3 - Testicular position in _Insl3⁻/⁻_, _Ar_/Y and _Ar_/Y _Insl3⁻/⁻_ male mice. _(For a color version of this figure, see page 161.)_
_(A)_ Dissected abdominal region of a 4-week-old wild-type mouse shows the testes (t), which were already descended into the scrotal sac, adjacent to the bladder (b). _(B)_ Genital tract of a 4-week-old wild-type female shows the position of ovaries (o) adjacent to the kidneys (k), uterine horns (u) and ovarian arteries (oa). _(C)_ Free moving genital tract in the abdomen of 4-week-old Insl3-deficient male. The Wolffian duct derivatives are normally differentiated into epididymis (e), vas deferens (v) and accessory glands (not shown). Note the torsion (arrowhead) of the vas deferens and testicular artery (ta). _(D)_ Testes of 3-week-old _Ar_/Y mouse located above the bladder and attached to the abdominal wall with cranial suspensory ligament and gubernaculum. _(E)_ Testes of 4-week-old _Ar_/Y _Insl3⁻/⁻_ male situated adjacent to the kidneys in a comparable position as ovaries in wild-type mouse (B).

vs.9.8 ± 0.9 (n=14) control females). Normal folliculogenesis was observed in the ovaries of the Insl3-deficient females (data not shown), suggesting that the Insl3 factor is not essential for female germ cell development or folliculogenesis.

Morphological abnormalities were only observed in male _Insl3⁻/⁻_ mice, which were infertile despite normal sexual behavior towards female mice and production of copulation plugs. Anatomical examination of the male _Insl3⁻/⁻_ mice revealed that the Wolffian duct derivatives had differentiated normally into vas deferens, epididymis and accessory glands

and no Müllerian duct derivatives were present (Figure 3.3A,C). However, all $Insl3^{-/-}$ males exhibited bilateral cryptorchid testes located high in the abdomen (Figure 3.3A,C). The testicular arteries arose from the abdominal aorta below the renal arteries and ran just below the kidneys in an ovarian vasculature-like fashion. No tight attachment of the testis and epididymis to the inguinal region was found, similar as in normal female mice. Therefore, gubernaculum development could be affected in these mutant mice. Torsion of the vas deferens and testicular artery and localization of the right testis in the contralateral position did occur in some $Insl3^{-/-}$ mice, presumably due to absence of tight attachment of the testes to the inguinal region in combination with regression of the CSL.

Roles of Androgen and Insl3 in Development of the CSL and the Gubernaculum Are Independent

The structural abnormalities contributing to cryptorchidism in $Insl3^{-/-}$ male mice are different from those observed in male mice with testicular feminization. The development of CSL and gubernaculum in Ar/Y mice disrupts normal testis descent. Consequently, testes of these mice are located at an intermediate position of ovaries and testes in wild-type mice (Figure 3.3D). To address the question whether the function of androgen and Insl3 in the development of both ligaments is independent, we have generated double mutant male mice in which the action of both factors is eliminated. Testes in Ar/Y $Insl3^{-/-}$ mice are completely undescended (Figure 3.3E) and tight attachment of the testes to the inguinal region is absent. The male external and internal genitalia are not virilized, and Müllerian and Wolffian duct derivatives are absent. In contrast to $Insl3^{-/-}$ mutant mice, the testes are situated adjacent to the kidney in a comparable position as ovaries in wild-type mice (Figure 3.3B,E), and attached to the dorsal abdominal wall via well-developed CSLs.

Insl3 Is Required for Normal Development of the Gubernaculum during Transabdominal Descent of the Testis

The transabdominal descent of the testis coincides with the regression of the CSL, the shortening of the gubernacular cord and the outgrowth of the gubernacular bulb including the differentiation of its outer mesenchymal layer into myoblasts (Radhakrishnan *et al.*, 1979; Wensing, 1988; Emmen *et al.*, 1998). Analysis of E17.5 wild-type males by scanning electron microscopy reveals that the gubernaculum shows swelling (Figure 3.4A), whereas the gubernaculum of the $Insl3^{-/-}$ male and control female displays a small bulb and an elongated cord (Figure 3.4B, C). In order to investigate whether the cryptorchidism found in the $Insl3^{-/-}$ male mice may result from an affected development of the gubernaculum, we have analyzed transverse sections from fetuses at stages prior (E15.5) and during (E17.5) the transabdominal descent of the testes. At E15.5, the gubernacular bulb of wild-type males and females and $Insl3^{-/-}$ males is similar in size and contains loose mesenchymal cells (data not shown). At E17.5, the gubernacular bulb in wild-type males is enlarged and well developed into mesenchyme in the center and myoblasts in circumferential layers (Figure 3.4D). In contrast, the gubernacular bulb in the $Insl3^{-/-}$ males and in the wild-type females is poorly developed as indicated by the lack of structural organization into outer and inner
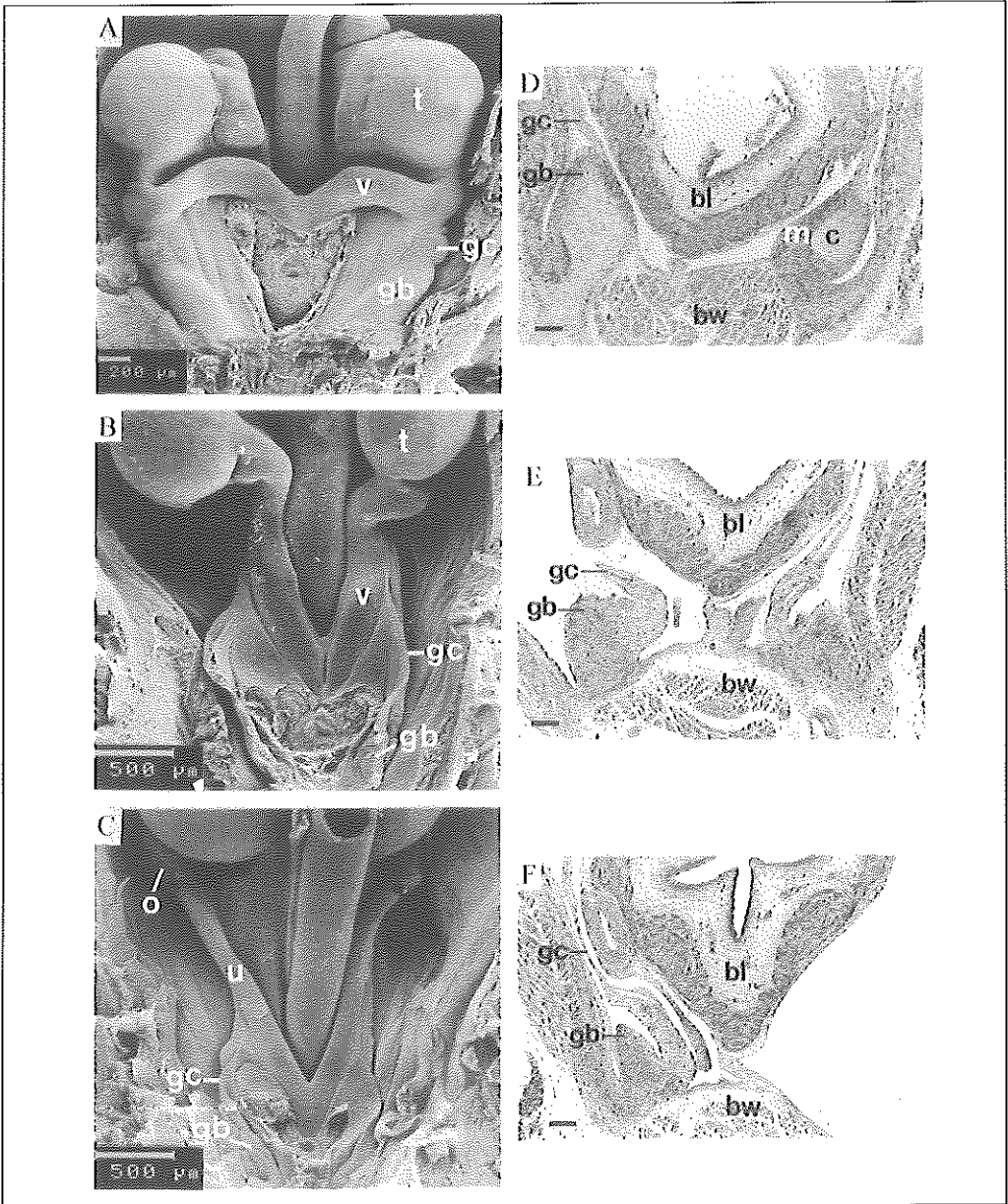
Figure 3.4 - Impaired gubernaculum development in E17.5 *Insl3*$^{-/-}$ male
(A-C) Scanning electron microscopy of the reproductive tract revealing a swollen gubernaculum in control male (wild-type or *Insl3*$^{+/-}$) (A), and thin, elongated gubernaculum in *Insl3*$^{-/-}$ male *(B)* and control female *(C)*. *(D-E)* Histological analysis of inguinal abdomen at E17.5 shows a well-developed gubernacular bulb in control male *(D)*, as indicated by marked differentiation into a mesenchymal core surrounded by a muscular outerlayer; whereas in *Insl3*$^{-/-}$ male *(E)* and control female *(F)* the gubernacular bulb is undifferentiated. bl, bladder; bw, abdominal body wall; c, mesenchymal core; gb, gubernacular bulb; gc, gubernacular cord; m, myogenic outer layer; o, ovary; t, testis; u, uterus; v, vas deferens. Scale bar: D-F, 100 μm.
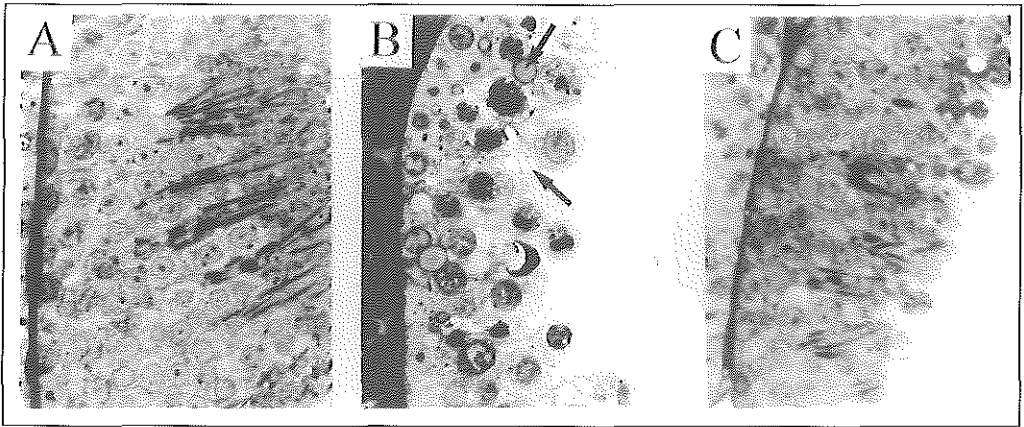
Figure 3.5 - Spermatogenesis in cryptorchid and surgically descended testes of *Insl3*-/- mice
(A) Histology of a descended testis from a 3-month-old adult control shows normal spermatogenesis.
(B) Section through a seminiferous tubule of cryptorchid testis from a 3- month-old *Insl3*-/- male
revealing karyolysis of pachytene spermatocytes and the absence of spermatids or spermatozoa.
Sertoli cells contain lipid-filled or empty vacuoles (arrows). *(C)* Three months after operation, the
descended testis from an *Insl3*-/- male shows normal spermatogenesis and presence of mature sper-
matids in most of the seminiferous tubules. Magnification: A- C, X 490.

layer (Figure 3.4E, F). These observations suggest that Insl3 stimulates the gubernaculum
development in male mice.

Insl3 Is Not Essential in Male Germ Cell Development

Histological analyses of the testes of 3-month-old *Insl3*-/- mice revealed abnormal sper-
matogenesis (Figure 3.5A, B). All seminiferous tubules showed a reduced number of sper-
matogonia, karyolysis of most of the primary spermatocytes and vacuolization of Sertoli
cell cytoplasm. Most notably, there was a complete absence of post-meiotic cells such as
spermatids and spermatozoa. Electron microscopy documented a normal appearance of
Leydig cells (data not shown). Furthermore, the testes of *Insl3*-/- mice at 5 days of age
showed an intact tubular structure with normal development of Sertoli and spermatogenic
cells (Figure 3.6A, B). Clear signs of germ cell depletion were observed at two weeks of age
when the first wave of spermatogenic cells undergoes the meiotic divisions (Figure 3.6C,
D). The fact that there is an increase in the testicular expression level of the *Insl3* after
the third week of postnatal development raises the question whether germ cell depletion
reflects a primary or a secondary defect. The cryptorchid testes of 3-week-old *Insl3*-/- mice
were surgically displaced and fixed in the inguinal canal. Three months after the opera-
tion, mating of four *Insl3*-/- male mice with wild-type females failed to produce any off-
spring, and no spermatozoa were detected in uteri of the females possessing vaginal plugs.
Histological examinations of the surgical descended testes of the *Insl3*-/- mice revealed
occurrence of normal spermatogenesis in most seminiferous tubules (Figure 3.5C) and
the presence of sperm in the epididymis (data not shown). Taken together, these observa-
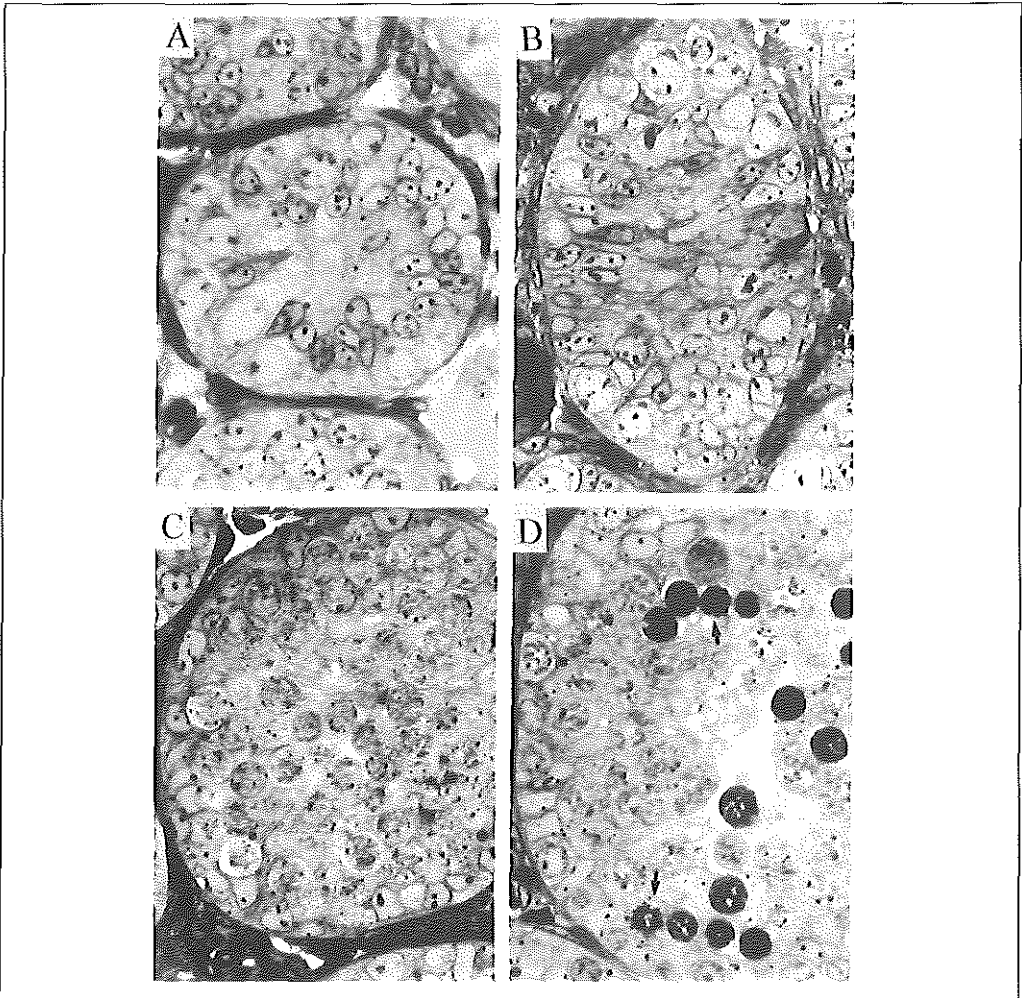
Figure 3.6 - Testes histology of wild-type and *Insl3*$^{-/-}$ mice at 5- and 15-days postpartum
(A,B) Histology of a testis from a wild-type (A) and an *Insl3*$^{-/-}$ mouse (B) at 5-day-old showing the presence of immature Sertoli cells and spermatogonia. *(C,D)* Section through a testis from a wild-type (C) and an *Insl3*$^{-/-}$ mouse (D) at postnatal day 15 showing a few degenerated spermatocytes with condensed and darkly stained nuclei (arrow) in the center of a seminiferous tubule of *Insl3*$^{-/-}$ testis. Magnification: A-D, X800.

tions demonstrate that Insl3 is not essential for male germ cell development. The germ cell depletion in abdominal testes of *Insl3*$^{-/-}$ mice might be attributed to a higher testis temperature, which is known to affect spermatogenesis.

Discussion

To address the biological role of the Insl3 in sexual differentiation and fertility, we gener-

ated mice lacking the *Insl3* gene. All *Insl3⁻/⁻* male mice have bilateral cryptorchid testes and presumably as a consequence are infertile, whereas the *Insl3⁻/⁻* female mice are fertile. This striking phenotype was displayed in all *Insl3⁻/⁻* male mice regardless the genetic background. Thus, Insl3 plays an essential role in the process of testis descent.

Transabdominal descent of the testis from the posterior abdominal wall to the inguinal region occurs in the mouse during the fetal life as a result of outgrowth of the gubernaculum and regression of the CSL (van der Schoot and Emmen, 1996; Hutson *et al.*, 1997). Lack of gubernaculum development and localization of the testis adjacent to the kidney in E17.5 mutant males demonstrate that arrest of the testis descent in the *Insl3⁻/⁻* mice takes place during the transabdominal phase. Furthermore, a successful initiation of early stages of the transabdominal descent is evidenced by proliferation of the gubernacular bulb and the differentiation of its outer mesenchymal layer into myoblasts (Radhakrishnan *et al.*, 1979). Histological analysis of E17.5 male mutant showed the lack of structural organization of the gubernacular bulb into an outer layer of myoblasts and an inner mesenchymal layer in both E17.5 male mutant and control female. These observations and the absence of *Insl3* gene expression in female mice during fetal life suggest that Insl3 stimulates the outgrowth and differentiation of the primordium of the gubernaculum in male mice. Whether the *Insl3* exerts its role in gubernaculum development by direct signaling, through activation of downstream genes that are required for mesenchymal cell proliferation and development, remains to be determined.

The involvement of a third testicular hormone in testis descent has been described by several research groups (Fentener van Vlissingen *et al.*, 1988; van der Schoot, 1993a; Visser and Heyns, 1995). In an *in vitro* analysis of testicular hormone action on the pig fetal gubernaculum, AMH, inhibin or androgen could not stimulate the proliferation of gubernaculum cells (Fentener van Vlissingen *et al.*, 1988). Normal outgrowth of the gubernaculum in *Ar*/Y mice and full descended testes in the homozygous *AMH* and *AMH* type II receptor mutant mice (Hutson, 1986; Behringer *et al.*, 1994; Mishina *et al.*, 1996) support the idea that neither androgen nor AMH but a third testicular factor is involved in prenatal development of the gubernaculum. Both androgen and AMH are still potentially involved in postnatal regression/inversion of the gubernaculum during the inguino-scrotal phase (Lyet *et al.*, 1996). We hypothesize that the Insl3 factor is the so far unidentified testicular factor, which is specifically involved in gubernaculum development. Full virilization of the male external genitalia, normal differentiation of the Wolffian duct derivatives into vas deferens, epididymis and accessory glands and absence of Müllerian duct derivatives in *Insl3* deficient mice are a strong indication that failure of gubernaculum development in *Insl3* mutant male mice is not due to absence of androgen- and *AMH*- mediated activities during fetal life.

The ovary-like position of the testes in the *Ar*/Y *Insl3⁻/⁻* double mutant mice, which lack the androgen- and Insl3- mediated activities during prenatal development as is the situation in wild-type females, demonstrates that the testicular factors androgen and Insl3 are essential for the establishment of sexual dimorphic position of the gonads via regulation of CSL regression and gubernaculum development, respectively. Normal regression of the CSL in the male *Insl3* mutants indicates that the action of androgen on CSL regression

does not require *Insl3*. Furthermore, the development of the gubernaculum in male *Tfm*/Y mice, which lack androgen mediated activity, demonstrates that the function of Insl3 in gubernaculum development is independent from androgen.

Although the pattern of *Insl3* expression during postnatal development of testis and ovary showed a correlation with spermatogenesis and folliculogenesis (Zimmermann *et al.*, 1997), normal spermatogenesis and follicle development were observed in the surgical descended testes of *Insl3*$^{-/-}$ mice and in ovaries of Insl3-deficient mice, respectively. These results suggest that Insl3 is not essential for germ cell development. The germ cell depletion in abdominal testis of *Insl3*$^{-/-}$ mice might be attributed to the higher testis temperature, which is known to affect spermatogenesis (Nishimune *et al.*, 1978). The infertility of the *Insl3*$^{-/-}$ male mice with surgically descended testis may be due to anatomical alteration of the reproductive organs during the operation, which mechanically obstructed the transfer of the sperm along their normal pathway from the epididymis to the uteri of the female mice, which had a vaginal plug.

The insulin-like family ligands are structurally related to each other, mediate many of the biological effects on cellular metabolism, growth and differentiation through binding and activation of their receptors, which are also structurally very similar (Blundell and Humbel, 1980; Ullrich *et al.*, 1986). It is known that insulin can bind to the insulin-like growth factor-1 receptor (IGF-1R), and the insulin-like growth factor-I and II (IGF-I and -II) to the insulin receptor (IR), albeit with lower affinities. The result of targeted mutagenesis of genes encoding members of insulin-like family ligands and receptors exhibit a growth deficiency in mouse embryos carrying a null mutation of the gene encoding IGF-I and II and IGF-1R, while mice homozygous for a null allele of the insulin-I and-II and insulin receptor are born with apparently normal intrauterine growth but die within hours after birth as a result of diabetic ketoacidosis (DeChiara *et al.*, 1990; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993; Accili *et al.*, 1996; Duvillie *et al.*, 1997). The striking phenotype of the *Insl3* mutant mice described suggests that the action of Insl3 on the gubernaculum development is specific and that other members of the insulin-like family do not compensate for the lack of the Insl3 during the fetal development of the male *Insl3*$^{-/-}$ mice. However, it remains to be investigated whether the action of *Insl3* on the gubernaculum development is mediated through an interaction with its own receptor, which has not yet been identified, or through crosstalk with other members of the insulin-like receptor family located in the gubernaculum primordia.

Cryptorchidism is the most common disorder of sexual differentiation in human males, with an incidence of 3.4% in the term newborn, decrease to 0.8% at 1 year of age. Severe complications of cryptorchidism are infertility and an increased risk for testicular malignancy (Elder, 1988). The complex process of testicular descent involves a series of hormonal and mechanical factors. Since the *INSL3* gene is also present in human genome (Burkhardt *et al.*, 1994a), INSL3 could be one of these factors and mutations in the gene encoding INSL3 could be a new etiology of cryptorchidism in humans.

Acknowledgements

Bilateral cryptorchidism is not associated with *Insl3* gene alterations

Bilateral cryptorchidism in the human is not associated with *INSL3* gene alterations

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Summary

Cryptorchidism is the most common disorder of male sex differentiation and various causative factors have been proposed, including impaired action of two testicular hormones, anti-Müllerian hormone (AMH) and testosterone. Recently, a third testicular factor, insulin-like factor3 (Insl3) has been shown to be involved in testis descent in mice. The aim of the present study was to investigate whether mutations in the *INSL3* gene might be associated with bilateral cryptorchidism in man.

Genomic DNA isolated from peripheral blood cells of 18 patients with bilateral cryptorchidism was analyzed for mutations in the *INSL3* gene. Two base pair substitutions were found; one base substitution (A→G) was a silent mutation in codon 18, whereas the other base substitution (A→G) resulted in a missense mutation at codon 36 (Ala→Thr). Both substitutions were also found in the *INSL3* gene of control individuals and are therefore considered to be polymorphisms. It can be concluded that mutations in the *INSL3* gene are not a frequent cause of bilateral cryptorchidism in man.

INTRODUCTION

Cryptorchidism, or failure of descent of the testis, is the most common disorder of sex differentiation in man and is closely associated with infertility and an increased risk of testicular malignancy (Frey and Rajfer, 1982; Swerdlow *et al.*, 1997). The incidence of cryptorchidism in boys at 3 months of age is 0.97 - 1.78 %, of which generally 15 % has bilateral cryptorchidism (Scorer, 1964; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Berkowitz *et al.*, 1993). Although in many patients the etiology of cryptorchidism cannot be fully explained, clinical and experimental evidence indicates that hormonal disturbances can result in undescended testes (Clarnette *et al.*, 1997).

Two hormones secreted by the fetal testis during male sex differentiation are anti-Müllerian hormone (AMH) and testosterone. Failure of action of either of these hormones is related to undescended testes, but until now their precise role in testis descent and the target structures involved have yet to be elucidated (Hutson *et al.*, 1997). Moreover, several investigators have suggested or confirmed involvement of a third testicular hormone in testis descent (Fentener van Vlissingen *et al.*, 1988; van der Schoot *et al.*, 1995; Visser and Heyns, 1995). It is commonly accepted that the gubernaculum, running between the testis and inguino-scrotal area, plays an important role in testis descent. Outgrowth of the gubernaculum during the transabdominal phase of testis descent is proposed to be regulated by the third testicular hormone.

In 1993, the cDNA encoding the porcine insulin-like factor 3 (Insl3) was cloned and this factor was identified as a new member of the insulin-like hormone superfamily (Adham *et al.*, 1993). Insl3 has also been named Leydig insulin-like (Ley I-L) or relaxin-like factor (RLF) (Ivell, 1997). The human gene encoding INSL3 (*INSL3*) was characterized and mapped to the region p13.2-p12 of chromosome 19 (Burkhardt *et al.*, 1994a). The *INSL3* gene is a single-copy gene, comprising only two exons with the single intron interposing within the open reading frame encoding the C (connecting) peptide. *INSL3* is predominantly expressed in the Leydig cells of the testis (Ivell *et al.*, 1997), but expression is also found in corpora lutea of the ovary and in trophoblast tissue (Tashima *et al.*, 1995). INSL3 probably functions as a paracrine factor in male and female reproductive tissues, but INSL3 is also present in human serum and may exert additional functions (Bullesbach *et al.*, 1999). All actions of Insl3 may involve binding to a membrane bound receptor (Bullesbach and Schwabe, 1999).

Analysis of *Insl3* gene expression in mice revealed that Insl3 is not only expressed in the prepubertal and adult gonads but also in the fetal testis (Zimmermann *et al.*, 1997). By creating *Insl3* knockout mice (*Insl3⁻/⁻* mice) through targeted deletion of the *Insl3* gene, the importance of Insl3 in male sex differentiation has been shown (Zimmermann *et al.*, 1999). Male *Insl3⁻/⁻* mice exhibit bilateral undescended testes in adulthood, associated with lack of gubernaculum development during the prenatal period. Both androgen and AMH action appears not to be affected in these mutant mice, since they are completely virilized and no Müllerian duct remnants could be identified (Zimmermann *et al.*, 1999). Similar findings were reported by Nef and Parada (1999).

It is hypothesized that mutations in the *INSL3* gene or its receptor might be an etiol-

ogy of bilateral cryptorchidism in humans. The aim of the present study was to investigate whether mutations in *INSL3* gene might also be associated with bilateral cryptorchidism in man.

MATERIALS AND METHODS

Patients

This study includes 18 male patients with bilateral cryptorchidism who underwent bilateral orchidopexy or had already undergone orchidopexy/orchiectomy up to several years before. There were 3 patients with prune-belly syndrome, including bilateral intra-abdominal testes, 12 patients had isolated bilateral intra-abdominal testes and 3 patients had bilateral impalpable testes of which the exact position was unknown. Of the 12 patients with isolated bilateral intra-abdominal testes, 2 were brothers with a cousin who also had the same anomaly but of whom no genomic DNA was available. The patients' protocol was approved by the Ethical Review Committee of the hospital, and informed consent was obtained from each patient or his parents. Forty individuals without a history of cryptorchidism were included as control group.

DNA analysis

Genomic DNA was extracted from peripheral blood cells according to standard procedures and served as a template for the PCR (Miller *et al.*, 1988). Exons 1 and 2 of the *INSL3* gene, including the exon/intron boundaries, were individually amplified using different pairs of primers (Table 4.1), designed according to the published sequence of the *INSL3* gene (Burkhardt *et al.*, 1994a) (Genbank/EMBL Data Bank under accession number X73637).

Table 4.1
Sequence of primers used for screening of the human *INSL3* gene

Primer	Sequence	Annealing Temperature °C	Orientation	Location
Hly6F	5' GGTGACAGAGTGAACTCCATC 3'	55	forward	5' flanking region
Hly6R	5' AACTTCTCACGCATCTCTG 3'		reverse	
Ley1A	5' GTCCTGAAGAATGTTCTGTC 3'	58	forward	exon1
Ley1B	5' CACGATCTGTGCACGCAG 3'		reverse	
HlyF1	5' GTCCTGAAGAATGTTCTGTCC 3'	55	forward	exon1
HlyR1	5' TCATGCATGCAAACCTGC 3'		reverse	
Ley2A	5' GATTACAGGTGTAAGCCACTG 3'	62	forward	exon2
Ley2B	5' CCTCAGGAGCTCACCAGAC 3'		reverse	
HlyF5	5' GCATGTCCTCTGTCGTTTCCGTTC 3'	55	forward	exon2
HlyR5	5' ATTCTGCAGTTGACTCCACAG 3'		reverse	

The PCR mixture contained 100 ng genomic DNA, 200 ng of each primer, 200 μM of each dNTP, 1 mM MgCl$_2$, 10% DMSO and 5 units Super Taq DNA polymerase (Enzyme Technology, UK) in a final volume of 50 μl. The PCR program included 30 cycles of denaturation for 1 min at 95°C, primer annealing for 2 min at different temperatures (Table 4.1) and elongation for 1 min at 72°C. Aliquots of 10 μl PCR-amplified product were loaded onto a 2% agarose gel containing ethidium bromide and visualized under UV-light to confirm amplification. Template-free conditions were always included.

For mutation screening via direct sequencing, PCR-amplified products were first purified with the *High Pure* PCR product purification kit (Boehringer Mannheim, Germany) The sequencing reaction was conducted by using the Thermo Sequenase cycle sequencing kit version 2.0 (Amersham, UK) and one of the PCR primers as sequencing primer, according to the manufacturer's instructions. The cycling sequencing reaction included 25 cycles consisting of 30 s at 95°C, 15 s at 50°C, and 4 min at 60°C.

Restriction enzyme analysis

One base pair substitution abolished an *EagI* site. Restriction digestion with *EagI* was performed on 5 μl purified PCR product, and products were analyzed on a 2% agarose gel.

```
Position

  1  CGCCACCCACCACC AGT GAC CCC CGT CTG CCC GCC TGG GCG CTG GTG CTG CTG GGC CCT
                   Met Asp Pro Arg Leu Pro Ala Trp Ala Leu Val Leu Leu Gly Pro
                   -24                      Signal peptide


 60 GCC CTG GTG TTC GCG TTG GGC CCC│GCG CCC ACC CCA GAG ATG CGT GAG AAG TTG TGC
    Ala Leu Val Phe Ala Leu Gly Pro│Ala Pro Thr Pro Glu Met Arg Glu Lys Leu Cys
                               -1 │ 1                      B-chain

                              A
117 GGC CAC CAC TTC GTA CGC GCG CTG GTG CGC GTG TGC GGG GGC CCC CGC TGG TCC ACC
    Gly His His Phe Val Arg Ala Leu Val Arg Val Cys Gly Gly Pro Arg Trp Ser Thr
    11


                          A   o Thr
174 GAA GCC│AGG AGG CCT GCG GCC GGA GGC GAC C gtgagtggggacgggcagggacagcgctctggg
    Glu Ala│Arg Arg Pro Ala Ala Gly Gly Asp
    30     │   C-peptide        EagI
```

Figure 4.1 - Location of base pair substitutions in exon 1 of the *INSL3* gene found in patients with bilateral cryptorchidism and in control male individuals
The nucleotide sequence of exon 1 is shown in upper case and the intronic sequence in lowercase. The amino acid sequence of the signal peptide, B-chain and part of the C-peptide is also indicated, with the putative processing sites as vertical lines; numbering is according to Burkhardt et al. (1994a). The *EagI* consensus sequence abolished by the G→A substitution at position 192 is underlined (GGCCG). The silent mutation (G → A) at codon 18, and the missense mutation (G → A) resulting in the Ala → Thr substitution at codon 36 are highlighted in bold. An insertion of a guanine at position 214 is a correction on the previously published sequence.

Table 4.2
Evaluation of the missense mutation in the *EagI* site

	Homozygous	Heterozygous	Total
Controls			
40	5	19	24 (60 %)
(80 alleles)	(10 alleles)	(19 alleles)	(29 alleles , (36 %))
Patients			
18	3	6	9 (50 %)
(36 alleles)	(6 alleles)	(6 alleles)	(12 alleles , (33 %))

RESULTS

A total of 18 patients with bilateral cryptorchidism were screened for mutations in the *INSL3* gene. Sequence analysis revealed two base pair substitutions in exon 1, one silent and one missense mutation (Figure 4.1). The silent mutation was an adenine to guanine transition in codon 18. This base pair substitution was found in patients but also among controls. The missense mutation was a transition of base pair 192 ($G \rightarrow A$), resulting in a substitution of an alanine residue by a threonine residue encoded by codon 36. This mutation abolished an *EagI* restriction site, which is unique in this exon. By means of digestion of the PCR products of genomic DNA with *EagI*, the presence of this mutation in exon 1 of 18 patients and 40 control individuals was analyzed. The results of this analysis are shown in Table 4.2. The missense mutation was found in 9 out of 18 patients with cryptorchidism but also in 24 of the 40 control individuals. Since both the silent and the missense mutation were found in patients as well as in the controls, these base pair substitutions were considered to be polymorphisms. No mutations were found in exon 2.

DISCUSSION

By means of PCR followed by direct sequencing, two base pair substitutions in the *INSL3* gene were identified. The A140G mutation does not result in an amino acid change, since the codons CTG and CTA both encode leucine. However, the G192A mutation does lead to substitution of an alanine residue by a threonine residue in codon 36. The amino acid residue encoded by codon 36 is located in the C-peptide of INSL3 and is not well-conserved (Ivell, 1997). Like other members of the insulin-like hormone superfamily, INSL3 is synthesized as a precursor, containing a signal peptide, B-chain, connecting C-peptide, and A-chain (Adham *et al.*, 1999). It is expected that bioactive INSL3 is formed after enzymatic removal of the C-peptide, similar as for insulin and relaxin. Although the physiological significance of the C-peptide is not known, the G192A missense mutation seems to have no major consequences for the functionality of INSL3 since this change is also present within the normal population.

Three of the 18 investigated patients had prune-belly syndrome, which includes bilateral undescended testes, usually located in the abdomen (Wheatley *et al.*, 1996). Failure of testis descent in prune-belly patients has been related to inadequate abdominal pressure due to absence of abdominal wall musculature, but also to failure of gubernaculum development (Elder, 1987). However, Nunn and Douglas Stephens (1961) reported that the gubernaculum is elongated but its structure is normal. Although it is not evident whether affected gubernaculum development is one of the factors causing cryptorchidism in this syndrome, the cryptorchidism appears not to result from alterations in the *INSL3* gene.

Since all patients included in the present study were normally virilized, no defect in androgen action is expected. In addition, previous results from Wiener *et al.* (1998) suggest that mutations in the gene encoding the androgen receptor appear not be a causative factor of isolated cryptorchidism. There are also no indications of loss of AMH function in any of these patients, since no retention of Müllerian duct derivatives was discovered at surgery; such retention has been described by Josso *et al.* (1993).

That genetic factors might be involved in isolated cryptorchidism is indicated by the fact that testis maldescendent has been shown to involve familial and inherited factors (Czeizel *et al.*, 1981; Savion *et al.*, 1984). Two of the patients included in the present study group were brothers, but it is not known whether other patients had a family history of cryptorchidism. Genetic analysis of cryptorchidism has been recently performed by screening for mutations in the *HOXA-10* gene of cryptorchid boys (Kolon *et al.*, 1999). Possible involvement of HOXA-10 in testis descent is based on the observation that targeted disruption of the *hoxa-10* gene in mice results in uni- or bilateral undescended testes (Rijli *et al.*, 1995; Satokata *et al.*, 1995). Preliminary data indicate that mutation of the *HOXA-10* gene in humans might be related to undescended testes (Kolon *et al.*, 1999). Analysis of the *HOXA-10* gene in the present group of patients will be of interest.

After molecular analysis of the coding parts of the *INSL3* gene, two base pair substitions were found in both patients and control individuals and therefore considered to be frequent polymorphisms and not pathogenic mutations. Mutations in the *INSL3* gene might be extremely rare, like mutations in genes encoding gonadotropins and their receptors, since these mutations will be effectively eliminated from the genetic pool due to their adverse effect on fertility (Huhtaniemi *et al.*, 1999). In addition, either factors regulating *INSL3* gene expression or the activity of the unknown INSL3 receptor, or downstream factors in INSL3 signaling might be affected in patients with cryptorchidism. Although *INSL3* gene alterations appear not to be frequently involved in bilateral cryptorchidism in the human, impairment and/or dysregulation of INSL3 action cannot be excluded yet. There might be a difference in the role of INSL3 across species. As testis descent in man and large mammals differs from the process in rodents in several aspects, the role of regulatory factors such as INSL3 also may involve subtle or more essential species-specific mechanisms (Heyns and Hutson, 1995). Consequently, mutations in the *INSL3* gene in the human would lead to another phenotype.

Recently, INSL3 was detected in human serum and the circulating levels appeared to change at puberty (Bullesbach *et al.*, 1999). In children, the INSL3 serum level was almost identical in boys and girls. At puberty, the INSL3 level in boys started to increase, resulting

in a significantly higher level in postpubertal males then in females and children. Similarly, the concentration of testosterone in plasma increases at puberty in normal boys (Plant, 1994). Since both testosterone and INSL3 are produced by testicular Leydig cells, such a correlation between testosterone and INSL3 levels might reflect a coordinated control of their production. Experiments with Insl3-deficient mice demonstrated that Insl3 is involved in gubernaculum development during male sex differentiation (Zimmermann *et al.*, 1999; Nef and Parada, 1999). Although the Leydig cells continue to express *Insl3*, its subsequent function in the male in not known (Zimmermann *et al.*, 1997; Balvers *et al.*, 1998; Zimmermann *et al.*, 1999). Consistent with a role of Insl3 in testis descent is the finding that mice ovaries do not produce Insl3 until after birth (Zimmermann *et al.*, 1997). *Insl3* is expressed during the process of folliculogenesis, with higher levels of expression in the follicular than in the luteal phase, suggesting that Insl3 might be involved in follicle development. However, Insl3 does not seem to play a dominant role as Insl3-deficient females are fertile, although smaller litter sizes were observed (Nef and Parada, 1999). Although *Insl3* is also expressed in human ovary and trophoblast and can be measured in serum of postpubertal females, nothing is known about a possible function of Insl3 in the female (Tashima *et al.*, 1995; Bullesbach *et al.*, 1999).

It is anticipated that hormone action requires interaction of this ligand with a plasma membrane receptor. Recently, specific, high affinity INSL3 binding sites have been described (Bullesbach and Schwabe, 1999). Although nothing is known yet about ligand binding and activation of the INSL3 receptor, functional mutations in this receptor gene may result in impaired INSL3 action and probably also in cryptorchidism. When mutation of the INSL3 receptor would inactivate its signaling to a limited extent, this could result in milder forms of INSL3 dysfunction and another phenotype in patients carrying such a mutation. Cloning of the INSL3 receptor would provide a tool to study the molecular regulation of INSL3 signaling, and eventually lead to a better understanding of the specific effect of INSL3 in development and function of the reproductive tissues, including the gubernaculum.

Note
After this paper was submitted, a publication containing similar data appeared (Krausz *et al. Mol Hum Reprod* (2000)6: 289-302).

Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism

Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism

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Summary

Recently, it has been shown that targeted inactivation of the *Insl3* gene in male mice results in cryptorchidism. The *Insl3* gene encodes insulin-like factor 3 (Insl3), which is expressed in fetal Leydig cells. The testicular factor Insl3 appears to play an important role in the transabdominal phase of testis descent, which involves development of the gubernaculum. Other studies have demonstrated that in utero exposure to diethylstilbestrol (DES), a synthetic estrogen, can lead to cryptorchidism both in humans and in animal models.

The present study was undertaken to investigate whether prenatal DES-exposure might interfere with testicular *Insl3* mRNA expression. Furthermore, the effect of DES on steroidogenic factor 1 (*Sf1*) mRNA expression level was determined, since it has been shown that SF1 plays an essential role in transcriptional activation of the Insl3 gene promoter. Timed pregnant mice were treated with DES (100 µg/kg body weight) or vehicle alone on days E9 (gestational day 9) through E17. Control and DES-exposed mouse fetuses were collected at E16, E17 and E18, when transabdominal testis descent is taking place. Lack of gubernaculum development in DES-exposed animals was confirmed by histological analyses at E17. Expression of *Insl3* and *Sf1* mRNAs was studied in testes of control and DES-exposed fetuses at E16 and E18 by RNase protection assay. Prenatal DES-exposure resulted in a three-fold decrease in *Insl3* mRNA expression level ($P < 0.005$), at both E16 and E18. In contrast, DES treatment had no effect on the expression of *Sf1* mRNA. These results support our hypothesis that DES may interfere with gubernaculum development by altering *Insl3* mRNA expression, providing a possible mechanism by which DES may cause cryptorchidism.

INTRODUCTION

Diethylstilbestrol (DES) is a non-steroidal synthetic estrogen, which has been used as estrogen therapy to prevent abortion, mainly in the 1950s and 1960s (Newbold and McLachlan, 1996). The usage of DES was banned in 1971, when it was found that *in utero* exposure to diethylstilbestrol (DES) is associated with urogenital tract abnormalities in female and male offspring. One of the abnormalities found in DES-exposed male offspring was cryptorchidism.

A biphasic model for the hormonal regulation of testis descent has been proposed (Hutson, 1985). During the first or transabdominal phase, the testis moves from its initial position near the kidney to the abdominal bottom. In mice, this phase takes place between embryonic days E15.5 and E17.5 (Hadziselimovic *et al.*, 1980). The transabdominal movement of the testis is dependent on the differential development of two ligaments, the cranial suspensory ligament (CSL) which is running between the gonad and the diaphragm, and the gubernaculum, which connects the caudal pole of the testis to the bottom of the abdomen (van der Schoot and Emmen, 1996; Hutson *et al.*, 1997). During the transabdominal phase, the male gubernaculum is growing and differentiating whereas outgrowth of the CSL is lacking. It has been shown in animal models that exposure to exogenous estrogens, including DES, disrupts the first phase of testis descent, leading to maldescent (Raynaud, 1958; Hadziselimovic *et al.*, 1980; Shono *et al.*, 1996). It has been suggested that the cryptorchidism induced by DES is due to failure of gubernaculum development (Raynaud, 1958; Shono *et al.*, 1996). The mechanism by which estrogens inhibit gubernaculum development is poorly understood.

Insulin-like factor 3 (Insl3), also designated Leydig insulin-like factor (Ley I-L) or relaxin-like factor (RLF), belongs to the insulin-like hormone family (Ivell, 1997; Adham *et al.*, 2000). The *Insl3* gene is specifically expressed in pre- and postnatal Leydig cells of the testis and in postnatal theca cells of the ovary (Zimmermann *et al.*, 1997). Recently, *Insl3* knockout mice, homozygous for a targeted inactivation of the *Insl3* gene (*Insl3^{-/-}* mice), have been generated (Zimmermann *et al.*, 1999; Nef and Parada, 1999). As a conspicuous aspect of the phenotype, male *Insl3^{-/-}* mice show disturbed testis descent resulting in cryptorchidism. Histological analysis of male *Insl3^{-/-}* fetuses revealed that the development of the gubernaculum is severely affected. This indicates that Insl3 might be an important factor for gubernaculum development in the mouse, during the transabdominal phase of testis descent. The position of the gonads in neonatal male *Insl3^{-/-}* mice (Zimmermann *et al.*, 1999; Nef and Parada, 1999) is strikingly similar to that of neonatal male mice prenatally exposed to DES (Shono *et al.*, 1996); the testes are located high in the abdomen, near the lower poles of the kidneys and gubernaculum development is lacking. This led us to suggest that *Insl3* expression might be impaired in testes of DES-exposed male fetuses, thereby providing a mechanism for DES-induced testis maldescent.

Steroidogenic factor-1 (SF1) is an orphan nuclear receptor, which is essential for gonadal development and sex differentiation (Luo *et al.*, 1994). Both Sertoli and Leydig cells in the fetal testis express *Sf1* (Majdic *et al.*, 1997). It has been shown that SF1 plays an essential role in transcriptional activation of a number of genes, including an action on the *Insl3*

gene promoter (Zimmermann *et al.*, 1998). Thus, altered *Insl3* gene expression might be a consequence of a changed level of SF1.

We studied the effects of DES on transabdominal testis descent via histological analysis and determined whether DES alters *Insl3* and *Sf1* mRNA expression during this phase by RNase protection assay.

MATERIALS AND METHODS

Animals, treatment and collection of tissue

Adult mice (FVB strain) were housed under standard animal housing conditions in accordance with NIH Guide for the Care and Use of Laboratory Animals. To obtain timed pregnancies, female mice were placed in individual cages with male mice and the morning a vaginal plug was found was designated day 0 (E0) of pregnancy. Pregnant mice were injected subcutaneously on days E9 through E17, with diethylstilbestrol (DES; 100 µg/kg body weight; Janssen Chimica, Beerse, Belgium) in olive oil or with olive oil alone (controls). Pregnant mice were sacrificed on E16, E17 or E18 by cervical dislocation and fetuses were quickly removed. Fetuses at E16 and E18 were examined under a dissection microscope, and after establishing the position of the testes, these were isolated, frozen in liquid nitrogen and stored at -80°C until RNA isolation. E17 fetuses were used for histological analysis. These were fixed in 10% formalin and embedded in paraffin. Serial sections of 7 µm were cut and stained with hematoxylin-eosin.

RNA analysis

Total tissue RNA was isolated from pooled testes of 3 control or 3 DES-exposed fetuses using a modified guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987). For ribonuclease (RNase) protection assay (Baarends *et al.*, 1994), antisense cRNA probes were prepared by *in vitro* transcription using T7 RNA polymerase and [^{32}P]UTP (400 Ci/mmol; Amersham, Buckinghamshire, United Kingdom). A mouse *Insl3* cDNA fragment (Zimmermann *et al.*, 1997), a mouse *Sf1* cDNA fragment (Lala *et al.*, 1992) and a rat glyceraldehyde 3-phosphate dehydrogenase (*GAPD*) cDNA fragment were used as templates. An amount of 10 µg total RNA was hybridized with the purified labeled probes of GAPD and Insl3 or Sf1 (5 x 10^4 cpm each) for 16 h at 55°C. After hybridization, samples were treated with RNase-A/T1 mixture for 1 h at 30°C and phenol/chloroform extracted. After precipitation, samples were resuspended in formamide loading buffer, and run on acrylamide-urea denaturing gels. Quantification of protected RNA fragments was performed using PhosphorImage analysis (Molecular Dynamics, B & L systems, Zoetermeer, The Netherlands). The mRNA levels of *Insl3* and *Sf1* were normalized to *GAPD* to correct for differences in the amounts of RNA that were hybridized with the probes.

Statistics

Statistical analysis was performed by Student's two-tailed *t* test.
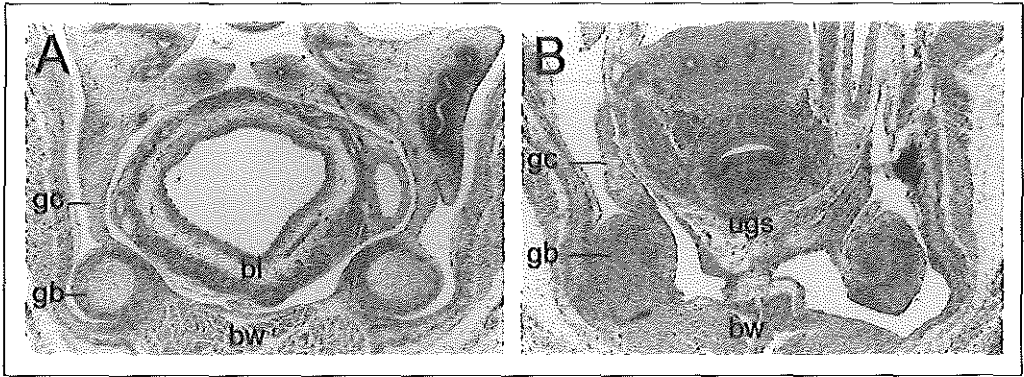
Figure 5. 1 - Inhibition of gubernaculum development by DES
Morphology of the lower abdomen of control and DES-treated fetuses at E17. In the control male fetus *(A)*, the gubernacular bulb is well-developed, with an inner mesenchymal core and muscular outerlayer. In contrast, in the DES-treated male fetus *(B)* the gubernaculum appears to be undifferentiated. bw, body wall; bl, bladder; gb, gubernacular bulb; gc, gubernacular cord; ugs, urogenital sinus.

RESULTS

Morphology

Male fetuses at E16 and E17 were examined under a dissection microscope to study the position of the testes, followed by dissection of the testes for RNA analysis. In control male fetuses at E16, the transabdominal descent of the testis was taking place; the testes were located well below the kidneys, just above the top of the bladder. However, in all DES-treated males at E16, the testes were found at a relatively high abdominal position, and in the majority of these animals (11/15), the testes were still located at the lower pole of the kidneys. At E18, the testes of control fetuses were located next to the bladder, at the bottom of the abdomen. In DES-treated male fetuses at the same fetal age, the testes were always positioned higher in the abdomen when compared to the controls, ranging from firm attachment to the posterior pole of the kidneys to a location well above the bladder.

Histological analysis of control and DES-treated fetuses at E17 showed an undifferentiated, female-like gubernaculum in DES-treated animals (Figure 5.1). The Müllerian ducts had almost completely regressed in the control fetuses, but were still present in the DES-exposed male fetuses (not shown).

RNase protection assay

The expression of *Insl3* and *Sf1* mRNAs in testes of control and DES-exposed fetuses at E16 and E18 was examined by RNase protection assay (Figure 5.2). PhosphorImage analysis of a representative RNase protection assay for *Insl3* and *GAPD* mRNAs is shown in Figure 5.2A. Quantitative analysis revealed that, at both E16 and E18, *Insl3* mRNA expression in testes of DES-exposed fetuses was decreased by 70% compared with controls, as
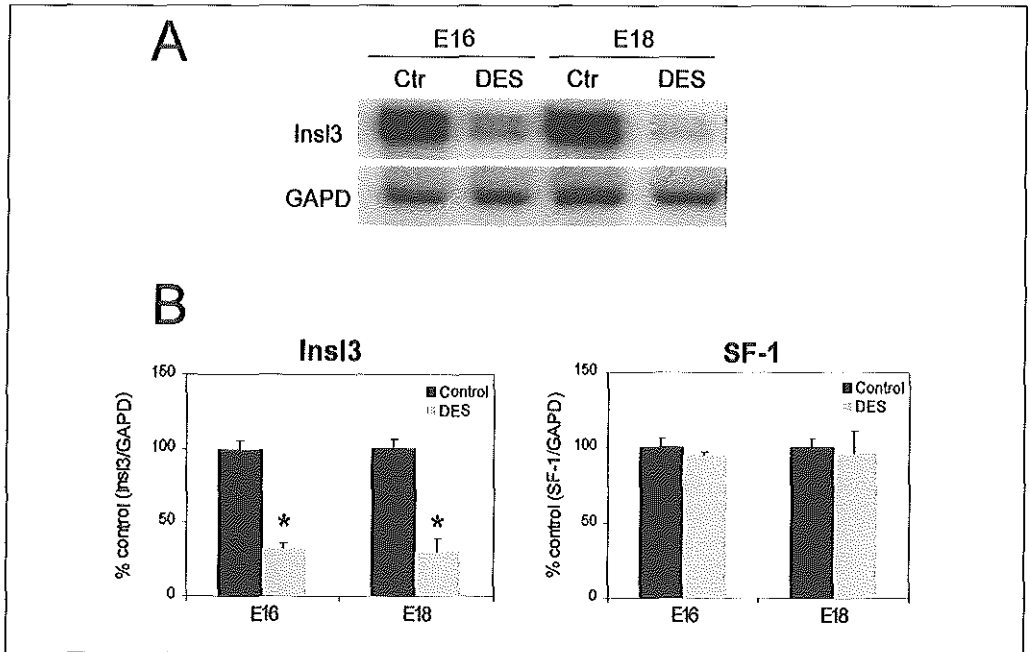
Figure 5.2 - Effect of DES on testicular expression of *Insl3* and *Sf1* mRNAs
(A) PhosphorImage analysis of a representative RNase protection assay for *Insl3* and *GAPD* mRNA expression at E16 and E18. *GAPD* mRNA analysis was included as a control for RNA loading.
(B) Quantification of *Insl3* and *Sf1* mRNA levels in DES-exposed fetuses at E16 and E18. Levels of *Insl3* and *Sf1* mRNA were normalized to that of *GAPD* mRNA. Values are expressed as percentage of control (considered 100% at each time point) and are the mean ± SEM of at least two independent experiments performed in duplicate. *, $P < 0.005$ vs. control.

illustrated in Figure 5.2B. In testes from control fetuses at E16, *Insl3* mRNA expression was similar to the expression level of this mRNA at E18. With regard to the possible involvement of SF1 in *Insl3* expression, the present results show that *Sf1* mRNA expression in testes of DES-treated fetuses was not significantly different from its expression in testes of control fetuses, at both E16 and E18.

DISCUSSION

In the present study, prenatal exposure of male mouse fetuses to DES inhibits gubernaculum development during the transabdominal phase of testis descent, which is in agreement with published data (Shono *et al.*, 1996). To date, the mechanism by which estrogens inhibit gubernaculum development has remained unclear. Hadziselimovic *et al.* (1980) demonstrated that estrogens inhibit Leydig cell development and consequently the secretion of testosterone. It was proposed that estrogens suppress the function of the fetal pituitary gland. However, fetal development of Leydig cell function is independent of pitui-

tary function (El-Gehani et al., 1998; O'Shaughnessy et al., 1998). The observation that the testes of hypogonadal (hpg) mice, lacking gonadotropin-releasing hormone, descend to the abdominal bottom with normal gubernaculum development excludes a role of the fetal pituitary gland in transabdominal testis descent (Grocock et al., 1988). Moreover, the role of estrogens in the ontogeny of undescended testes also appears to be independent of their effects on androgen action (Hutson, 1987). Although mice with complete androgen resistance (testicular feminization, Tfm) are cryptorchid, the transabdominal phase of testis descent is not affected in these mice (Hutson, 1986). Exposure of Tfm mice to estrogen inhibits the development of the gubernaculum (Hutson, 1987). In addition, exposure of wild-type fetal mice to estrogen in combination with testosterone does not reverse the estrogen-induced cryptorchidism (Hutson and Watts, 1990).

Hutson and Donahoe (1986) have suggested a possible role for anti-Müllerian hormone (AMH) in regulation of the first phase of testis descent, although direct evidence for such a role is lacking. It has been proposed that estrogens might interfere with AMH action. However, studies from Majdic et al. (1997) and Visser et al. (1998) could not demonstrate an effect of DES on AMH mRNA expression in fetal testicular tissue around the time of transabdominal descent.

In the present study, prenatal exposure to DES from E9 through E17 decreased the expression of Insl3 mRNA in mouse testis, both at E16 and E18. Recently developed Insl3 knockout mouse models demonstrated the requirement of Insl3 in the transabdominal descent of the testis (Zimmermann et al., 1999; Nef and Parada, 1999). It was proposed that Insl3 is a third testicular factor, next to androgens and AMH, which is involved in male sex differentiation. Insl3 is clearly involved in gubernaculum development, either directly or indirectly. Whether or not Insl3 acts directly on the gubernaculum, reduced expression of Insl3 mRNA in fetal testes by exposure to exogenous estrogens might be the mechanism by which estrogens inhibit gubernaculum development and cause testis maldescent. Under the present experimental conditions, prenatal exposure to DES did not affect Sf1 mRNA expression in mouse testis, measured at E16 and E18. Based upon a previous study done by Visser et al. (1998), an effect of DES on the general development of DES-exposed fetuses can probably be excluded as a possible explanation for the decrease of Insl3 mRNA. This is strengthened by the present observation that Sf1 mRNA expression is not affected. In contrast, Majdic et al. (1997) demonstrated a decrease in Sf1 mRNA in testis of DES-exposed rat fetuses. However, the latter result is based on the use of rats and a different DES dose and treatment schedule.

It remains to be determined whether DES directly represses transcriptional activity of the Insl3 gene in fetal Leydig cells, or causes nonspecific dysfunction of fetal Leydig cells which indirectly leads to impaired Insl3 production. Furthermore, a direct action of DES upon the gubernaculum cannot be excluded.

Cryptorchidism can be experimentally induced in animals by prenatal exposure to DES, but also occurs in sons whose mothers have been given DES during pregnancy (Newbold and McLachlan, 1996). In humans, the INSL3 gene has been identified and characterized (Burkhardt et al., 1994a), and in situ hybridization on human testis sections demonstrated that Insl3 is exclusively expressed in Leydig cells (Ivell et al., 1997). It is of extreme interest

to obtain information on the role of INSL3 in testis descent in the human and such a study could lead to direct analysis of the effect of DES on *INSL3* expression in human males. This would not only give insight into the role of DES in the etiology of cryptorchidism, but would also be useful in view of the present concerns about the possible effects of environmental estrogens on fetal development of the reproductive system (Sharpe and Skakkebaek, 1993).

Hormonal control of gubernaculum development during testis descent;

gubernacular outgrowth *in vitro* requires both Insl3 and androgen

Hormonal control of gubernaculum development during testis descent; gubernaculum outgrowth *in vitro* requires both Insl3 and androgen

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Submitted

Summary

The gubernaculum connects the gonad to the inguino-scrotal region and is involved in testis descent. It rapidly develops in the male fetus whereas development in the female fetus is lacking. Possible factors involved in gubernaculum development are androgens, anti-Müllerian hormone (AMH) and insulin-like factor 3 (Insl3). Sexual dimorphism in gubernaculum development correlated with the mitotic activity of cells in the gubernacular bulbs from male and female fetuses. Androgen receptor expression was restricted to the mesenchymal core of the gubernacular bulb, whereas skeletal muscle was detected in its outerlayer. In organ culture system, devised to study gubernaculum development *in vitro*, morphology of gubernacular explants grown in the presence of testes was comparable with that of gubernacula developed *in vivo*. Testicular tissue or medium containing R1881, a synthetic androgen, had a growth stimulatory effect on gubernacular explants when compared to ovarian tissue or basal medium only. Moreover, $Amh^{-/-}$, $Amh^{+/-}$ and $Insl3^{+/-}$ testes stimulated growth of gubernacular explants to the same extent as control testes. $Insl3^{-/-}$ testes, however, did not produce such an activity. This study reveals an essential role for both androgens and Insl3 in the gubernaculum outgrowth during transabdominal testis descent.

INTRODUCTION

Testis descent is the process by which the developing testis moves from its initial position high in the abdomen into the scrotum. The process is generally subdivided into two phases (Hutson, 1985). During the first or transabdominal phase, which takes place before birth, the testis gains a position at the bottom of the abdomen. The second or inguino-scrotal phase involves movement of the testis from the abdominal bottom to the base of the scrotum. Two ligaments appear to play an important role in determining the position of the gonad; the gubernaculum, which develops below the gonad in the inguinal area of the abdominal cavity, and the cranial suspensory ligament (CSL), which develops between the gonad and the dorsal abdominal wall, near the last rib (Figure 6.1) (van der Schoot and Emmen, 1996; Hutson *et al.*, 1997). During the transabdominal phase, the male gubernaculum is developing whereas outgrowth of the CSL is lacking, resulting in a position of the testis close to the bladder neck.

Experimental studies oriented towards understanding the mechanism of testis descent often involve observations on gubernaculum outgrowth and regression (Heyns and Hutson, 1995). In rodents, the gubernaculum can be divided into a cranial part, the gubernacular cord, and a caudal part called the gubernacular cone or bulb. The gubernacular bulb consists of a mesenchymal core with a muscular cover. In larger mammals, including the pig, ungulates, and man, the gubernaculum only consists of mesenchymal cells (Wensing, 1986). Fetal orchiectomy prevents gubernaculum outgrowth, suggesting involvement of fetal testicular factors (Baumans *et al.*, 1983). The specific hormone mediating this process appeared not to be androgen, since testosterone was unable to counteract the effects of orchiectomy. This is further supported by the observation that gubernaculum outgrowth
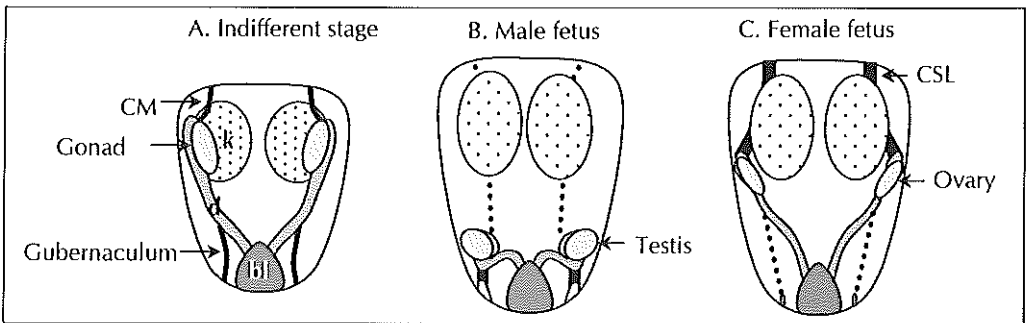

Figure 6.1 - Transabdominal phase of testis descent in the rat fetus
(A-C) Schematic drawings of the gonadal position at E16, the indifferent stage, and at E19 in the male and female rat fetus. *(A)* During the indifferent stage, the developing gonad of both male and female fetuses is positioned on the ventromedial aspect of the kidney (k). Gonads and ducts (d) are attached to the abdominal body wall by two ligaments, the cranial mesentery (CM) and the gubernaculum. *(B)* In the male, the testis gains a position lateral to the bladder (bl), connected to the inguinal region via a developing gubernaculum. Cranial suspensory ligament (CSL) development is lacking. *(C)* In the female, the ovary is positioned lateral to the kidney, attached to the last rib via a well-developed CSL. The gubernaculum does not further differentiate.

normally occurs in both mice and humans with complete androgen resistance (Hutson, 1986). Androgens might play a role in initiation of gubernaculum regression, occurring during the inguinoscrotal phase (Baumans *et al.*, 1983; Hutson and Donahoe, 1986). A possible role for anti-Müllerian hormone, AMH, in control of the first phase of testis descent was hypothetized (Hutson and Donahoe, 1986), although direct experimental evidence is lacking. A strong argument against a role of AMH in testis descent is the observation that AMH-deficient male mice show normal testis descent (Behringer *et al.*, 1994). Concerning gubernaculum development, involvement of a third testicular factor has been suggested (Fentener van Vlissingen *et al.*, 1988; van der Schoot *et al.*, 1995; Visser and Heyns, 1995).

Recently, data became available indicating that insulin-like factor 3 (Insl3), also designated Leydig insulin-like factor (Ley I-L) or relaxin-like factor (RLF), might be an important factor for gubernaculum development in mice (Adham *et al.*, 2000). *Insl3* is specifically expressed in Leydig cells of the pre- and postnatal testis and in theca cells of the postnatal ovary (Zimmermann *et al.*, 1997). *Insl3* knockout mice, homozygous for a targeted inactivation of the *Insl3* gene (*Insl3*$^{-/-}$ mice), have been generated and it was observed that male *Insl3*$^{-/-}$ mice have undescended testis (Zimmermann *et al.*, 1999). Histological analysis of male *Insl3*$^{-/-}$ fetuses revealed that the development of the gubernaculum is severely affected. Similar findings were reported by Nef and Parada (1999).

Although studies with mutant mouse models have provided more insight into the complicated regulation of gubernaculum development, it still remains to be determined whether the targeted factors are directly involved in control of gubernaculum development. In the present study, development of the rat gubernaculum during the first phase of testis descent was studied *in vivo* with respect to cell differentiation and proliferation. Furthermore, an organ culture technique for *in vitro* culturing of gubernacula is established in order to study the effects of different testicular hormones/factors on rat gubernaculum development.

MATERIALS AND METHODS

Animals and tissue collection

Adult rats (Wistar) and mice were kept under standard animal housing conditions. Female animals were placed in individual cages with males and the morning a vaginal plug was found was designated day 0 (E0) of pregnancy.

For immunohistochemical analysis of the rat gubernaculum, fetuses were removed from the uterus on E17 and E19 and fixed in 10% neutral buffered formalin for 24 h. After fixation, fetuses were embedded in paraffin, sectioned at 7 µm and sections were selected for immunohistochemistry.

Cell proliferation in the fetal rat gubernaculum *in vivo* was assessed using 5'-bromodeoxyuridine (BrdU), a thymidine analogue which is incorporated into the DNA during the S-phase of the cell cycle. Pregnant rats were injected intraperitoneally with a single pulse of BrdU (Boehringer Mannheim, Germany) dissolved in saline (100 mg/kg body weight) on E17. Two hours after injection, fetuses were removed from the uterus, fixed in 10%

neutral buffered formaline and further processed for immunohistochemical detection of incorporated BrdU.

Organ cultures

For each culture, fetal rat gubernacula and mouse gonads were isolated. Gubernacula were obtained from E17 male rat fetuses. After the mothers were killed, the rat fetuses were quickly removed and placed on ice until dissection. Fetuses were examined under a dissecting microscope, and gubernacula were obtained from male fetuses and placed in phosphate-buffered saline under sterile conditions. Urogenital ridges were also removed.

Gonads were obtained from neonatal mice (FVB strain) at postnatal day 6. Additionally, two different mutant mouse models were used; *AMH* mutant mice (B6 strain; Behringer *et al.*, 1994) and *Insl3* mutant mice (129/SV strain; Zimmermann *et al.*, 1999) mice. Homozygous $AMH^{-/-}$ or $Insl3^{-/-}$ female mice were bred with heterozygous $AMH^{+/-}$ or $Insl3^{+/-}$ female males, respectively, to obtain homozygous and heterozygous neonates. The macroscopic anatomy of the neonatals was determined with a dissection microscope, and homozygous *AMH* and *Insl3* mutant neonates could be reliably distinguished from heterozygous ones based on their phenotypes. Still, tails were collected for genotyping by PCR. After dissection, testes were decapsulated.

The culture technique used was similar to the one utilized by Cooke *et al.* (1987) for mouse neonatal bulbourethral gland. Briefly, tissues were transferred onto Millipore CM filters (Millipore Corp., Bedford, USA) and floated on 1 ml of medium in four-well plates (Nunc, Roskilde, Denmark). The culture medium used was DMEM/F12 (Gibco, Life Technologies, Breda, The Netherlands) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), fungizone (0.6 µg/ml), insulin (10 µg/ml) and transferrin (10 µg/ml; all from Sigma, St Louis,USA). The medium was supplemented with 2% stripped fetal calf serum (Greiner). In part of the cultures, the synthetic androgen methyltrienolone (R1881;10^{-8} M) was added to the medium. The culture dishes were placed in a humidified incubator at 37°C in a 5% CO_2 atmosphere, and the medium was changed daily.

Gubernacula were cultured for 5 days with or without added gonads. The gonads were placed in close proximity to the gubernacula on a Millipore filter at the ratio of 1 gonad: 1 gubernaculum. After 5 days of culture, some explants were chosen randomly for immunohistochemical analysis. Tissues were fixed in neutral-buffered formalin, embedded in paraffin and serial sections of 5 µm were cut.

Thymidine incorporation

The proliferation of [^{3}H]thymidine into DNA was measured on culture day 5. At the end of day 4, an amount of 5 µCi/ml of methyl-[^{3}H]thymidine (specific activity 48 Ci/mmol, Amersham, Little Chalfort, UK) was added to the culture medium. After incubation overnight, the cultured tissues were removed, rinsed with 10 % ice-cold trichloroacidic acid (TCA) and centrifuged. The precipitate was washed twice with cold 5% TCA and hydrolyzed in 25 µl NaOH (68°C; 30 min). The radioactivity of the supernatant was measured using a liquid scintillation counter, and the incorporation of radioactive thymidine was calculated as dpm/gubernaculum.

Immunohistochemistry

Selected sections were mounted on silane-coated slides. After deparaffinization, sections were treated with 3% H_2O_2/methanol solution to block endogenous peroxidase activity.

To study gubernaculum development *in vivo* and *in vitro*, polyclonal antibodies against the rat androgen receptor (SP197; Bentvelsen *et al.*, 1995) were used as marker for the mesenchymal core, and monoclonal antibodies against sarcomeric myosin (MF20, obtained as hybridoma from the Developmental Studies Hybridoma Bank; Bader *et al.*, 1982) as a marker for the skeletal muscle outerlayer. The procedure used was based upon that described by Janssen *et al.* (1994). Briefly, sections were placed in 10 mM citrate buffer and microwaved for 3x5 min at 700 W. Sections were preincubated with normal goat serum, followed by incubation with the primary polyclonal antibodies SP197 (diluted 1:7000) or monoclonal antibody MF20 (diluted 1:100) overnight at 4°C. The antibodies were detected using biotinylated goat anti-rabbit antibody (dilution 1:400) or biotinylated goat anti-mouse antibody (diluted 1:50), respectively, by incubation for 30 min at room temperature, followed by treatment with streptavidin-biotin-peroxidase complex. The peroxidase activity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). In samples processed without the primary antibody, no cells with nuclear (AR) or cytoplasmic (myosin) immunostaining were found.

The BrdU staining procedure has been described previously (Emmen *et al.*, 1998). Briefly, labelling was detected in 7-µm sections, pretreated with pronase 0.1% for 30 min and 2 N HCl for 30 min, followed by 1 h incubation with a mouse monoclonal anti-BrdU antibodies (diluted 1:25, Sigma), all at 37°C. Sections were then incubated with peroxidase-conjugated goat-anti-mouse (diluted 1:100) for 30 min at room temperature. BrdU-labeled nuclei were visualized with DAB. Slides were counterstained with haematoxylin.

Controls and statistical analysis

The proliferation index of the rat gubernaculum was estimated by determining the proportion of BrdU-labeled nuclei per total number of cells (x100) per fetus. Statistical significance was assessed by using Student's two-tailed *t* test.

All culture experiments were performed in triplicate and the data are expressed relative to [^{3}H]thymidine incorporation induced in gubernaculum cultured with R1881 (10^{-8} M) in the same experiment. All the data shown were collected in at least two independent experiments. Statistical analysis was performed using one-way analysis of variance supplemented with Hochberg's GT2 test. All data are presented as mean ± SEM.

RESULTS

Sexual dimorphic development of the gubernaculum

Histological analysis of rat fetuses at E17 and E19 showed sexual dimorphic development of the gubernacular bulb (Figure 6.2). The sexual dimorphic development of the gubernaculum was further examined by determination of the proliferation index and by study-
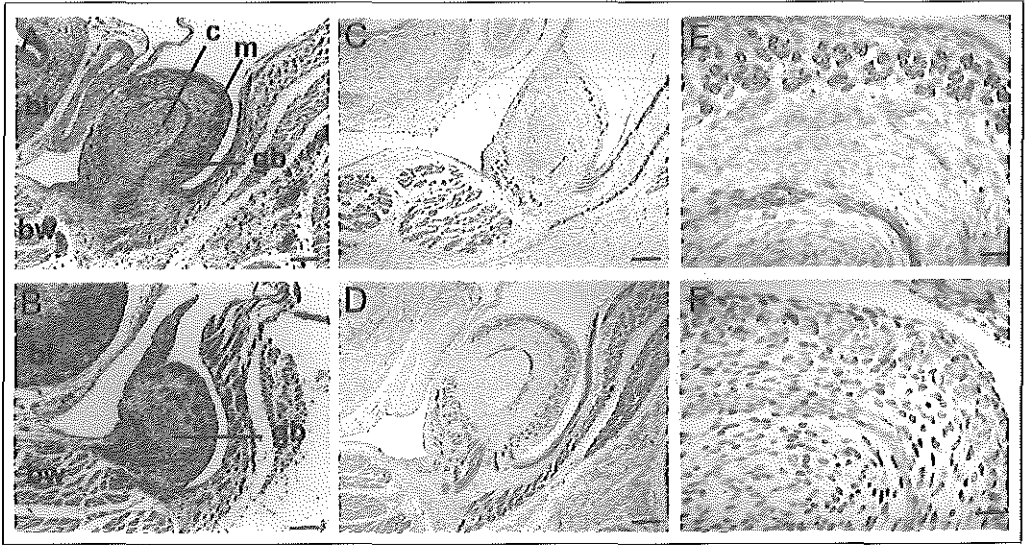
Figure 6.2 - Histology and immunohistochemistry of gubernaculum development in the rat fetus *in vivo*. (For a color version of this figure, see page 164.)

(A-B) Histological analysis of the gubernaculum at E19 in the male (A) and female rat fetus (B). In the male gubernaculur bulb (gb), the muscular layer (m) and mesenchymal core (c) can be discriminated. Although the gubernacular bulb of the female fetus also consists of myoblasts and mesenchymal cells, it is smaller and less well-organized when compared to the male bulb. bl, bladder; bw, body wall. *(C-F)* Immunohistochemical localization of myosin (C-E) and androgen receptor (F) in the developing male gubernacular bulb of the rat. At E17, (C) the muscular layer of the gubernacular bulb can be clearly distinguished by myosin immunostaining. At E19, (D) the muscular layer is more pronounced when compared to E17, as demonstrated by a strong antin-myosin immunoreaction. Detail (E) of the muscular layer from section (D), showing differentiating myoblasts which are myosin-positive. (F) is a section from the same male rat fetus (D, E), but stained with antibodies against the androgen receptor (AR), showing that the mesenchymal cells are AR-positive (F). Scale bar = (A, B, C, D) 100 μm, (E, F) 25 μm.

ing the organization of the gubernacular bulb into mesenchymal cells and myoblasts by use of two different cell differentiation markers. At E17, the bulb of both sexes is organized in a mesenchymal core and outer muscular layer, but the male bulb is enlarged when compared to the female gubernacular bulb. The proliferation index for the male and female gubernaculum at E17 was estimated using the BrdU-labelling technique. BrdU incorporation was significantly higher in the male gubernacular bulb (15.8 % ± 0.5, n = 4) than in the female bulb (7.2 % ± 1.8, n = 4) (P < 0.005). The enlargement or swelling of the male gubernacular bulb progresses to E19, whereas in the female fetus no further development occurs (Figure 6.2A,B).

Immunohistochemical analysis of the gubernaculum demonstrated expression of sarcomeric myosin in the perifery of the gubernacular bulb of both sexes, on E17 and E19 (Figure 6.2). However, in the male fetus, the muscular layer expands between E17 and E19 and the myoblasts start to differentiate (Figure 6.2C-E). Androgen receptor (AR) expression was restricted to the mesenchymal cells of the gubernacular bulb (Figure 6.2F). AR

expression in the male gubernaculum was consistently higher than in the female gubernaculum, at both time points (not shown).

An *in vitro* model of gubernaculum development

To analyze hormonal control of gubernaculum development, the possibility of using a culture system was studied. Since the *in vivo* studies demonstrated that gubernaculum development is characterized by rapid cell proliferation, it was decided to follow gubernaculum development *in vitro* by measurement of the incorporation of [³H]thymidine into DNA. Development of the Wolffian duct *in vitro* is also marked by rapid proliferation when cultured in presence of a testis or androgen (Tsuji *et al.*, 1991). Therefore, the Wolffian duct was used in each culture experiment as a control androgen responsive tissue.

Initial studies indicated that E17 rat gubernacular explants did respond to the presence of testicular tissue in the culture by increased incorporation of [³H]thymidine. To determine the effects of gonads from *AMH*$^{-/-}$ and *Insl3*$^{-/-}$ mice on gubernaculum development *in vitro*, a series of culture experiments with mouse gubernacula and gonads was carried out. However, in contrast to fetal rat gubernacular explants, fetal mouse gubernacular explants
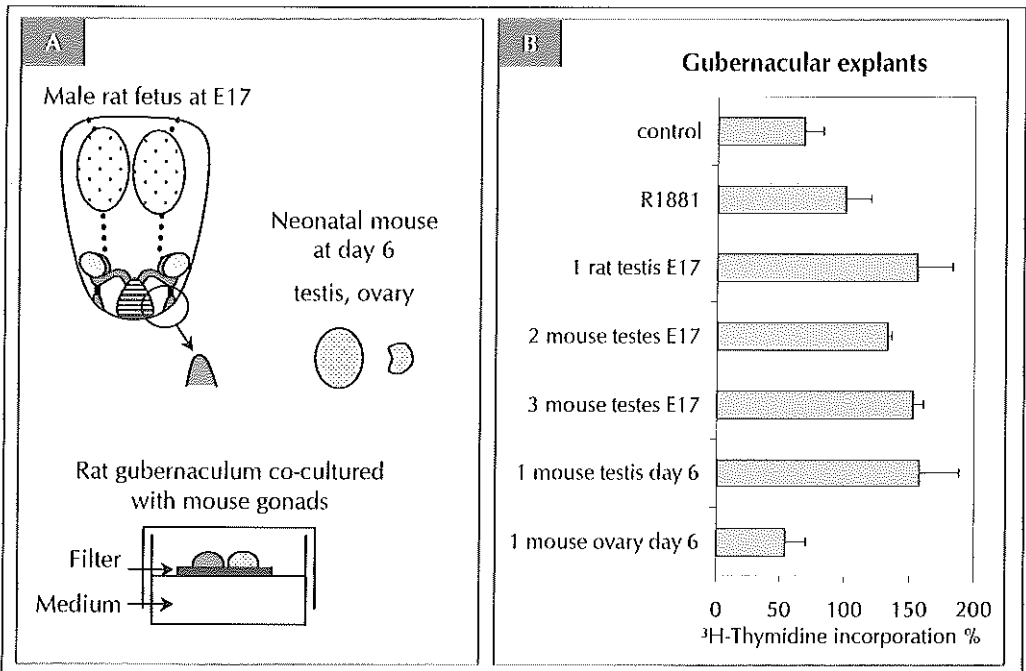

Figure 6.3 - *In vitro* culture of gubernacular explants
(A) Schematic outline of the organ culture system used for co-culture of rat gubernaculum explants and mouse gonads, as described in Materials and Methods. *(B)* A representative experiment of [³H]-thymidine incorporation by rat gubernacular explants cultured without hormone (control) or in presence of R1881 (10⁻⁸M), testicular tissue or ovarian tissue. Testis was derived from E17 rat fetuses, E17 mouse fetuses or postnatal day 6 mice. Ovarian tissue was only derived from postnatal day 6 mice. Values are expressed relative to the effect of R1881 (set at 100%).

did not show any indication of a proliferative response to testicular tissue. It was there-fore decided to perform a co-culture of rat gubernaculum with mouse gonads, outlined in Figure 6.3A, which turned out to be successful. Maximal differences in [³H]thymidine uptake per rat gubernaculum cultured in the presence or absence of a mouse testis were observed after 5 days of culture. After 5 days of culture, [³H]thymidine uptake by the gubernacular explants was enhanced when cultured in the presence of a testis or R1881, the effect of a testis being greater than that of R1881 (Figure 6.3B). Ovarian tissue had no effect on gubernaculum growth. Since a prenatal mouse testis is much smaller then a prenatal rat testis, three prenatal mouse testes per rat gubernaculum were necessary to give a growth response that was similar to that of one prenatal rat testis. However, replacing these three fetal mouse testes by one neonatal mouse testes, dissected at postnatal day 6, induced a growth response in a E17 rat gubernaculum which compared well with that of E17 rat testis.

At the light microscopical level, the explants were studied after 5 days of culture to determine the viability of the tissues (Figure 6.4A,B). Addition of 2% fetal calf serum and insulin (10 μg/ml) to the medium enhanced the viability of gubernaculum and gonadal tissue during culturing. At day 0, the gubernacular bulb was organized into a muscular
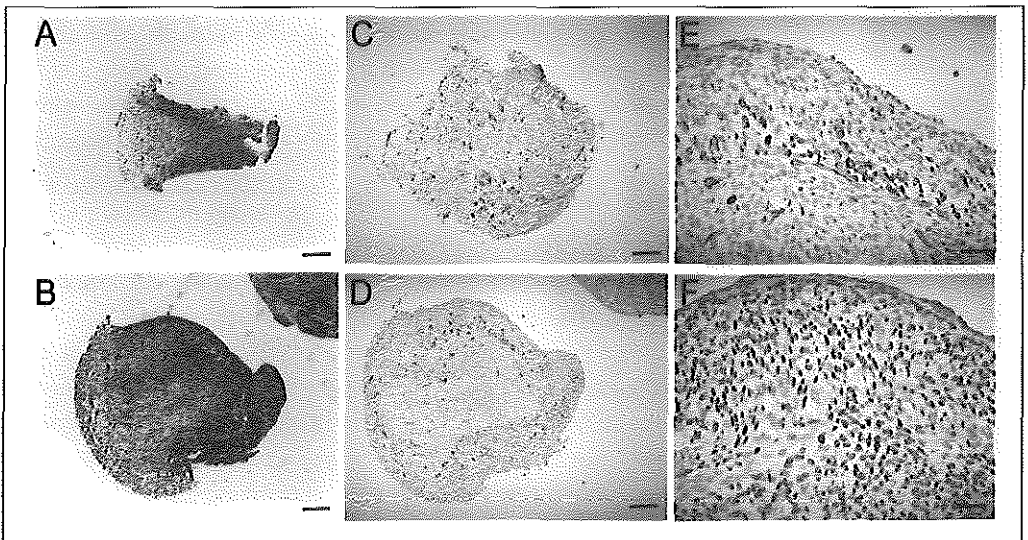

Figure 6.4 - Histological and immunohistochemical features of gubernacular explants, prior to and after 5 days of culture. (For a color version of this figure, see page 165.)
(A-B) Morphology of gubernacular explant prior to culture (A) and after 5 days of culture in pres-ence of mouse testicular tissue (B). Note the pronounced increase in size of the gubernacular bulb after culturing. (C-F) Immunolocalization of myosin (C-E) and androgen receptor (AR); (F) after cultur-ing in the presence of R1881 (10⁻⁸M); (C) or testis (D-F). Both gubernacular explants (C,D) demon-strate immunostaining with myosin. In the gubernacular explant cultured in presence of R1881 (C), myosin-positive cells can be seen across the whole explant. In contrast, the gubernacular explant cultured in presence of a testis is showing a myosine-positive outerlayer. Detail (E) of the muscular layer from section (D), showing myoblasts which are myosine-positive. (F) is a section from the same explant (D, E), but stained with antibodies against the AR, showing that the mesenchymal cells are AR-positive (F). Scale bar = (A, B, C, D) 100 μm, (E, F) 25 μm.

outlayer and inner mesenchymal core (Figure 6.4A). By day 5, the entire gubernaculum had enlarged. The organization into muscular outerlayer and mesenchymal core was preserved in the gubernacular bulb cultured in the presence of a testis (Figure 6.4B).

Immunohistochemistry was performed to study the expression of two differentiation markers, androgen receptor (AR) and myosin, in cultured gubernacula (Figure 6.4C-F). Myosin was expressed in the gubernaculum under all culture conditions, including in gubernacula cultured without hormone or in the presence of ovarian tissue. AR expression was detected in gubernacular explants cultured in the presence of either R1881 or testis (Figure 6.4F). The organization of the gubernacular bulb into a muscular outerlayer and inner AR-positive mesenchymal core, as seen *in vivo*, was also observed in gubernacular explants *in vitro* but only when cultured in the presence of a testis (compare Figure 6.4E,F with Figure 6.2E,F). However, the muscular layer was thinner and less differentiated when compared to *in vivo* development (compare Figure 6.4E to 6.2E). In all other cultures, myosine-positive cells were found all across the gubernaculum (Figure 6.4C).

Effects of testes from different mutant mice

The culture system described above was used to study the stimulatory effects of gonads from different mutant mice on gubernacular explants. Two different mutant mouse models were available: $AMH^{-/-}$ and $Insl3^{-/-}$ mice and also the heterozygous animals. Although *in vivo* analysis of heterozygous and homozygous AMH and $Insl3$ mutant male mice did not indicate any defect in androgen production by the testis, R1881 was added to the culture medium to compensate a possible deficiency in androgen production *in vitro*. In Figure 6.5, the effect of testicular tissue from the different mutants on [³H]thymidine uptake by gubernacular explants is shown. $AMH^{-/-}$, $AMH^{+/-}$ and $Insl3^{+/-}$ testicular tissue stimulated [³H]thymidine incorporation by the gubernacular explants to the same extent as control testis. In contrast, in the presence of a $Insl3^{-/-}$ testis the [³H]thymidine incorporation was significantly reduced when compared with the effect of the control testis ($P<0.05$). The effect of $Insl3^{-/-}$ testicular tissue on gubernaculum growth was diminished to a level similar to that of R1881 alone. Heterozygous and homozygous mutant ovarian tissue did not induce any increase in gubernaculum proliferation when compared to proliferation of gubernacular explants alone (results not shown).

As mentioned previously, Wolffian duct was used in each culture experiment as a control androgen responsive tissue. After 5 days of culture, [³H]thymidine uptake by rat Wolffian duct explants was enhanced when cultured in the presence of testis or R1881, the effect of the testis being similar to that of R1881 (Figure 6.6). Ovarian tissue did not stimulate growth of Wolffian duct explants. In contrast to the results obtained with gubernacular explants, the stimulatory effect of $Insl3^{-/-}$ testes on Wolffian duct growth *in vitro* was not different from that of control or $Insl3^{+/-}$ testes.

DISCUSSION

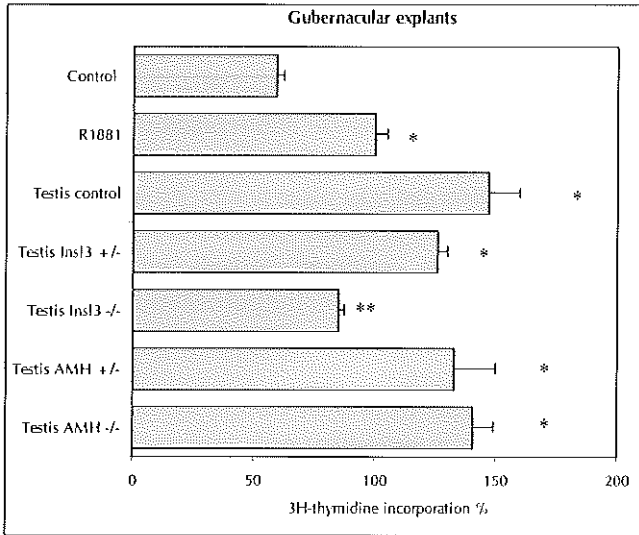In the present study, an organ culture system has been established which provides an

Gubernacular explants

Control
R1881 *
Testis control *
Testis Insl3 +/- *
Testis Insl3 -/- **
Testis AMH +/- *
Testis AMH -/- *

0   50   100   150   200
3H-thymidine incorporation %

Figure 6.5 - Effects of testes from *Insl3*- and *Amh*-deficient mice on gubernaculum growth *in vitro*
[³H]-thymidine incorporation by gubernacular explants cultured without hormone (control), in the presence of R1881 (10^{-8}M) or testis from control, *Insl3*+/-, *Insl3*-/-, *Amh*+/- or *Amh*-/- mice. Values are expressed relative to the effect of R1881 (set at 100%) and are mean ± SEM of at least two independent experiments performed in triplicate. *, $P < 0.05$ vs. control; **, $P < 0.05$ vs. control testis.

Wolffian duct explants

Control
R1881
Testis control
Ovary control
Testis Insl3 +/-
Testis Insl3 -/-

0   50   100   150
3H-thymidine incorporation %
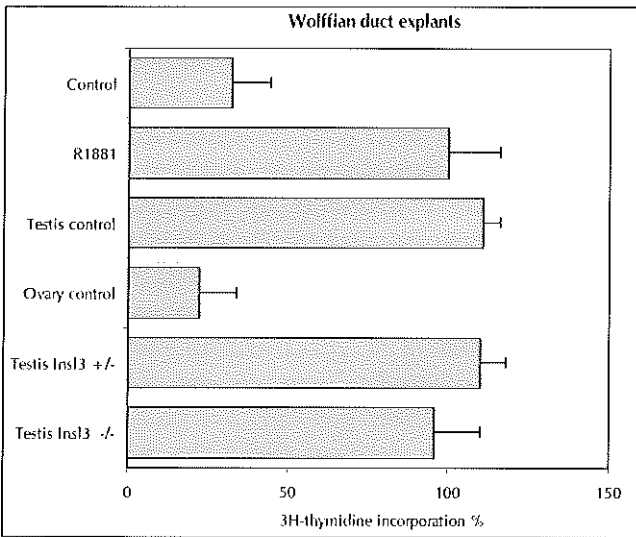
Figure 6.6 - *In vitro* culture of Wolffian duct explants
A representative experiment of [³H]-thymidine incorporation by rat Wolffian duct explants cultured without hormone (control) or in the presence of R1881 (10^{-8}M), testicular tissue or ovarian tissue. Testis tissue was derived from control, *Insl3*+/- or *Insl3*-/- postnatal day 6 mice. Ovarian tissue was derived from postnatal day 6 control mice. Values are expressed relative to the effect of R1881 (set at 100%).

unique opportunity to study hormonal regulation of the fetal rat gubernaculum under precise and defined conditions. The organ culture system used was similar to the one used by Cooke *et al.* (1987) for the mouse neonatal bulbourethral gland. Co-culture of a rat gubernaculum with a mouse testis induces a growth response, illustrating that the factors controlling gubernaculum outgrowth in these rodents are most likely similar, to be recognized across species barriers. This is also observed for other structures of the male urogenital tract such as the prostate and seminal vesicles (Cunha *et al.*, 1983; Higgins *et al.*, 1989). Establishment of an organ culture with mouse gubernacula failed, since no growth stimulatory effect could be induced by addition of mouse testis. The reason for this is not clear.

Immunohistochemical studies presented herein showed that both male and female rat gubernacula express myosin, a marker for myoblasts, *in vivo*. Furthermore, myosin was also

expressed *in vitro*, under all different culture conditions. However, the muscular layer of the gubernaculum *in vitro*, even after co-culture with a testis, was thinner when compared to the *in vivo* situation. Similarly, Radhakrishnan and Donahoe (1981) limited their *in vitro* studies to the muscular layer and did not observe induction of muscle differentiation in cultured rat gubernacular tissue by using whole testis. Studies on *in vitro* skeletal muscle development showed that only a limited degree of differentiation can take place *in vitro*, probably due to the lack of innervation, growth factors and extracellular matrix (Muntz, 1990). Although myoblasts can differentiate in the absence of nerves, continuing growth and maturation definitively require innervation (Muntz, 1990). Upon isolation, the gubernaculum is separated from the body wall musculature and nerves, which could explain the retarded muscular development *in vitro*.

Immunostaining of the AR *in vivo* demonstrated AR positive cells in the mesenchymal layer of the gubernaculum of both sexes, which is in agreement with published data (Husmann and McPhaul, 1991; Bentvelsen *et al.*, 1995). The level of AR expression increases in the male but decreases in the female gubernaculum, indicating an intial androgen independent expression followed by a hormone-dependent expression (Bentvelsen *et al.*, 1994). In agreement with the *in vivo* situation, the AR was only highly expressed in explants which were cultured in presence of androgen, either as R1881 added to the medium or in co-cultures with testicular tissue.

Generally, gubernacular tissue cultured *in vitro* showed a more condensed structure than gubernacular tissue *in vivo*. This could be due to the flattening of the gubernacular explants when cultured on the two-dimensional filter layer. Furthermore, the organization of the gubernaculum into the mesenchymal core and muscular outerlayer, as seen in the male gubernaculum development *in vivo*, was only observed in the gubernacular explants cultured in the presence of a testis. Apparently, testicular factors other then androgens are necessary to maintain the male specific organization of the gubernaculum during culture.

At E17, the male gubernaculum *in vivo* was enlarged substantially when compared to the female, which is in agreement with histological observations reported previously (Radhakrishnan *et al.*, 1979; Wensing, 1986; van der Schoot and Elger, 1993). This difference can be explained by a difference in cell proliferation, as determined by BrdU labelling indices. This is in agreement with observations in the pig fetus, in which gubernaculum outgrowth during the transabdominal phase of testis descent is also characterized by rapid cell proliferation (Heyns *et al.*, 1986; Fentener van Vlissingen *et al.*, 1989; Heyns *et al.*, 1990). The regulation of proliferation was used as parameter for further analysis of hormonal control of gubernaculum development.

Surprisingly, R1881 added to the culture medium clearly increased [^{3}H]thymidine uptake by gubernacular explants. In two earlier *in vitro* studies using isolated porcine gubernaculum cells, androgens did not stimulate proliferation (Fentener van Vlissingen *et al.*, 1988; Visser and Heyns, 1995). Since the major difference between rat and porcine gubernaculum proper is the presence of a muscular outerlayer in the rat (Wensing, 1986), the increased proliferation might reflect an increase of the muscular component of the rat gubernaculum. Given the fact that expression of the AR, which is a prerequisite for androgen activity, is detected in the mesenchymal core of both the rodent and porcine gubernaculum

(Heyns and Pape, 1991; Husmann and McPhaul, 1991; Bentvelsen and George, 1993), it is suggested that androgens may act directly on the mesenchymal cells which in turn elicit a growth response on the myogenic cells through paracrine mechanisms. In several androgen responsive tissues including prostate, seminal vesicles and bulbourethral glands, testicular androgens act via AR in the mesenchymal cells (Cunha *et al.*, 1992). In addition, influences of the mesenchyme on the muscular layer and *vice versa* might be necessary for complete gubernaculum development. Such a mechanism of action might also hold true for the process of gubernaculum outgrowth in the pig. Although the porcine gubernaculum proper only consists of mesenchymal cells, it is in close contact with the cremaster muscle, which is the equivalent of the muscular outerlayer of the rat gubernaculum (Wensing, 1986). The present *in vitro* data are in apparent contradiction with the currently available data from *in vivo* studies that more strongly support a role of androgens in gubernaculum regression then outgrowth (Heyns and Hutson, 1995). However, both Spencer *et al.* (1991) and Cain *et al.* (1995) demonstrated a decrease in gubernacular bulb development upon anti-androgen exposure of male rat fetuses. This indicates the complexity of gubernaculum development *in vivo* and underlines the usefulness of organ culture for further studies on the role of androgen.

The presence of testicular tissue during the culture period exceeded the stimulatory effect of androgen on [³H]thymidine uptake by gubernacular explants, whereas ovarian tissue did not stimulate [³H]thymidine uptake. This implies that a testicular factor, different from androgens, is also involved in gubernaculum growth. This is in contrast to the Wolffian ducts, which are stimulated to the same extent by testicular tissue as by androgens alone. Two factors that are secreted by the fetal testis at the time of transabdominal testis descent are AMH and Insl3 (Münsterberg and Lovell-Badge, 1991; Zimmermann *et al.*, 1997). Since both factors were not available for direct studies, co-culture experiments were designed in which gonads of *AMH* and *Insl3* mutant mice were tested. Culturing gubernacula in the presence of $Amh^{-/-}$ testes did not decrease the uptake of [³H]thymidine, when compared to the effect of control or $Amh^{+/-}$ testes. This is consistent with the observed lack of a stimulatory effect of AMH on cultured porcine gubernaculum cells (Fentener van Vlissingen *et al.*, 1988; Visser and Heyns, 1995). $Insl3^{-/-}$ testes, however, were not competent to further increase the stimulatory effect of androgens on [³H]thymidine uptake by gubernacular explants. This result consolidates the putative role of testicular Insl3 in gubernaculum development *in vivo* and indicates that the primary defect of impaired gubernaculum development observed in Insl3 deficient mice appears to be intrinsic to the testis. In contrast, Insl3 appeared not to be essential for growth of the Wolffian ducts.

Whether Insl3 is secreted by the testis and acts directly on gubernaculum cells, remains to be determined. Recently, however, INSL3 has been demonstrated in the human circulation using antibodies (Bullesbach *et al.*, 1999), and specific, high affinity Insl3 receptors have been identified (Bullesbach and Schwabe, 1999), indicating a possible endocrine role of Insl3. According to the predicted structure, the biologically active Insl3 protein consists of a B-chain (3.3 kDa) and an A-chain (Adham *et al.*, 2000). Fentener van Vlissingen *et al.* (1988) and Visser and Heyns (1995) have previously demonstrated that low molecular weight factors, < 30k and < 3.5k respectively, isolated from the fetal pig testis stimulated

growth in cultured gubernaculum cells. Since the *Insl3* gene has also been identified in the pig and porcine Leydig cells express *Insl3* mRNA (Burkhardt *et al.*, 1994), the growth stimulatory effect observed by these investigators might have been related to Insl3 activity.

In the pig fetus, gubernaculum growth is determined by cell proliferation but also by changes in the extracellular matrix (ECM), resulting in true swelling of the gubernaculum due to water uptake (Heyns *et al.*, 1989). Whether changes in ECM are important during transabdominal testis descent in rodents remains a question to be answered. Interestingly, however, primary structure analysis revealed that Insl3 is closely related to relaxin (Bullesbach and Schwabe, 1995). Furthermore, synthetic human INSL3 peptide augmented the activity of relaxin in the mouse pubic symphysis bioassay, indicating relaxin-like properties of Insl3 (Bullesbach and Schwabe, 1995). Relaxin is a hormone which plays a significant role in promoting growth and softening of the cervix and loosing of the pubic symphysis prior to parturition, by remodelling connective tissue (Samuel *et al.*, 1998; Sherwood *et al.*, 1993). A similar mechanism of Insl3 action in the male fetus during gubernaculum development and testis descent would be an attractive working hypothesis for further research.

Further analysis of gubernaculum development *in vivo* and *in vitro* in relation to regulation of *Insl3* gene expression might lead to a better understanding of the process of testis descent. A major goal of future studies will be to define downstream genes through which the action of Insl3 on gubernaculum development is mediated.

ACKNOWLEDGEMENTS

General discussion

MALE SEX DIFFERENTIATION is implemented by the actions of fetal testicular hormones, AMH and testosterone, on the undifferentiated fetal reproductive tract (Jost, 1953; Wilson *et al.*, 1981). One obvious aspect of sex differentiation, however, was not included in this model being the sex dimorphic development of gonadal position. Most importantly, it was not explained which factors trigger and control testis descent. Investigations on the process of testis descent proposed involvement of testosterone and AMH as endocrine regulators. The exact mechanisms by which these hormones exert their effects on testis descent has remained an interesting puzzle. Recently, an important missing piece of that puzzle was discovered, namely the gonadal factor Insl3, which is produced by fetal testes and not by fetal ovaries. Insl3 can be considered a third testicular hormone produced by the fetal testis in addition to AMH and testosterone, involved in control of male sex differentiation.

The objective of this thesis was to study the transabdominal phase of testis descent in relation to two possible structures, the cranial suspensory ligament (CSL) and the gubernaculum, and their regulating hormones, AMH, testosterone and Insl3. The number of reports on Insl3 is rapidly increasing, which include studies on the expression of Insl3 in several animal species, the possible role of Insl3 in development and function of endocrine tissues and its structure and mechanism of action (Ivell, 1997; Adham *et al.*, 2000). The current knowledge on Insl3 will be briefly summarized and issues for future research will be addressed. Furthermore, the results obtained in this thesis in relation to the present understanding of testis descent will be discussed.

ANDROGEN ACTION

Development of the CSL in the outer border of the cranial mesentery can be considered a sex-dimorphic process, occurring in female rat fetuses whereas development in male fetuses is absent (Chapter 2). We observed that prenatal exposure of female rat fetuses to androgens inhibited CSL development, which is in agreement with published data (van der Schoot and Elger, 1992; Barthold *et al.*, 1994; Shono *et al.*, 1994; Cain *et al.*, 1995). Up till now, the only other sex-dimorphic process known in which androgens suppress development rather then exerting a stimulatory effect, is regression of the nipple primordia of the mammary glands in rat and mouse (Goldman *et al.*, 1976; Kratochwil, 1977). It has been shown that the mammary gland mesenchyme is responsive to androgens, whereas the epithelium exhibits hormone-dependent cell death. Despite the fact that this androgen-dependent mesenchymal-epithelial tissue interaction eventually leads to tissue degeneration, its characteristics are similar to what is observed for androgen-dependent development of the male urogenital tract (Cunha *et al.*, 1992). This mechanism of mesenchymal-epithelial interaction, however, cannot be applied to suppression of CSL development due to the fact that the cranial mesentery does not have an epithelial component. Using immunohistochemistry, androgen receptor (AR) expression was shown in the primordial cells of the CSL, suggesting that androgens act directly upon these cells (Chapter 2). These primordial cells differentiate into smooth muscle cells in the female fetus, whereas differentiation is

lacking in the male fetus. A role of androgens in the suppression of cell differentiation seems likely, but remains speculative.

In the experiments described in Chapter 2, androgens had no effect upon development of the rat CSL from gestational day 19 (E19) onwards, indicating that the time period during which the CSL primordium is sensitive to androgens has ended. This critical period for CSL suppression coincides with the period during which anti-androgens can affect testis descent (Husmann and McPhaul, 1991; Spencer *et al.*, 1991). This provides further evidence that prevention of CSL development might be an important factor in the determination of gonadal position. However, an additional testicular factor, probably Insl3, is necessary as no ovarian descent occurs after prenatal exposure to androgens (Chapter 2).

Results obtained from *in vitro* culture experiments indicated another possible target of androgenic action during the first phase of testis descent; the rodent gubernaculum bulb (Chapter 6). Previous *in vitro* studies with porcine gubernaculum cells did not show any growth response to androgen (Fentener van Vlissingen *et al.*, 1988; Visser and Heyns, 1995). Since the porcine fetal gubernaculum only consists of mesenchymal cells, the muscular outerlayer of the rodent gubernacular bulb should be considered the androgen-responsive structure. However, expression of the AR in the mesenchymal cells and not in the muscular cells, as shown by Husmann and McPhaul (1991a), Bentvelsen *et al.* (1995) and in Chapter 6, further complicates the interpretation of the androgenic effects. Thus, it is the mesenchymal core that responds to testosterone, even though it is the muscular outerlayer that exhibits the hormone-dependent cell proliferation. This might also be true for the process of testis descent as it occurs in several large mammalian species. Although the gubernaculum proper only consists of mesenchyme, it is in close contact with the cremaster muscle, which is the equivalent of the muscular layer of the rodent gubernacular bulb. Such a role of androgens in the development of the gubernaculum is speculative. However, the search for the mechanism of hormone action should not only be concentrated on the apparent target cell. Androgens may mediate developmental processes either by acting directly on the target cells where the physiological effect takes place, or by acting on cells which translate the androgenic signal into a signal that acts locally on nearby target cells for the final developmental effect.

Combining the above results, androgens are involved in the transabdominal phase of testis descent by acting on both the CSL and the gubernaculum. The obtained results seem to be in contradiction with the generally accepted biphasic model in which androgens do not act during the first phase of testis descent (Hutson and Donahoe, 1986; Husmann and Levy, 1995). Apparently, changes induced by androgens during the first phase of testis descent become noticeable at a later phase of the process.

THE FIRST PHASE OF TESTIS DESCENT

Based on the result obtained from the work presented in this thesis, the following conclusions can be drawn in relation to the three testicular factors:
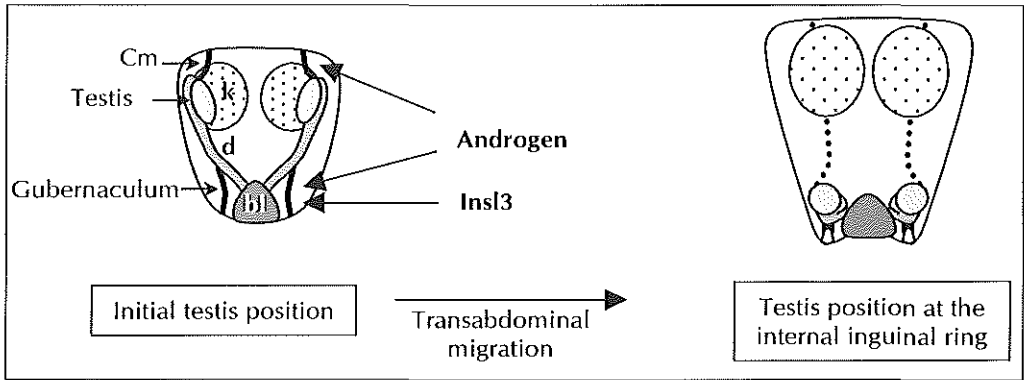
Figure 7.1 - First phase of testis descent in rodents
Due to outgrowth of the gubernaculum and the lack of CSL development, the testis gains a position at the bottom of the abdomen. Gubernaculum outgrowth is regulated by both Insl3 and androgens, whereas development of the CSL in the outer border of the cranial mesentery is prevented by androgens. bl, bladder; cm, cranial mesentery; d, duct; k, kidney.

AMH
There are no indications for a role of AMH during transabdominal testis descent in mice.

Androgens
Androgens affect development of the CSL. In addition, androgens stimulate growth of the gubernaculum, probably through action on the muscular component of the rodent gubernacular bulb.

Insl3
Insl3 stimulates growth of the rodent gubernacular bulb. In this respect, Insl3 can be considered to be a third testicular hormone produced by the testis, in addition to AMH and androgens, which plays a role in male sex differentiation. However, it cannot be excluded that the primary site of action of Insl3 is within the testis, and that such a local action of Insl3 might result in a cascade of events leading to production of (an) unknown testicular factor(s) acting directly on the gubernaculum.

A schematic representation of the first phase of testis descent with the most important structures and hormones involved is shown in Figure 7.1. This model is based on the data presented in this thesis, combined with data from the literature.

IN VITRO MODEL

Studies on the effects of hormones on the male genital tract *in vivo* are often difficult to interpret because the action of a hormone on a specific target structure cannot be assessed separately. To better define the role of Insl3, but also AMH and androgens, in the developmental regulation of the gubernaculum, an *in vitro* organ culture system was developed (Chapter 6).

In the initial experiments, fetal mouse gubernacula were cultured to devise a system which could be used to study the effect of testes from different mouse models on gubernaculum development. However, no growth response could be induced, neither by testicular tissue nor by androgens. As rat gubernacula did respond when cultured in the presence of mouse testes, it was concluded that mouse gubernacula appeared to be non-responsive in the *in vitro* culture system, under the present conditions (see below).

Analysis of gubernaculum development in fetal pig demonstrated a rapid increase in the amount of DNA per gubernaculum in the outgrowth phase, whereas no increase was measured during the regression phase (Fentener van Vlissingen *et al.*, 1989). Thus, cell proliferation in the porcine gubernaculum completely arrests when the structure starts to regress. Transabdominal descent in mice occurs within a short period of time, between E15.5 and E17.5. In the experiments described in this thesis, mouse gubernacula were dissected at E16, when gubernaculum outgrowth had started. It might be possible that during the culture period of at least two days, gubernaculum outgrowth is terminated and cell proliferation has ceased. Thus, by the time the growth response assay was performed at the end of the culture period, no increase in the growth rate could be determined anymore. In contrast, the period of gubernaculum outgrowth in the fetal rat is longer, as transabdominal descent occurs between E16 and E20. This may explain why we could demonstrate a growth response using rat gubernacula.

The developed *in vitro* model has been very useful in studying effects of AMH, androgens and Insl3 on gubernaculum development, measured by cell proliferation. The *in vitro* culture system would also be a suitable system for solving the question whether Insl3 acts either directly on the gubernaculum itself or indirectly via a gene product downstream of Insl3 action. Until now, this question could not be addressed due to lack of Insl3 protein and specific antibodies against Insl3 that could block Insl3 action. Both Fentener van Vlissingen *et al.* (1988) and Visser and Heyns (1995) have previously demonstrated that a low molecular weight factor, isolated from the fetal pig testis, stimulated growth in cultured gubernaculum cells. Since Insl3 has also been identified in the pig and porcine Leydig cells express *Insl3*, it seems that the growth stimulatory effect observed by these investigators was related to Insl3 activity.

It was previously shown *in vivo* that gubernaculum outgrowth in the fetal pig is marked by both rapid cell proliferation and changes in extracellular matrix (ECM) composition (Fentener van Vlissingen *et al.*, 1989; Heyns *et al.*, 1990). The *in vitro* culture system could be further improved if in addition to cell proliferation also changes in the ECM could be monitored, in particular since Insl3 might play a role in promoting changes of the ECM (see below).

INSL3

The role of Insl3 during development

Insulin-like factor3 (Insl3) is a member of the insulin-like hormone superfamily which comprises insulin, relaxin, and the insulin-like growth factors I and II (IGF-I and -II)

(Adham *et al.*, 2000). Insl3 was originally identified as a product of Leydig cells from the porcine testis and initially named Leydig cell insulin-like factor (Ley I-L) (Adham *et al.*, 1993). This factor is also named relaxin-like factor. Insl3 is a single gene product, and the gene consists of 2 exons. In line with other members of the insulin-like hormone super-family, Insl3 is synthesized as a prepropeptide consisting of a signal peptide, a B-chain, a connecting C-peptide and an A-chain (Adham *et al.*, 2000). It is expected that bioactive Insl3 is formed after enzymatic removal of the C-peptide, similar as for insulin and relaxin. In contrast, pro-IGF-I and pro-IGF-II contain a small C-peptide, which is maintained in the active proteins. Insl3 probably mediates its action by binding to a specific receptor on the membrane of the target cell. In mice, *Insl3* mRNA is specifically expressed in the Leydig cells of the fetal and adult testis and in the theca cells of the postnatal ovary (Pusch *et al.*, 1996; Zimmermann *et al.*, 1997). Production of Insl3 by fetal mouse Leydig cells is independent of gonadotropin stimulation (Balvers *et al.*, 1998). Due to the identification of functional SF1 sites in the *Insl3* promoter, SF1 is probably a mediator of *Insl3* gene transcription during male sex differentiation (Zimmermann *et al.*, 1998). Gonadotropins become essential for the maintenance of *Insl3* expression in the postnatal and adult testis, as supported by the observation that *hpg* mice lack *Insl3* expression in adulthood (Balvers *et al.*, 1998). Insl3 is thought to function primarily in close proximity to its site of produc-tion in the fetus and as a paracrine factor in the gonads, although the presence of Insl3 in human serum might indicate additional functions of a more endocrine nature (Bullesbach *et al.*, 1999).

Because little was known about a possible role and function of Insl3, mice were gener-ated with a targeted deletion of the *Insl3* gene, to provide insight into the primary functions of Insl3 (Chapter 3). Female mice lacking Insl3 appear to suffer no adverse consequences from the loss of this gene product. However, the loss of *Insl3* gene product affected male reproduction profoundly. Male mice deficient for Insl3 had bilateral undescended testes, probably due to failure of gubernaculum development. This phenotype was unexpected, but confirmed the previously proposed involvement of a third testicular factor in the proc-ess of gubernaculum development (Fentener van Vlissingen *et al.*, 1988). The generation of Insl3-deficient mice as reported in this thesis, and several months later by Nef and Parada (1999), has linked the *Insl3* gene to the process of testis descent. This finding has been a major step towards understanding the molecular and cellular mechanism of testis descent and will stimulate further research.

The pattern of *Insl3* expression in mice is consistent with a role in gubernaculum devel-opment (Zimmermann *et al.*, 1997). *Insl3* shows a male-specific pattern of expression in the fetal testis from E13.5 onwards. In contrast, the ovaries do not produce Insl3 until after birth. However, unscheduled activity of the Insl3 pathway throughout female development might occur due to, for example, an activating mutation in the membrane-bound Insl3 receptor or ectopic expression through a mutation in the *Insl3* gene promoter. This would cause gubernaculum development in the female fetus, maybe even leading to partial ovar-ian descent. The round ligament, also called uterine teres ligament, is considered the female homolog of the male gubernaculum (Attah and Hutson, 1991). This ligament is probably the ligament found in the inguinal hernia sac of girls, which can contain Fallopian tubes

and/or ovaries (Boley *et al.*, 1991). In a recent study by Ozbey *et al.* (1999), the round ligament of girls who underwent inguinal hernia repair was evaluated, and it was concluded that an ovary in a hernia sac might not be simply prolapsed but has to be considered a descended gonad. Therefore, inguinal hernia in girls might conceivably be the human phenotype of ectopic *INSL3* expression or activation of the Insl3 signal transduction pathway. Since there are no indications from mouse studies that Insl3 interferes with production of androgens or AMH (Chapter 3), these girls will be otherwise normal female children, without masculinization. The generation of Insl3 transgenic mice, showing expression of *Insl3* in fetal ovaries, will be a very useful tool to study the effect of aberrant *Insl3* expression in the female fetus.

The human gene encoding INSL3 has been characterized and is mapped to the region p13.2-p12 of chromosome 19 (Burkhardt *et al.*, 1994). It is to be expected that INSL3 will also play a role in testis descent in the human, although evidence for such a role in the human remains to be presented. Up till now there are no indications that INSL3 deficiency is a frequent etiological factor in bilateral undescended testes in men (Chapter 4). The function of INSL3 in men might differ from that in mice. Consequently, mutations in the *INSL3* gene in the human would lead to another, maybe milder phenotype. It can also be speculated that impaired INSL3 function might occur in the human due to target organ insensitivity, caused by mutations in the gene encoding the as yet unidentified receptor. Recently, specific, high affinity INSL3 binding sites have been described (Bullesbach and Schwabe, 1999). The cloning of a Insl3 receptor is eagerly awaited, since this would be a very useful tool to further explore the function of Insl3 in testis descent.

Possible function of Insl3 in the adult gonad

The *Insl3* gene is expressed in both testis and ovary of the human, mouse, sheep, cow and marmoset monkey (Tashima *et al.*, 1995; Bathgate *et al.*, 1996; Roche *et al.*, 1996; Ivell *et al.*, 1997; Zimmermann *et al.*, 1997; Zarreh-Hoshyari-Khah *et al.*, 1999).

The defect in spermatogenesis seen in the Insl3-deficient mice appears to be secondary to the intra-abdominal position of the testis. By bringing the testis into the scrotum at the day of birth, spermatogenesis can occur in *Insl3* knockout males (Chapter 3). This finding suggests that Insl3 is probably not directly involved in spermatogenesis. Moreover, *Insl3* expression may not require ongoing spermatogenesis, since it has been observed that INSL3 protein remains highly expressed in the human testis even in patients with severely disturbed spermatogenesis (Ivell *et al.*, 1997). In addition, sterile mutant mice (lacking a functional kit ligand/receptor system), caused by a defect in the embryonic migration of primordial germ cells resulting in a complete lack spermatogenesis, normally express *Insl3* mRNA (Balvers *et al.*, 1998). However, these data do not exclude a possible role of Insl3 in the testis. The *Insl3* gene is abundantly expressed in the adult testis, being restricted to the Leydig cells (Ivell, 1997). Exposure of the adult rat testis to ethane dimethyl sulphonate results in destruction of Leydig cells, which coincides with the loss of *Insl3* mRNA expression. As Leydig cells start to reappear, expression of *Insl3* mRNA is also detectable again (Teerds *et al.*, 1999). Recently, it has been shown that expression of *Insl3* mRNA is downregulated in human Leydig cell hyperplasia and adenoma (Klonisch *et al.*, 1999). In order to

gain more insight in the possible role of Insl3 in the adult testis, conditional inactivation of the *Insl3* gene in male mice after completion of testis descent would provide a very helpful animal model to study this.

Although *Insl3* is predominantly expressed in the testis, *Insl3* is also expressed in the ovary in growing follicles and the corpus luteum (Bathgate *et al.*, 1996; Roche *et al.*, 1996; Zimmermann *et al.*, 1997). Insl3 does not seem to play a prominent role in female mice, as Insl3-deficient females are fertile (Chapter 3), although smaller litter sizes were reported by Nef and Parada (1999). *INSL3* is also expressed in the human ovary and in trophoblast tissue and can be measured in serum of postpubertal females, but nothing is known about a possible function of Insl3 in the female (Tashima *et al.*, 1995; Bullesbach *et al.*, 1999). It has been demonstrated in a mouse pubic symphysis assay, that Insl3 acts in synergy with relaxin on widening of the mouse pubic symphysis (Bullesbach and Schwabe, 1995). Relaxin is a family member of Insl3 and has a broad range of biologic activities in the female reproductive tract, which are all related to parturition, including the inhibition of uterine contractile activity, relaxation of the pubic ligaments during pregnancy, preparation of the endometrium and softening of the cervix before delivery (MacLennan, 1981). No *relaxin* gene has been identified in ruminants so far, but *Insl3* is highly expressed in the follicles and corpus lutea of both the bovine and ovine ovary (Bathgate *et al.*, 1996; Roche *et al.*, 1996). On the basis of these findings, a relaxin-like role of Insl3 in certain animal species has been proposed (Ivell, 1997).

Mechanism of Insl3 action

Primary structure analysis revealed that Insl3 is more related to relaxin then to insulin or the IGFs (Bullesbach and Schwabe, 1995). As discussed above, it has even been proposed that Insl3 might be a physiological substitute for relaxin in certain animals (Ivell, 1997). This suggests that Insl3 and relaxin might have a very similar mechanism of action. Relaxin is best known for its action on the softening of the tissues of the birth canal in preparation for delivery, and is named after this action. Relaxin is thought to play a significant role in remodelling of connective tissue of most target tissues (MacLennan, 1981). Findings on changes in the biochemical composition of the cervix and the pubic symphysis have been derived from studies on animal models (Sherwood *et al.*, 1993; Samuel *et al.*, 1998). During pregnancy, the collagen content and solubility of collagen in the cervix and symphysis increases. In addition, increased synthesis of extracellular glycosaminoglycans (GAG) is observed. Although the combined concentration of the three predominant GAG dermatan sulfate, hyaluronic acid and heparan sulfate, does not change, there are changes in the relative concentrations of the individual GAG. The increase in GAG is greater then the net increase in collagen, which may contribute to remodelling of the connective tissue. The observed changes in extracellular matrix were associated with increased water content. There is strong evidence that the above-described changes are promoted by relaxin. Gubernaculum outgrowth is also characterized by the synthesis of both GAG and collagen (Fentener van Vlissingen *et al.*, 1989; Heyns *et al.*, 1989; Heyns *et al.*, 1990). Relaxin can also exert mitogenic activity (MacLennan, 1981). It is therefore tempting to speculate that Insl3 is directly involved in gubernaculum outgrowth by inducing both cell growth and changes

in the extracellular matrix. Clearly, the possible role of induced changes in the extracellular matrix in Insl3 action deserves further study.

ESTROGENS AND TESTIS DESCENT

There is an increasing amount of evidence that endogenous estrogens do play a role in the normal functioning of the reproductive tract in the adult male, mainly based on observations from estrogen receptor α and β knockout models (Couse and Korach, 1999). However, there is no apparent role for estrogens in the development of the male reproductive tract, including the process of testis descent. Analysis of the process of testis descent in estrogen receptor α deficient mice revealed an excessive development of the cremaster muscle in these mice, without a direct effect on the process of testis descent (Donaldson et al., 1996).

The adverse effect of prenatal exposure to a supra-physiological amount of exogenous estrogen on testis descent has been shown in many animal experiments but also in men exposed to the synthetic estrogen diethylstilbestrol (DES) before birth via their mothers (Newbold and McLachlan, 1996). The male offspring of mothers taking DES during pregnancy demonstrate many urogenital tract abnormalities, which include epididymal cysts, microphallus, testicular hypoplasia and cryptorchidism (Cosgrove et al., 1977; Stillman, 1982). The incidence of cryptorchidism, hypospadias and testicular cancer in the general population has been reported to be increased in the last decades (Giwercman and Skakke-baek, 1992; John Radcliffe Hospital Cryptorchidism Study Group, 1992). There is a general concern that chemicals with estrogenic activity in our environment might act as endocrine disrupters, and thereby attribute to the increasing incidence of male reproductive tract abnormalities, similar to the abnormalities observed in DES exposed males (Sharpe and Skakkebaek, 1993; Cheek and McLachlan, 1998). The mechanisms by which estrogens induce these effects are largely unknown, although it has been suggested that estrogens might affect follicle-stimulating hormone (FSH) secretion by the pituitary and/or normal production of AMH by Sertoli cells (Sharpe and Skakkebaek, 1993).

During analysis of the male phenotype of Inls3-deficient mice, similarities between this phenotype and the phenotype of prenatally estrogen-exposed male mice became apparent; intra-abdominal position of the testes and lack of gubernaculum development. Based on this observation, we postulated that exposure to exogenous estrogens might interfere with fetal Insl3 expression. Therefore, expression of Insl3 mRNA in control and DES-exposed fetuses was studied during the phase of transabdominal descent (Chapter 5). Since SF1 might be a transciptional regulator of Insl3 expression in the fetal gonad, the effect of DES on SF1 expression in the fetal testis was also determined. SF1 expression in the fetal testis was not changed after exposure to DES. In contrast, expression of Insl3 was significantly reduced in the DES-exposed testes when compared to testes of control fetuses. Thus, the undescended testes observed in DES-exposed fetuses might be related to diminished Insl3 expression. Future studies will need to focus on the mechanism by which estrogens decrease Insl3 expression. Estrogens might directly repress Insl3 gene transcription or could

have an inhibitory influence on Leydig cell function which would indirectly lead to a decrease in *Insl3* gene expression. Alternatively, a direct effect of estrogens on the gubernaculum might also occur. A recent report showed the presence of estrogen receptor α in the gubernaculum of the fetal pig (Barthold *et al.*, 1999).

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Summary
Samenvatting

SUMMARY

The migration of the testis from its site of origin in the upper body cavity towards the bottom of the abdomen and finally into the scrotum is called testis descent. In many mammalian species, the testis needs a lower temperature for spermatogenesis to take place, and a testis temperature several degrees centigrade below body temperature can be reached in the scrotum. Mammals (most, but not all mammalian species) are the only animals in which the testis descends. The total process of testis descent can be generally divided into two phases: the first or transabdominal phase, which involves movement of the testis from its initial, high abdominal position to the bottom of the abdomen. During the second or inguino-scrotal phase, the testis moves from the abdominal bottom, through the inguinal canal, into the scrotum. The complex process of testis descent involves a highly complex interplay of different factors and structures, which is far from being completely understood.

The transabdominal movement of the testis depends on the differential development of two ligaments, the gubernaculum and the cranial suspensory ligament (CSL). The gubernaculum is a structure that develops between the gonad and the abdominal bottom. In rodents, the gubernaculum can be subdivided into two parts: a cranial part, which is called the gubernacular cord and a caudal part that is called the gubernacular bulb. The gubernacular bulb consists of a mesenchymal core with a muscular cover. The CSL is a cord-like structure in the border of the cranial (mesonephric) mesentery of the gonad, which connects the gonad cranially to the abdominal body wall, at a position near the last rib. The gonadal position appears to be determined by the opposing development and actions of the CSL and the gubernaculum. In the male fetus, the gubernaculum rapidly develops whereas differentiation of the CSL primordium is lacking, and the testis gains a position low in the abdomen, near the inguinal region. By contrast, the ovary is held high in the abdomen by the well-developed CSL and no gubernaculum outgrowth is observed.

Since exogenous hormones can impair testis descent, it is assumed that endogenous hormones normally control testis descent. The fetal testis produces two hormones that are essential for male sex differentiation, testosterone and anti-Müllerian hormone (AMH). Testosterone is generally believed to play a role in testis descent, although its precise mechanism of action is unclear. A site of androgen action is the CSL primordium; exposure of this primordium to androgen prevents outgrowth. Gubernaculum outgrowth is postulated to be under AMH control, although no positive experimental evidence for such a role has been provided so far. Involvement of a third testicular factor/hormone in the first phase of testis descent was proposed, specifically with regard to gubernaculum development.

This thesis focuses on the hormonal control of the first phase of testis descent, with the gubernaculum and the CSL as the main target structures. In the General Introduction (Chapter 1) an overview is given of male sex differentiation, with particular emphasis on the process of testis descent.

In Chapter 2, a study is presented where exogenous androgens were administered to pregnant rats during different gestational stages to investigate if inhibition of the outgrowth of the fetal CSL is limited to a critical period of sex differentiation. Pregnant rats

were daily injected with 5α-dihydrotestosterone propionate and exposed female fetuses were evaluated for the presence of the CSL at birth or in adulthood. CSL development was only prevented when female fetuses were exposed to androgens before E19 (19 days after conception), at the early stages of genital development. Immunohistochemical detection of the androgen receptor (AR) was performed to investigate whether this androgen responsive phase was paralleled by AR protein expression within the cranial mesentery, which contains the CSL primordium. Continuous, high expression of AR was found in the cranial mesentery of male rat fetuses. In female fetuses, AR expression varied at different prenatal days, with highest expression at E17. The gradual decrease in AR expression in the mesenteric tissue in the female fetus from E18 onwards coincided with the appearance of a differentiated CSL in the border of the mesentery, as evidenced by the expression of two cell differentiation markers: α-smooth muscle actin and desmin. The difference in CSL development between the sexes was not related to differences in mitotic activity or apoptosis of cells in the cranial mesentery. These results indicate that prevention of outgrowth of the CSL primordia by androgens occurs during early genital development. The underlying mechanism appears not to be inhibition of growth but rather may involve an inhibition of differentiation.

Insulin-like factor 3 (Insl3) is a member of the insulin-like hormone family. The *Insl3* gene is specifically expressed in Leydig cells of the pre- and postnatal testis and in the theca cells of the postnatal ovary. Hence, during fetal development, only the testis but not the ovary produces Insl3. To investigate the role of Insl3 in mammalian sex differentiation, Insl3-deficient mice were generated (Chapter 3). Male mice homozygous for targeted disruption of the *Insl3* gene (knockout mouse model) exhibited bilateral undescended testes, which were freely mobile within the abdominal cavity. Histological analysis of male *Insl3* knockout mice fetuses revealed that development of the gubernaculum was severely affected. There were no indications that Insl3 interfered with either AMH or androgen function. *Insl3* knockout female mice were apparently unaffected. This suggests that the Insl3 factor might be the as yet unidentified testicular factor, which is specifically involved in gubernaculum development. In *AR* and *Insl3* double knockout mice, which also lack a functional AR in addition to Insl3, the testes became located in a position that is comparable to that of ovaries in wild-type female mice. The testes of these double knockout mice were positioned adjacent to the kidneys, attached to the abdominal wall via well-developed CSLs without any indication of gubernaculum development. These results indicate that both androgens and Insl3 are essential for establishment of the sex dimorphic position of the gonads.

Cryptorchidism, or failure of the testis to descend into the scrotum, is the most common disorder of male sex differentiation. In the majority of affected boys, cryptorchidism is an isolated anomaly of which the pathogenesis is unknown. Since the *INSL3* gene is present in the human genome and Leydig cells in the human testis express *INSL3*, it is suggested that INSL3 might also be involved in testis descent in the human. The aim of the study described in Chapter 4 was to investigate whether bilateral cryptorchidism in the human might be associated with mutations in the *INSL3* gene. After molecular analysis of the coding parts of the *INSL3* gene, two base pair substitutions were found in several indi-

viduals with bilateral undescended testes. However, these substitutions were also found in control individuals and are therefore considered to be frequent polymorphisms and not pathogenic mutations. Up till now there are no indications that INSL3 deficiency is a frequent cause of bilateral cryptorchidism in men. These findings, however, do not conclusively exclude a role for INSL3 in testis descent in the human. Impaired INSL3 function might also occur due to target cell insensitivity, caused by mutations in the gene encoding the as yet unidentified INSL3 receptor.

It is known for many years that unscheduled exposure to estrogens during gestation can cause undescended testes. Findings from studies with laboratory animals indicated that exogenous estrogens, including the synthetic estrogen diethylstilbestrol (DES), disrupts the first phase of testis descent probably by affecting gubernaculum outgrowth. Although the effect of DES on fetal development has been the subject of numerous studies, the mechanism of action has remained elusive. In Chapter 5, a study is presented, aimed to investigate whether maternal exposure to DES might affect *Insl3* expression in the developing male mouse fetus at the time of transabdominal testis descent. Since steroidogenic factor 1 (SF1) is a candidate transcription factor to be involved in regulation of the expression of the *Insl3* gene in the fetal testes, the effect of DES on Sf1 mRNA expression was also determined. Histological analysis of the fetal male genital tract confirmed that prenatal exposure to DES has an adverse effect on gubernaculum development. A marked and significant decrease in the amount of *Insl3* mRNA was observed in DES-exposed testes when compared to control testes. In contrast, DES had no effect on *Sf1* mRNA expression in the fetal testis. The present data support the idea that estrogens might interfere with normal gubernaculum development and consequently testis descent, through reduction of expression of *Insl3* mRNA in the fetal testis.

Chapter 6 describes the establishment of an organ culture technique for in vitro culturing of gubernacula. This provided a unique opportunity to study hormonal control of development of the fetal gubernaculum under precise and defined conditions. Rat gubernacular explants from E17 fetuses were cultured in basal medium for up to 5 days, either in the presence or absence of mouse gonads. The heterologous rat-mouse system was chosen to overcome problems with the mouse-mouse system. A testis had a growth stimulatory effect on gubernacular explants in comparison with an ovary or basal medium. The morphology of gubernacula explants grown in the presence of a testis was maintained and comparable with the histological appearance of gubernacula differentiated in vivo. These results indicate a direct involvement of testicular factors in gubernaculum differentiation. In addition, AR expression was evident in the mesenchymal core of the developing gubernacular bulb in vitro and in vivo, suggesting a role of androgens in gubernaculum development. Indeed, addition of R1881, a synthetic androgen, to the culture medium increased cell proliferation in gubernacular explants, although this effect did not exceed the effect induced by a testis. By means of this organ culture system, the growth stimulatory effects of gonads from AMH- or Insl3-deficient mice on gubernacular explants was studied. Testes from $Amh^{-/-}$, $Amh^{+/-}$ and $Insl3^{+/-}$ animals ($^{-/-}$ stands for knockout and $^{+/-}$ for heterozygous mutant animals) stimulated growth of gubernacular explants to the same extent as control testes. $Insl3^{-/-}$ testes, however, did not produce such an activity. These findings sug-

gest that both androgens and Insl3, directly and/or indirectly, are involved in gubernacular development in the mouse.

Chapter 7 presents a general discussion of the findings described in this thesis. In addition, suggestions for further research are given. In summary, both androgens and Insl3 appear to be important during the transabdominal phase of testis descent in the rodent model, with the CSL and gubernaculum as target structures. No indications were obtained that AMH is involved in this part of the process. It can be predicted that future research will reveal the precise role of Insl3 in the process of testis descent in mammals. Cloning of the Insl3 receptor would provide a useful tool to study the molecular mechanism of Insl3 signaling. Moreover, generation of transgenic mice overexpressing Insl3 may further help to define the specific role of Insl3 in the development and function of the reproductive system. Eventually, this research will lead to further knowledge about endogenous and exogenous factors leading to cryptorchidism in the human.

Samenvatting

De migratie van de testis (testikel, zaadbal of teelbal) vanuit zijn oorspronkelijke positie hoog in de buikholte naar het scrotum wordt testisindaling genoemd. Zoogdieren zijn de enige diersoorten waarbij de testikels indalen. De positie van de testis in het scrotum is door de enigszins lagere temperatuur van essentieel belang voor de zaadcelproductie. Het gehele proces van testisindaling kan worden opgedeeld in twee fasen. Tijdens de eerste of trans-abdominale fase verplaatst de testis zich van zijn oorspronkelijke positie hoog in de buikholte naar de bekkenbodem. Tijdens de tweede of inguinale-scrotale fase daalt de testis vanaf de bekkenbodem, via het lieskanaal, af naar het scrotum. Het complexe proces van testisindaling wordt gecontroleerd door een wisselwerking van verschillende factoren en structuren, waarover nog weinig duidelijkheid bestaat.

De trans-abdominale fase van testisindaling is afhankelijk van de differentiële ontwikkeling van twee ligamenten (bindweefselbanden), het gubernaculum en het craniale suspensorische ligament (CSL). Het gubernaculum is een structuur die zich ontwikkelt tussen de gonade en de bekkenbodem. In knaagdieren wordt het gubernaculum onderverdeeld in twee delen: een craniaal deel, dat het gubernaculumkoord wordt genoemd, en een caudaal deel, dat de gubernaculum conus wordt genoemd. De gubernaculum conus bestaat uit een kern van mesenchymale cellen met daaromheen een spierlaag. Het CSL is een koordachtige structuur in de rand van het craniale mesenterium van de gonade, welke de gonade met de buikwand verbindt, ter hoogte van de onderste rib. De positie van de gonade lijkt bepaald te worden door de tegengestelde werkingen van het gubernaculum en het CSL. In een mannelijk embryo groeit het gubernaculum snel uit terwijl het CSL zich niet ontwikkelt, zodat de testis laag in de buikholte terecht komt, in de buurt van het lieskanaal. In tegenstelling tot de testis wordt het ovarium (de eierstok) hoog in de buikholte gehouden door het sterk ontwikkelde CSL, terwijl in de vrouwelijke foetus geen uitgroei van het gubernaculum waargenomen wordt.

Omdat exogene hormonen de testisindaling kunnen veranderen, wordt aangenomen dat testisindaling gereguleerd wordt door endogene hormonen. De testis in de mannelijke foetus produceert twee hormonen waarvan de werking essentieel is voor mannelijke geslachtsdifferentiatie, testosteron en het anti-Müllerse gang hormoon (AMH). Het wordt algemeen aangenomen dat testosteron (een androgeen steroidhormoon) een rol speelt in testisindaling, alhoewel het precieze werkingsmechanisme van testosteron niet duidelijk is. Een doelwitweefsel van androgenen is de aanleg van het CSL; blootstelling aan androgenen verhindert de uitgroei van het CSL. Uitgroei van het gubernaculum is verondersteld afhankelijk te zijn van de aanwezigheid van AMH, alhoewel dit tot nu toe nog niet experimenteel bewezen is. Er is gepostuleerd dat een derde hormoon of factor uit de testis belangrijk is voor regulatie van de eerste fase van testisindaling en met name de ontwikkeling van het gubernaculum.

Het onderzoek dat in dit proefschrift beschreven is, heeft zich gericht op de hormonale regulatie van de eerste fase van testisindaling, met het gubernaculum en het CSL als belangrijkste doelwitstructuren. In de algemene inleiding (Hoofdstuk 1) wordt een overzicht gegeven van de huidige kennis met betrekking tot mannelijke geslachtsontwikkeling,

waarbij de nadruk gelegd is op het proces van indaling.

In Hoofdstuk 2 wordt een studie beschreven waarin androgenen tijdens verschillende perioden van de zwangerschap aan ratten werden toegediend om te bepalen of de uitgroei van het CSL tijdens een specifieke periode van de geslachtsontwikkeling wordt geremd. Zwangere ratten werden dagelijks geïnjecteerd met het androgeen 5α-dihydrotestosteron propionaat en de foetaal blootgestelde vrouwtjes werden geëvalueerd op aanwezigheid van het CSL bij de geboorte of wanneer ze volwassen waren geworden. De ontwikkeling van het CSL kon voorkomen worden wanneer vrouwelijke foetussen blootgesteld waren aan androgenen voor dag 19 van de zwangerschap, tijdens de vroege fase van de geslachtsontwikkeling. Middels immunohistochemie werd nagegaan of tijdens de androgeen-gevoelige periode het androgeenreceptor eiwit waargenomen kon worden in het craniale mesenterium. In de androgeen-gevoelige periode werd in het mesenterium van mannelijke foetussen een constant hoog expressieniveau gevonden. In vrouwelijke foetussen daarentegen varieerde de hoeveelheid androgeenreceptor per dag van de zwangerschap en was er sprake van een maximale expressie op dag 17 van de zwangerschap. De geleidelijke afname van de hoeveelheid androgeenreceptor in het mesenterium weefsel van vrouwelijke foetussen ging gepaard met het verschijnen van een gedifferentieerd CSL in de rand van het mesenterium, aangetoond door de aanwezigheid van een tweetal celdifferentiatie eiwitten: actine en desmine. Het verschil tussen mannelijke en vrouwelijke foetussen in ontwikkeling van het CSL was niet gerelateerd aan verschillen in celdelingsactiviteit of geprogrammeerde celdood (apoptosis) van cellen in het craniale mesenterium van beide geslachten. Deze resultaten geven aan dat de uitgroei van de aanleg van het CSL door androgenen verhinderd wordt tijdens de vroege fase van de geslachtsontwikkeling. Het onderliggende werkingsmechanisme lijkt geen groeiremming te zijn, maar remming van de differentiatie.

Insuline-achtige factor 3 (Insl3) is een lid van de insuline-achtige hormoonfamilie. Het *Insl3* gen komt tot expressie in pre- en postnatale Leydigcellen van de testis en in de postnatale thecacellen van het ovarium. Om de rol van Insl3 in de mannelijke geslachtsontwikkeling na te gaan, werden er muizen gemaakt waarin het *Insl3* gen werd uitgeschakeld (*Insl3* knockout muizen). Mannelijke *Insl3* knockout muizen bleken tweezijdig niet-ingedaalde testes te hebben, welke zich vrij in de buikholte konden bewegen. Histologische analyse van mannelijke *Insl3* knockout muizenfoetussen toonde aan dat de ontwikkeling van het gubernaculum ernstig aangedaan was. Er waren geen aanwijzingen voor een verminderde werking van AMH of androgenen. Vrouwelijke *Insl3* knockout muizen hadden geen aantoonbare afwijkingen. Deze resultaten wijzen erop dat Insl3 de tot nu toe niet-geïdentificeerde testiculaire factor zou kunnen zijn die specifiek betrokken is bij de ontwikkeling van het gubernaculum. In dubbel-knockout muizen die zowel androgeen- als Insl3-gemedieerde functies missen, werden de testes in een positie aangetroffen die overeenkomt met de positie van de ovaria in normale vrouwelijke muizen. De testes van deze dubbel-knockout muizen liggen ter hoogte van de nieren, opgehangen aan de buikwand via sterk ontwikkelde CSL's, terwijl er geen enkele aanwijzing is voor ontwikkeling van het gubernaculum. Deze resultaten geven aan dat zowel androgenen als Insl3 een essentiële rol spelen bij de tot stand koming van het geslachtsverschil in de positie van de gonaden.
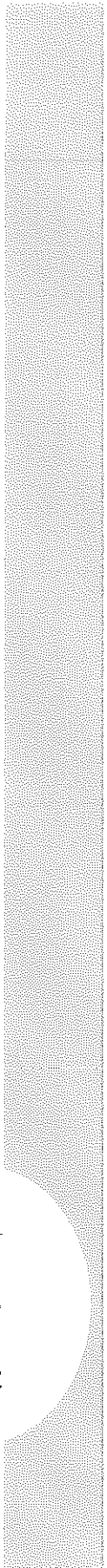
Cryptorchisme, of het niet indalen van één of beide testes, is de meest voorkomende stoornis in de mannelijke geslachtsontwikkeling. In de meerderheid van de patiënten is cryptorchisme een geïsoleerde afwijking waarvan de oorzaak niet bekend is. Omdat het *INSL3* gen aanwezig is in het menselijke genoom en de Leydigcellen van de humane testis dit gen tot expressie brengen, zou INSL3 mogelijk ook betrokken kunnen zijn bij het proces van testisindaling in de mens. Het doel van de studie beschreven in Hoofdstuk 4 was om te bepalen of bilateraal cryptorchisme (tweezijdig niet ingedaalde testes) mogelijk geassocieerd is met mutaties in het *INSL3* gen. Na moleculaire analyse van de coderende gedeelten van het *INSL3* gen werden twee verschillende nucleotide substituties gevonden in patiënten met tweezijdig niet ingedaalde testes. Echter, deze twee substituties werden ook aangetroffen in controlepersonen en worden daarom als veelvoorkomende polymorfismen beschouwd en niet als pathogene mutaties. Tot nu toe zijn er geen aanwijzingen gevonden dat INSL3-deficiëntie gestoorde indaling bij de mens zou kunnen veroorzaken. Echter, deze bevindingen sluiten nog niet uit dat INSL3 ook in de mens een rol zou kunnen spelen in testisindaling. INSL3-dysfunctie zou mogelijk ook kunnen optreden door INSL3-ongevoeligheid van de doelwitcellen, veroorzaakt door mutaties in het gen dat codeert voor de nog te identificeren INSL3 receptor.

Het is al sinds lange tijd bekend dat blootstelling aan oestrogenen gedurende de zwangerschap tot cryptorchisme kan leiden. Studies met proefdieren toonden aan dat verschillende oestrogenen, waaronder het synthetische oestrogeen diethylstilbestrol (DES), de eerste fase van testisindaling kunnen verstoren, waarschijnlijk doordat de uitgroei van het gubernaculum wordt geremd. Alhoewel de effecten van DES op de foetale ontwikkeling in vele studies onderzocht zijn, is het mechanisme dat eraan ten grondslag ligt nog niet opgehelderd. In Hoofdstuk 5 wordt een studie beschreven waarin onderzocht is of blootstelling van muizen aan DES tijdens de zwangerschap de aanwezigheid van *Insl3* mRNA in de testes van mannelijke foetussen tijdens de transabdominale fase van testisindaling beïnvloedt. Omdat de expressie van *Insl3* in de foetale testis mogelijk gereguleerd wordt door steroidogenetische factor 1 (SF1), werd ook het effect van DES op *Sf1* mRNA expressie bepaald. Middels histologische analyse van de mannelijke geslachtsorganen van muizenfoetussen werd bevestigd dat prenatale blootstelling aan DES de ontwikkeling van het gubernaculum negatief beïnvloedt. Er werd een significante afname van de hoeveelheid *Insl3* mRNA waargenomen in de testes van foetussen die blootgesteld waren aan DES ten opzichte van controle foetussen. DES had daarentegen geen effect op de hoeveelheid *Sf1* mRNA in de foetale testes. Deze resultaten geven aan dat blootstelling aan exogene oestrogenen de normale ontwikkeling van het gubernaculum en vervolgens testisindaling zou kunnen verstoren door de expressie van *Insl3* mRNA in de foetale testes te verlagen.

Hoofdstuk 6 beschrijft het opzetten van een orgaankweektechniek voor het *in vitro* kweken van gubernacula. Met behulp van deze orgaankweek kon de hormonale regulatie van het foetale gubernaculum onder meer gedefinieerde omstandigheden bestudeerd worden. Gubernacula van mannelijke rattenfoetussen, uitgeprepareerd op dag 17 van de zwangerschap, werden 5 dagen in basaal medium gekweekt, in aan- of afwezigheid van een muizengonade. Testisweefsel had een groeistimulerend effect op gubernacula, in vergelijking met een ovarium of alleen basaal medium. De morfologie van gubernacula gekweekt

in aanwezigheid van een testis was vergelijkbaar met de morfologie van *in vivo* gedifferentieerde gubernacula. Verder kon de aanwezigheid van androgeenreceptoren in de mesenchymale kern van de zich ontwikkelende gubernaculum conus worden aangetoond, zowel na de ontwikkeling *in vitro* als *in vivo,* hetgeen duidt op een mogelijke rol van androgenen in de ontwikkeling van het gubernaculum. Toevoeging van een synthetisch androgeen, R1881, aan het kweekmedium verhoogde het aantal celdelingen in de gubernacula, alhoewel dit effect niet groter was dan het effect van toevoeging van een testis. Met behulp van dit orgaankweeksysteem werden ook de effecten van testes van *AMH* en *Insl3* knockout muizen op de groei van het gubernaculum bestudeerd. Testes van *AMH* knockout muizen stimuleerden de groei van gubernacula in dezelfde mate als controle testes. Echter, testes van *Insl3* knockout muizen vertoonden een verminderde groeistimulatie. Op basis van deze resultaten kan geconcludeerd worden dat zowel androgenen als Insl3, direct en/of indirect, betrokken zijn bij de ontwikkeling van het gubernaculum.

Hoofdstuk 7 bevat een algemene discussie van de in dit proefschrift beschreven bevindingen. Ook worden aanbevelingen gedaan voor toekomstig onderzoek. Samengevat lijken zowel androgenen als Insl3 een belangrijke rol te spelen tijdens de transabdominale fase van testisindaling in het knaagdier-model, met het CSL en het gubernaculum als doelwitstructuren. Er zijn geen aanwijzingen gevonden dat AMH betrokken is bij dit proces. Verder onderzoek zal de functie van Insl3 bij de testisindaling verduidelijken. De verdere opheldering van het moleculaire werkingsmechanisme van Insl3 zal vergemakkelijkt worden na klonering en karakterisering van het gen dat codeert voor de Insl3 receptor. Verder zou de ontwikkeling van transgene muizen waarin *Insl3* tot verhoogde expressie gebracht is, een specifieke bijdrage kunnen leveren aan het verder definiëren van de rol van Insl3 in de ontwikkeling en functie van de voortplantingsorganen, inclusief het gubernaculum. Uiteindelijk kan dit onderzoek leiden tot meer kennis over endogene en exogene factoren die cryptorchisme bij de mens veroorzaken.

Dankwoord
List of publications
Curriculum vitae

DANKWOORD

LIST OF PUBLICATIONS

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J.M.A. Emmen, A. McLuskey, I.M. Adham, W. Engel, J.A. Grootegoed and A.O. Brinkmann. Hormonal control of gubernaculum development during testis descent; gubernaculum outgrowth *in vitro* requires both Insl3 and androgen. *Submitted.*

CURRICULUM VITAE

Judith Emmen was born on the 12th of January 1970 in Breda. Her secondary education started in 1982 at the Mencia de Mendoza Lyceum in Breda. She graduated in 1988. In the same year she started her study Biology at the University of Utrecht. Her three doctoral stages included a main subject in Comparative Endocrinology at the Department of Experimental Zoology, University Utrecht under supervision of Dr J.G.D. Lambert, entitled "Optimalization of a high-performance liquid chromatography system to localize dopamine and its metabolites in the brain of the African catfish". A second subject in Neuroethology was performed at the Department of Comparative Physiology, University Utrecht, under supervision of Dr. R.C. Peeters, on the topic "Effects of changes in plasma calcium concentration and a hypocalcemic hormone on electroreceptor organs in the American catfish". Furthermore, a subject in Cell Biology entitled "Use of pituitary cells in a perfusion system to study the effects of testosterone on the secretion of gonadotropin in the goldfish" was performed under supervision of Dr J.P. Chang at the Department of Zoology, University of Alberta, Edmonton, Canada. The Biology study was successfully completed in August 1994.

From October 1994 till October 1999, she worked on the research project presented in this thesis, at the Department of Endocrinology and Reproduction, Erasmus University Rotterdam, under supervision of Dr A.O. Brinkmann and Prof. Dr. J.A. Grootegoed. Within this time period, a fetal organ culture technique has been learned during a 3-month visit to the laboratory of Prof. Dr. G.R. Cunha, Department of Anatomy, University of San Francisco, California, USA.

Color illustrations

Chapter 2

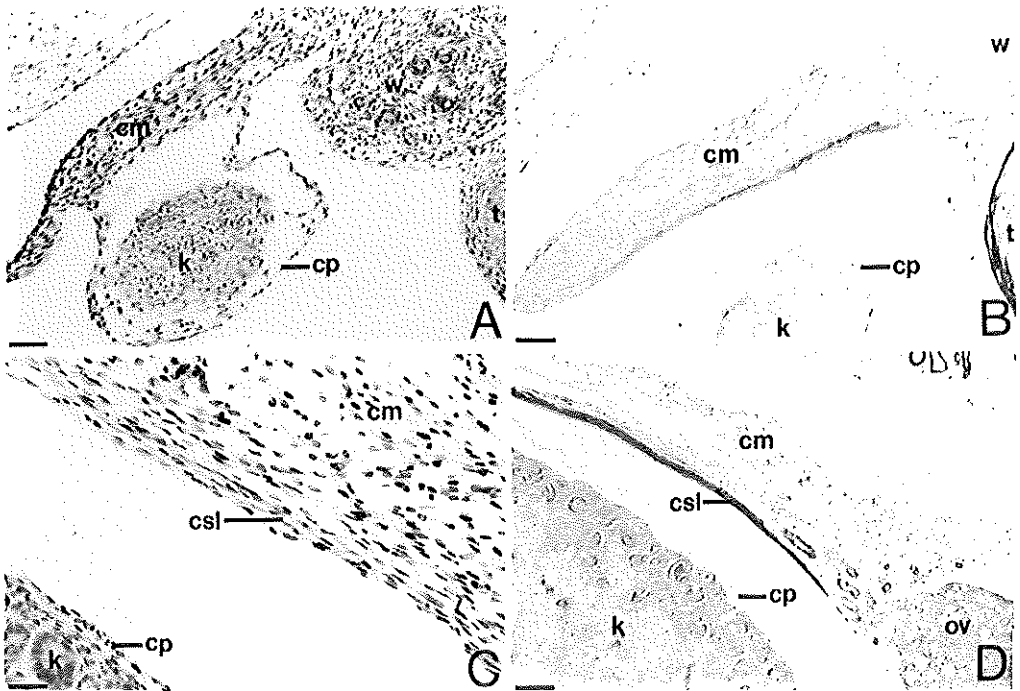*Androgen action during male sex differentiation includes suppression of cranial suspensory ligament development*

Figure 2.4 – Immunohistochemical localization of α-smooth muscle (α-SM) actin and androgen receptor (AR) in the cranial part of the urogenital system of male rat fetuses and female neonates. *(A, C)* AR immunostaining and *(B, D)* α-SM action immunostaining in E17 male fetuses (top) and female neonates on postnatal day 7 (bottom).

In the E17 male fetus, AR (A) is expressed in virtually all cells of the cranial mesentery (cm), whereas α-SM actin-positive cells (B) are mainly found in the outer border of the mesentery, the primordial cells of the CSL. Caudally, the Wolffian duct (w) is surrounded by AR positive cells (A). The tunica albuginea demonstrates a strong anti-actin immunoreaction (B). Detail of CSL (csl) of a neonatal female after postnatal exposure to androgen (C), shows that many cells in differentiated CSL and surrounding mesenchyme have high expression of AR. In overview (D), the CSL on postnatal day 7 is present as the outer border of the cranial mesentery, containing strands of actin-positive cells. The ovary (ov) is located at the caudal tip of kidney (k). t, testis; cp, kidney capsule. Scalebar = (A, B) 50 μm, (C) 25 μm, (D) 200 μm.

Chapter 3

Targeted disruption of the Insl3 *gene causes bilateral cryptorchidism*
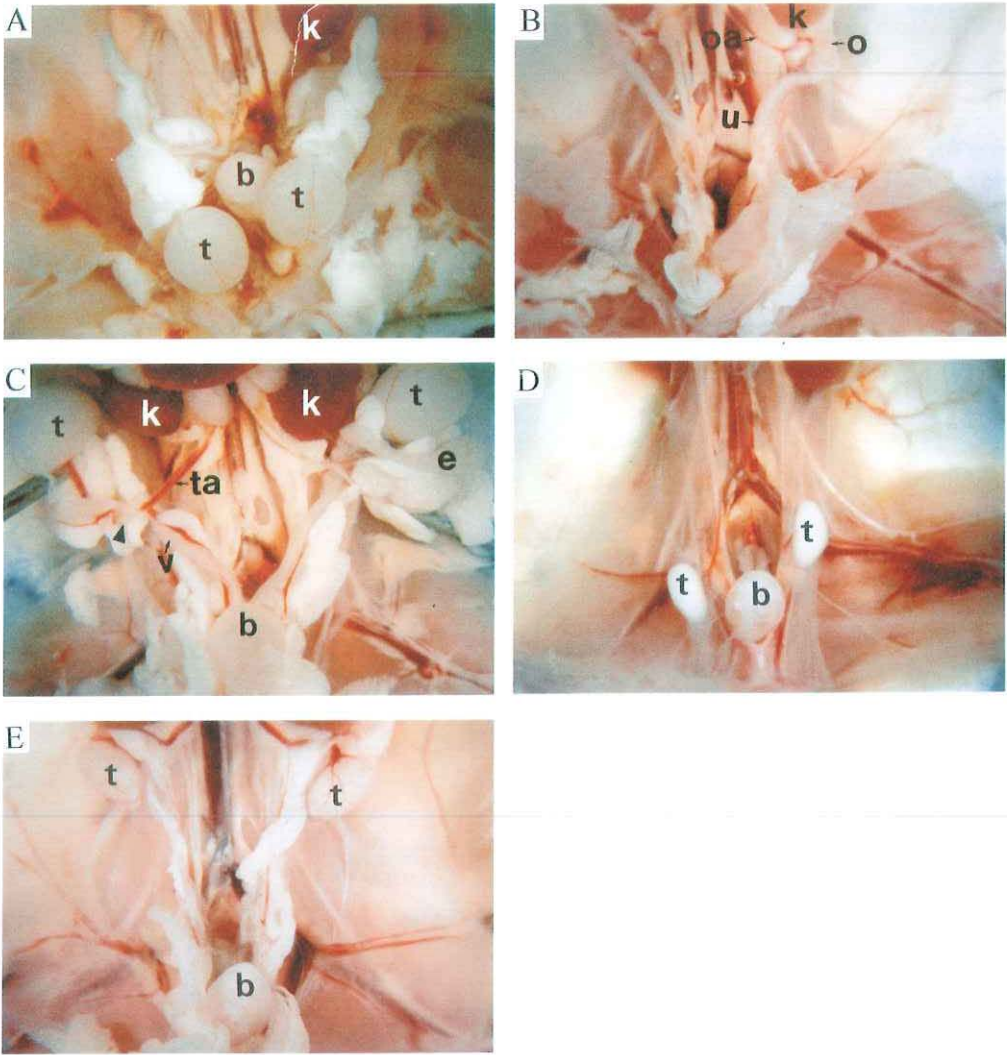
Figure 3.3 - Testicular position in *Insl3*^{-/-}, *Ar/Y* and *Ar/Y Insl3*^{-/-} male mice.
(A) Dissected abdominal region of a 4-week-old wild-type mouse shows the testes (t), which were already descended into the scrotal sac, adjacent to the bladder (b). *(B)* Genital tract of a 4-week-old wild-type female shows the position of ovaries (o) adjacent to the kidneys (k), uterine horns (u) and ovarian arteries (oa). *(C)* Free moving genital tract in the abdomen of 4-week-old Insl3-deficient male. The Wolffian duct derivatives are normally differentiated into epididymis (e), vas deferens (v) and accessory glands (not shown). Note the torsion (arrowhead) of the vas deferens and testicular artery (ta). *(D)* Testes of 3-week-old *Ar/Y* mouse located above the bladder and attached to the abdominal wall with cranial suspensory ligament and gubernaculum. *(E)* Testes of 4-week-old *Ar/Y Insl3*^{-/-} male situated adjacent to the kidneys in a comparable position as ovaries in wild-type mouse (B).

Chapter 6

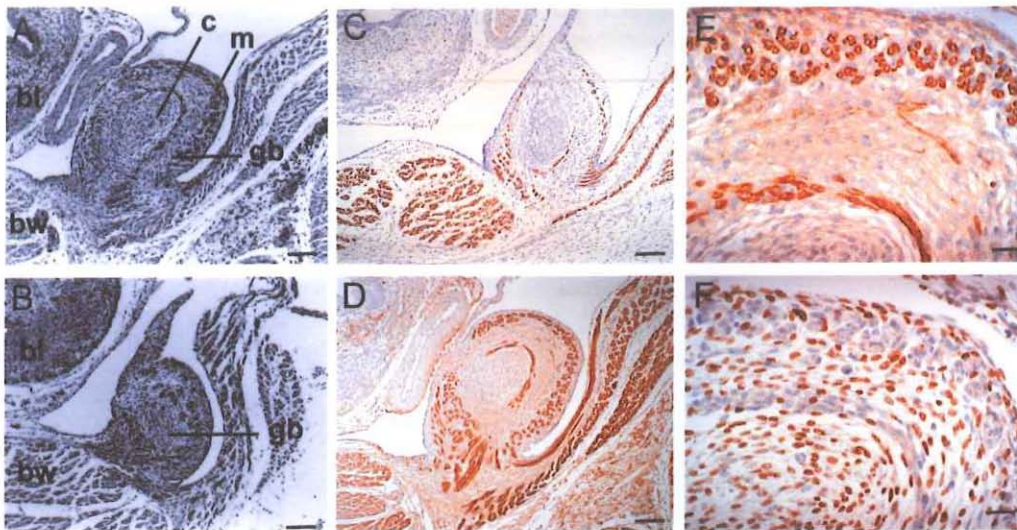*Hormonal control of gubernaculum development during testis descent*

Figure 6.2 - Histology and immunohistochemistry of gubernaculum development in the rat fetus *in vivo.*

(A-B) Histological analysis of the gubernaculum at E19 in the male (A) and female rat fetus (B). In the male gubernaculur bulb (gb), the muscular layer (m) and mesenchymal core (c) can be discriminated. Although the gubernacular bulb of the female fetus also consists of myoblasts and mesenchymal cells, it is smaller and less well-organized when compared to the male bulb. bl, bladder; bw, body wall. *(C-F)* Immunohistochemical localization of myosin (C-E) and androgen receptor (F) in the developing male gubernacular bulb of the rat. At E17, (C) the muscular layer of the gubernacular bulb can be clearly distinguished by myosin immunostaining. At E19, (D) the muscular layer is more pronounced when compared to E17, as demonstrated by a strong antin-myosin immunoreaction. Detail (E) of the muscular layer from section (D), showing differentiating myoblasts which are myosin-positive. (F) is a section from the same male rat fetus (D, E), but stained with antibodies against the androgen receptor (AR), showing that the mesenchymal cells are AR-positive (F). Scale bar = (A, B, C, D) 100 µm, (E, F) 25 µm.
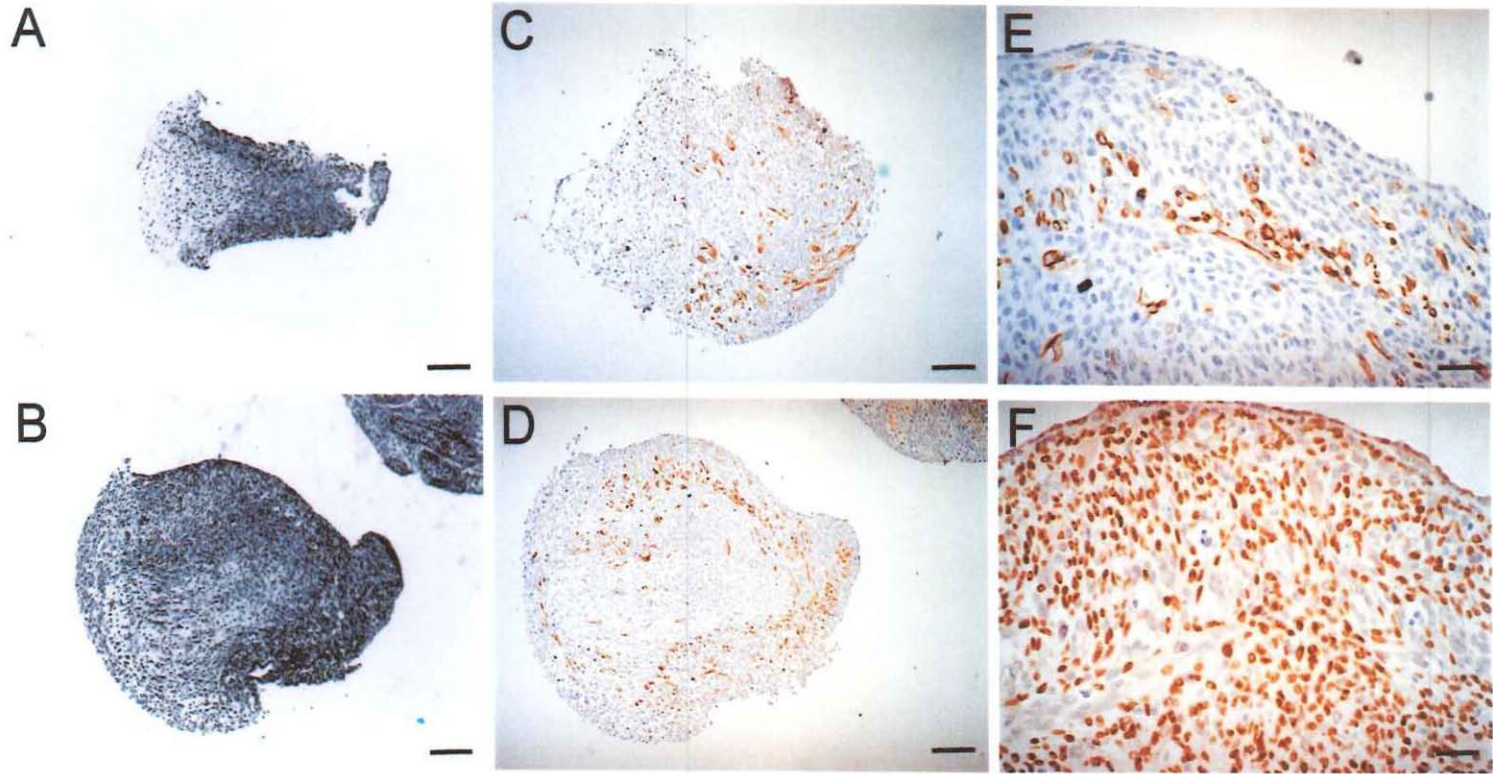
Figure 6.4 - Histological and immunohistochemical features of gubernacular explants, prior to and after 5 days of culture.

(A-B) Morphology of gubernacular explant prior to culture (A) and after 5 days of culture in presence of mouse testicular tissue (B). Note the pronounced increase in size of the gubernacular bulb after culturing. *(C-F)* Immunolocalization of myosin (C-E) and androgen receptor (AR) (F) after culturing in the presence of R1881 (10^{-8}M) (C) or testis (D-F). Both gubernacular explants (C,D) demonstrate immunostaining with myosin. In the gubernacular explant cultured in presence of R1881 (C), myosin-positive cells can be seen across the whole explant. In contrast, the gubernacular explant cultured in presence of a testis is showing a myosine-positive outerlayer. Detail (E) of the muscular layer from section (D), showing myoblasts which are myosine-positive. (F) is a section from the same explant (D, E), but stained with antibodies against the AR, showing that the mesenchymal cells are AR-positive (F). Scale bar = (A, B, C, D) 100 μm, (E, F) 25 μm.