Basic Studies in Andrology

Spermatogenesis and Sperm Maturation in Rodents and Men

Aan mijn ouders
Aan Sophie

ISBN 90-9012625-2

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Spermatogenesis and Sperm Maturation in Rodents and Men

Basale Studies in de Andrologie

Spermatogenese en Zaadcelrijping in het Knaagdier en de Mens

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr P.W.C. Akkermans, M.A.
en volgens besluit van het college voor promoties.
De openbare verdediging zal plaatsvinden op
woensdag 21 april 1999 om 11.45 uur

door

Jan Herman van Roijen
geboren te 's-Gravenhage

Promotiecommissie

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Dit proefschrift werd bewerkt binnen de afdeling Endocrinologie & Voortplanting, Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam en binnen de Polikliniek/Laboratorium Andrologie, Academisch Ziekenhuis Rotterdam, Dijkzigt.

De uitgave van dit proefschrift is mede mogelijk gemaakt dankzij een financiële bijdrage van Yamanouchi Nederland B.V. Merck Sharp & Dohme B.V., Hoechst Marion Roussel B.V., Byk Nederland B.V. en Christiaens B.V.

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List of Abbreviations

ABP	Androgen binding protein
ACTH	Adrenocorticotropic hormone
AIS	Androgen insensitivity syndrome
AMHR II	AMH type II receptor
AMH	Anti-Müllerian hormone
AR	Androgen receptor
AZF	Azoospermia factor
cAMP	Adenosine cyclic-3':5'-monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
CRE	cAMP-response element
CREM	cAMP-response element modulator
CRF	Corticotropin hormone releasing factor
DAZ	Deleted in azoospermia
DFFRY	*Drosophila fat facets* related Y gene
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
EDS	Ethane dimethane sulphonate
EGF	Epidermal growth factor
FSH	Follicle-stimulating hormone
FRA-1	Fos-related antigen
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone antagonist
GTP	Guanosine triphosphate
H2A	Histone 2A
H2B	Histone 2B
HCG	Human chorionic gonadotropin
HMG	High mobility group
HR6A	Homolog A of RAD6
HR6B	Homolog B of RAD6
ICSI	Intracytoplasmatic sperm injection
IGF	Insulin-like growth factor
i.p.	Intraperitoneally
IVF	*In vitro* fertilisation
kDa	Kilo Dalton
LH	Luteinizing hormone
MEP 10	Mouse epididymal protein 10

MESA	Microsurgical epididymal sperm aspiration
mRNA	Messenger ribonucleic acid
POMC	Pro-opiomelanocortin
PSA	Prostate-specific antigen
R1881	Methyltrienolone
RBM	Ribonucleic acid binding motif
rec-hFSH	Recombinant human FSH
RNA	Ribonucleic acid
ROS	Reactive oxygen species
s.c.	Subcutaneously
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SP197	Antibody targeting the androgen receptor
SRY	Sex-determining gene on the Y chromosome
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TDF	Testis-determining factor
TESE	Testicular sperm extraction
Tfm	Testicular feminization
TGF	Transforming growth factor
TH2A	Testis-specific histone 2A
TH2B	Testis-specific histone 2B
TP	Testosterone propionate
TSH	Thyrotropin-releasing hormone
WHO	World Health Organisation

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General Introduction: The male reproductive system

Sex Determination

During early embryonic development in mammals, there is no apparent phenotypic sex. Male and female embryos both have a double set of rudimentary genital ducts, and indifferent gonads that can evolve into either testes or ovaries. Gonadal sex determination involves a series of developmental changes that precedes differentiation of other primary sexual characteristics (for review see Hunter, 1995). In mammalian species, the initial step towards male or femaleness is distinctly genetic. It has been recognized since 1959 that individuals who have developed into males carry a Y chromosome, and that those who have developed as females do not (Ford *et al.*, 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959). Generally, human males display a 46,XY karyotype while females have two X chromosomes. The study of rare cases of human individuals with a 45,XO, 46,XX or 47,XXX karyotype (females) *versus* those with 46,XY, 47,XXY and 48,XXXY karyotypes (males) demonstrated that the presence of a single Y chromosome is not only a *conditio sine qua non* for male sex determination, but also that no amount of surplus X chromosomes can override its influence (Davis, 1981). As the male phenotype is the result of the actions of testis-derived hormones (testosterone and anti-Müllerian hormone), it was postulated that the Y chromosome contains a dominant locus determining the formation of a testis from the bipotential gonad. A small 35 kilobase chromosome fragment adjacent to the pseudoautosomal region of the Y chromosome was apparently able to induce maleness. Within this region, a gene named *SRY* in man and *Sry* in mice was located (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). Subsequently *Sry* was shown to induce maleness in genetic female mice when introduced into newly fertilized XX eggs (Koopman *et al.*, 1991). These experiments demonstrate that *SRY/Sry* is a genetic switch that induces male sex determination, and that all other genes necessary for the formation of the male phenotype can also be activated in XX females. An important exception is, however, spermatogenesis. Spermatogenesis requires not only the formation of testes, but also expression of a number of genes on the long arm of the Y chromosome (i.e. AZFa, AZFb and AZFc). Furthermore, spermatogenesis can occur only in the presence of one copy of the X chromosome (Koopman *et al.*, 1991; Vogt *et al.*, 1996; Vogt, 1997).

It has been shown that binding of *SRY* to DNA bends this DNA, thereby possibly creating a three-dimensional conformation facilitating the assembly of a transcription regulatory protein complex (Ferrari *et al.*, 1992; Giese *et al.*,

1992). *SRY*'s function as a switch in sex determination implicates a cascade of events 'downstream' from its activity. Although the DNA sequences that bind SRY with high affinity (Giese *et al.*, 1992; Harley *et al.*, 1992; Harley *et al.*, 1994) have been identified, there is still little information about genes which are regulated by this protein. As the events after male sex determination dictate the presence of factors that induce formation of the testis (Sertoli cells, Leydig cells, peritubular myoid cells etc.), the Wolffian duct-derived organs, and involution of the Müllerian ducts, downstream genes will invariably play an important role in these events. Indeed, a peptide corresponding to the HMG domain of SRY has been shown to bind with high affinity to the promoter regions of the genes encoding anti-Müllerian hormone (AMH) and P450 aromatase (Haqq *et al.*, 1993). Whether these *in vitro* observations actually have any bearing on the actual order of events during gonadogenesis remains to be elucidated. In addition, it has been proposed that *SRY* may act as an inhibitor of a male sex determination inhibitory gene (McElreavey *et al.*, 1993). In that scenario, SRY binding would result in direct competition with a 'female determining protein', for binding to specific DNA sequences.

Differentiation of the Male Gonad

Prior to gonadal sex determination, primordial germ cells, probably derived from the epiblast (Snow and Monk, 1983; Ginsburg *et al.*, 1990), move to the posterior extra-embryonic mesoderm at 7 days post coitum (pc) in the mouse (Ginsburg *et al.*, 1990). Subsequently, a number of primordial germ cells, having reached the yolk sac, migrate via the hindgut epithelium, mesenteries and the ventral mesonephros into the primitive, undifferentiated gonad (Witschi, 1948; Byskov and Hoyer, 1994). The number of primordial germ cells is initially very low [some authors have even suggested a population of only 8 cells at the 7 day pc stage in the mouse (Resnick *et al.*, 1992)], but during migration these diploid cells undergo a number of mitoses. On arrival at the primordial gonad, however, the mitotic rate increases such that at day 13 pc the mouse gonads may contain > 10,000 germ cells (Tam and Snow, 1981). Migration of the primordial germ cells depends on interaction of stem cell factor (SCF) with the Kit receptor: a transmembrane receptor with intrinsic tyrosine kinase activity. Activation of the Kit receptor in primordial germ cells results in stimulation of survival and proliferation (Fleischman, 1993).

The first morphological sign of male sexual development is the formation of testicular cords and the appearance of pre-Sertoli cells at 12.5 day pc in the mouse (Byskov and Hoyer, 1994). The primordial Sertoli cells, together with the germ cells, are rapidly enclosed by a basal lamina and a layer of peritubular myoid cells, which form the initial boundary between tubular and interstitial testis tissue (Pelliniemi, 1976; Magre and Jost, 1983; Wartenberg *et al.*, 1991). In the interstitial tissue, Leydig cells, derived from the mesonephric region, undergo differentiation and first show active steroidogenesis at day 13.5 pc (as monitored by 3ß-hydroxysteroid dehydrogenase activity) in the mouse (McLaren, 1991). Within the testicular cords, Sertoli cell numbers increase through mitosis, resulting in a tortuous cluster of elongated tubuli. Onset of spermatogenesis and meiosis in the germ stem cells enclosed in these tubuli does not occur until shortly before puberty (Byskov, 1986).

Testis formation is initially not dependent on gonadotropins or steroid hormones, but rather the activities of pre-Sertoli cells (which may include the secretion of a factor which activates the differentiation of Leydig cells) and peritubular myoid cells seem to be responsible. It is clear that the presence of germ cells is not a prerequisite for testis formation (Mintz and Russell, 1955; Merchant, 1975; McCoshen, 1982). In the series of events that follows testis determination and differentiation, however, testosterone produced by the Leydig cells and anti-Müllerian hormone (AMH) produced by the Sertoli cells are of critical importance in the development of the primary (and later secondary) male characteristics, including the prominent role of testosterone in the initiation of spermatogenesis at puberty and maintenance of spermatogenesis in the adult.

Differentiation of the Genital Ducts

AMH

The classic experiments of the late Alfred Jost have implied the existence not only of an active virilizing hormone, testosterone, but also of an active defeminization hormone that is instrumental in the regression of the Müllerian ducts (Jost, 1947). This latter factor, anti-Müllerian hormone, has been shown to be a 140,000 dalton protein composed of two identical subunits linked by disulphide bonds, produced by fetal Sertoli cells (Josso *et al.*, 1977; Vigier *et al.*, 1983). AMH has been shown to be responsible for the

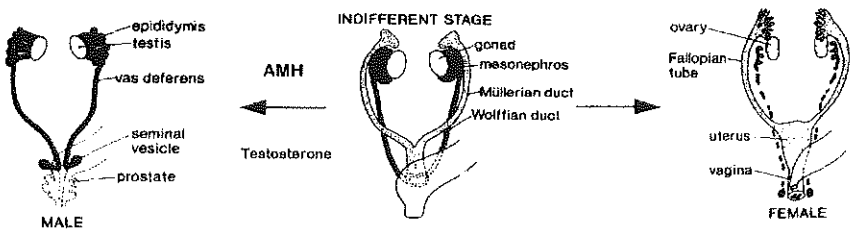

Figure 1. Schematic representation of the development of the male and female genital ducts. In the indifferent stage, the bipotential gonads are formed together with the Wolffian and Müllerian ducts. In the male, the gonads develop into testes which secrete anti-Müllerian hormane (AMH) and testosterone. AMH causes the regression of the Müllerian ducts, while testosterone stimulates the development and differentiation of the Wolffian ducts into the epididymides, the ductus deferens, and the seminal vesicles. In the female, lack of testosterone secretion results in regression of the Wolffian ducts, while the absence of AMH results in the development of the Müllerian ducts into Fallopian tubes, uterus, and upper part of the vagina.

regression of the Müllerian ducts, which in female embryos, in the absence of AMH production by the ovaries, evolve into the Fallopian tubes, the uterus, the cervix, and the upper portion of the vagina (Wilson *et al.*, 1981).
The testicular pre-Sertoli cells first produce AMH at day 12 of gestation in the mouse and at 7 weeks of gestation in man (Josso *et al.*, 1993; Taketo *et al.*, 1993). Its action, presumably mediated by a complex of transmembrane receptors of the serine/threonine kinase family (Baarends *et al.*, 1994; Di Clemente *et al.*, 1994; Grootegoed *et al.*, 1994), is responsible for the regression of the Müllerian ducts in the male fetus. Although indications exist concerning direct action of AMH on testis formation and function (Vigier *et al.*, 1987; Vigier *et al.*, 1989; Behringer *et al.*, 1990; Behringer *et al.*, 1994), the importance of this action is probably limited, as male patients lacking AMH activity due to mutation of one of the genes encoding ligand or receptor have fully developed testes (albeit not descended, resulting in spermatogenic impairment) (Knebelmann *et al.*, 1991). Interestingly, AMH secretion is quite protracted (ceasing at 4-20 days post partum in the rat, and at some point between the age of 2 years and the onset of puberty in man), while its action during a very narrow window of time (at 15 days pc in the rat and at 8 weeks of gestation in man) determines Müllerian duct regression. Exposure of the Müllerian ducts to AMH prior to or after this time window will not result in regression (Taguchi *et al.*, 1984). Even more puzzling in this

respect is the expression pattern of the AMH type II receptor mRNA (AMHRII) before and after birth in the rat. Before birth, the AMHRII mRNA is expressed in the genital ridges of the male (15 days pc) and female (15-16 days pc) rats. At 16 days pc, in the male rat, AMHRII mRNA expression has regressed to a small area including the cells associated with the degenerating Müllerian duct, in accordance with its role in Müllerian duct regression. However, AMHRII mRNA expression is also high in the prenatal ovaries, while expression in the prenatal testes is lower. About 7 days after birth, AMHRII mRNA expression in the rat testis shows a pronounced increase, when the AMH mRNA level is very low. In the postnatal rat ovary, AMH mRNA is produced at an increasingly high level (in the granulosa cells) in the continuous presence of a high AMHRII mRNA level (Kuroda.T *et al.*, 1990; Haqq *et al.*, 1992; Hirobe *et al.*, 1992; Baarends *et al.*, 1994; Baarends *et al.*, 1995; Baarends *et al.*, 1995). From this it has been suggested that there might be some role for AMH in the adult testis, and in particular in development and function of adult ovaries.

Testosterone

The production of testosterone by the testicular Leydig cells is instrumental in the development of the male genital ducts and other male characteristics (Greene *et al.*, 1939; Jost, 1947). The Wolffian duct develops into epididymis, vas deferens, and seminal vesicles. Further distal, the urogenital sinus develops into the prostate, the prostatic urethra, the penis and scrotum. Interestingly, while the Wolffian duct-derived accessory sex organs are directly dependent on testosterone for development, the urogenital sinus-derived organs and structures require modulation of the androgen signal through the enzyme 5α-reductase, transforming testosterone into dihydro-testosterone (DHT) (Griffin and Wilson, 1989). Testosterone and DHT bind to the same androgen receptor, albeit with different dynamics: DHT has a 10-fold greater potency *in vitro* (Grino *et al.*, 1990). Individuals with defective 5α-reductase activity, due to a 5α-reductase type 2 enzyme deficiency, display male pseudohermaphroditism and develop a syndrome called pseudovaginal perineoscrotal hypospadias: a partial or more complete failure in the virilization of the external genitalia while the Wolffian duct derivatives are well developed (Imperato-McGinley *et al.*, 1974; Walsh *et al.*, 1974). Why the presence of two hormones that bind to the same receptor is

necessary for formation of the male phenotype, is still somewhat of an enigma. It has been proposed that the close proximity of the Wolffian duct system to the testis results in the exposure to a higher testosterone level, as compared to a lower testosterone level in the more peripheral position of the urogenital sinus organs. Reduction of testosterone to DHT would thus serve to amplify the testicular virilization signal. Although one could argue that the seminal vesicles and the prostate develop in close proximity to each other, and would thus not require differential androgen stimulatory action, it has been shown that testosterone transport through the lumen of the Wolffian duct may be instrumental in creating a high local testosterone level (Veyssiere *et al.*, 1982). Indeed, human hermaphrodites with one testis develop only one vas deferens at the side of the testis (Grumbach and Wijk, 1955). In adulthood, all Wolffian duct-derived organs have been shown to contain 5α-reductase activity, and indeed to depend highly on DHT for adequate function (Robaire and Hermo, 1988).

Spermatogenesis

The adult testis has two major functions: one is its endocrine function as a gland that produces testosterone, necessary for the development and maintenance of the primary and secondary male sex characteristics. The other comprises the production of a huge number of highly specialized spermatozoa from a pool of undifferentiated stem cells (spermatogonia) that reside in the basal compartment of the seminiferous tubuli. The human male may produce as many as $2x10^{12}$ spermatozoa in his lifetime (Grootegoed, 1996). Not only in numbers is this an extraordinary feat, the testes and the epididymides nurse the development and maturation of the only cells that are designed to live and function outside the male body. The dual function of the testis, steroidogenic and gametogenic, is a uniquely fine-tuned process encompassing the interregulation of a considerable number of cells.

The hypothalamus-pituitary-testis axis

The intricately regulated processes mentioned above, depend on the highly controlled secretion of a number of messenger molecules (hormones) which will each generate their specific endocrine, paracrine and autocrine effects. At

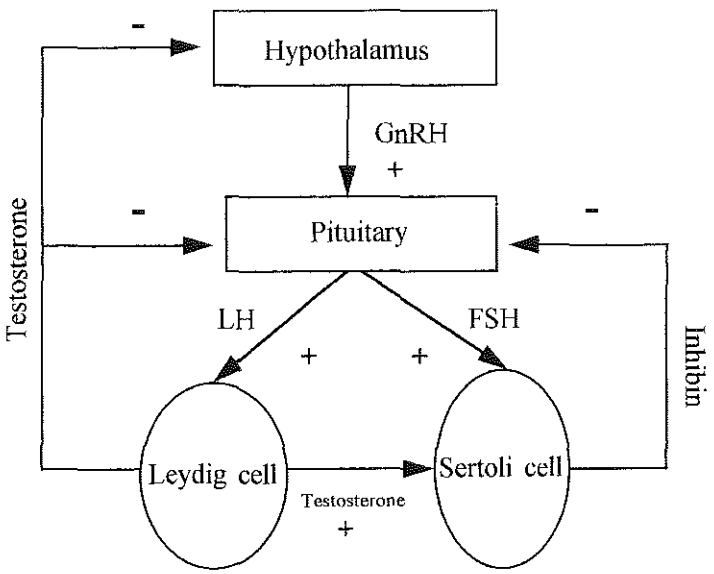

Figure 2. Schematic representation of the hypothalamus-pituitary-testis axis. Hypothalamic GnRH stimulates the pituitary secretion of LH and FSH. LH stimulates testicular Leydig cells, resulting in synthesis and secretion of testosterone. FSH and testosterone directly stimulate Sertoli cells, which in turn, regulate germ cell development. GnRH is under negative feedback control by testosterone, while pituitary secretion of LH and FSH is under feedback control by testosterone, and inhibin causes selective inhibition of FSH production.

the basis of this scheme of events is the hypothalamus-pituitary-testis axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which, through the portal vascular system, reaches the pituitary gland to stimulate production and secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH bind to cell surface trans-membrane receptors on different testicular cell types. Hypothalamus and pituitary functions are regulated via a feed-back mechanism, where testosterone (produced by the LH-stimulated Leydig cells) inhibits GnRH, and thereby LH and FSH secretion, and inhibin (produced, under control of FSH, by Sertoli cells) more specifically inhibits FSH secretion (Swerdloff and Wang, 1998).

Role of gonadotropins in spermatogenesis

FSH and LH are both members of the family of large heterodimeric glycoproteins, which also includes thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). Within a species, these hormones have an identical α-subunit that is associated with one of four different β-subunits. The resulting glycoprotein structures bind to specific membrane receptors (although LH and hCG are closely related and both bind to the LH receptor). The receptors specific for these hormones are also similar in structure: all have a large extracellular domain (encoded by 10 and 9 exons for the LH and the FSH receptors, respectively) that specifically binds its respective hormone, which involves proper positioning of the hormone in the 'binding pocket' formed by the extracellular and transmembrane domains (Willey and Leidenberger, 1989; Themmen *et al.*, 1994). The large transmembrane domain traverses the membrane seven times, and is followed by a short intracellular C-terminal domain that together with intracellular loops of the transmembrane domain can interact with G proteins. The transmembrane and intracellular domains of the LH and FSH receptors are encoded by a single exon. Activation of G proteins is effectuated by a conformational change of the receptor as a result of ligand binding. G proteins, when bound to GTP, activate adenylyl cyclase as the most important signal transduction pathway of this type of receptor. The activated cyclase converts ATP into the second messenger cAMP. Release of cAMP into the cytoplasm is followed by binding of cAMP to the regulatory subunit of cAMP-dependent protein kinase, dissociation of this regulatory subunit, and consequent activation of the catalytic subunit of the enzyme.

FSH

The role of FSH in the development of the testis, and the initiation and maintenance of spermatogenesis, has been widely studied. The only cell type expressing the receptor for FSH in the male mammal, is the Sertoli cell. During development FSH plays an important role as a regulator of Sertoli cell number and, indirectly, also of Leydig cell number (Huhtaniemi *et al.*, 1986; Dungen *et al.*, 1990; Sharpe, 1993). Proliferation of Sertoli cells occurs during a relatively short time: in the rat starting during the fetal phase and ending around day 15 after birth, while in man proliferation may continue through to the very early teens (for review see Sharpe, 1994). Cessation of the

proliferative activity of Sertoli cells coincides with maturation of these cells. As the number of Sertoli cells present in the seminiferous tubuli determines the length of the testicular tubules and hence the maximal capacity of spermatogenesis in adulthood, any defect in FSH secretion or action during the proliferative phase of the Sertoli cells will ultimately result in lower sperm output.

FSH responsiveness in immature rat Sertoli cells is quite well defined. In response to FSH stimulation, Sertoli cells have been found to upregulate androgen receptor (AR) expression, and also show increased production and secretion of seminiferous tubular fluid and proteins, such as transferrin, androgen binding protein (ABP), and inhibin (Blok *et al.*, 1989; Sharpe, 1993). Upon maturation, there is a clear reduction in the responsiveness of Sertoli cells to FSH, coinciding with an increase in responsiveness to androgens. Indeed, there seems to be a maturation switch from FSH- to androgen- dependence of the Sertoli cells (Sharpe, 1994).

In adulthood, the role of FSH remains an issue that has yet to be fully settled. Male mice deficient in the FSH-β subunit have small testes but are fertile (Kumar *et al.*, 1997). The genetic defect in these mice resulted in total absence of FSH activity throughout development, probably resulting in a deficient testis at the onset of puberty. In normally developed rodents, inhibition of FSH action has less far reaching implications. A number of studies have shown that immunization against FSH has little or no impact on spermatogenesis in rats (Davies *et al.*, 1979; Dym *et al.*, 1979), although careful scrutiny of the available data does show a 10% decrease in testis weight in these studies (Sharpe, 1994). FSH probably has a more prominent role in the maintenance of spermatogenesis in primates than in rodents, as immunization against FSH in primates results in a dramatic decrease (though not complete cessation) of spermatogenesis (Nieschlag *et al.*, 1975; Wickings and Nieschlag, 1980; Raj *et al.*, 1982). This discrepancy between primates and rats may be explained by the following: The available evidence suggests that in the adult testis, FSH may have a role in regulation of the number of spermatogonia that enter the spermatogenic pathway. In non-human primates, FSH is able to increase the number of B spermatogonia, spermatocytes and spermatids [mediated by a relative increase in the number of A-pale (A_p; see later this chapter) spermatogonia (Arslan *et al.*, 1992)]. Regulation of spermatogonial numbers in the rat seems to be controlled by density dependent degeneration: there is a maximum number of sperma-

togonia that can enter the spermatogenic pathway (i.e. can be supported by the Sertoli cells), and the rest degenerate (Huckins, 1978; Huckins and Oakberg, 1978; Rooij and Lok, 1987; De Rooij and Grootegoed, 1998). Thus even if FSH would have an effect on the proliferation of spermatogonia in the rat, this effect would be lost by this density dependent degeneration of spermatogonia. In man and non-human primates, however, the number of spermatogonia that is available to enter meoisis may not be maximal. In these species, there appears to be no density dependent degeneration as a consequence of a much lower rate of division of the undifferentiated spermatogonia (Clermont and Leblond, 1959; Clermont, 1972; Clermont and Antar, 1973; Rooij *et al.*, 1989). Hence, an FSH-stimulated increase in spermatogonial number entering the spermatogenic pathway is more readily discerned in primates (Sharpe, 1994). Recently, a number of men homozygous for an inactivating mutation in the FSH receptor have been studied. Although spermatogenesis was clearly negatively affected, these individuals did not have azoospermia, and some were fertile (Tapanainen *et al.*, 1997). The treatment of oligozoospermic males with regimens resulting in a higher serum FSH level, however, has given disappointing results, in terms of improvement of semen parameters (Baker, 1989).
The above data indicates that, in man and rodents, FSH action is required for normal testis development and probably for the maintenance of optimal spermatogenesis in adulthood, but that qualitative spermatogenesis evolves and can be maintained under conditions of suboptimal, or even absent, FSH action.

LH

Within the testis, the LH receptor is found exclusively on the Leydig cells. Previous studies have estimated the number of Leydig cells at 18 to $34x10^6$ per rat testis (Mori and Christensen, 1980; Ewing and Zirkin, 1983; Tapanainen *et al.*, 1984; Kerr *et al.*, 1987; Zirkin and Ewing, 1987; Mendis-Handagama *et al.*, 1988), accounting for about 3 percent of the total testis volume (Ewing and Keeney, 1993). The pituitary gland secretes LH in a pulsatile manner into the bloodstream, through which it reaches the Leydig cells within the testis. LH binds to the transmembrane receptor at the cell surface, resulting in stimulation of G protein and adenylyl cyclase, thereby increasing cAMP formation. Cyclic AMP subsequently activates protein

kinase A, which in turn effectuates the phosphorylation of specific proteins. Changes observed within the Leydig cell upon stimulation with LH *in vitro* are: 1) depletion of intracellular lipid droplets that store cholesterol esters, 2) stimulation of cholesterol hydrolyse to produce free cholesterol esters, 3) changes in cytoskeletal elements, 4) transport of cholesterol to the inner mitochondrial membrane, site of the $P450^{SCC}$ enzyme, and 5) stimulation of RNA and protein synthesis (Dufau, 1988; Hall, 1988; Ewing and Keeney, 1993). The conversion of cholesterol to pregnenolone has for many years been considered the rate-limiting step in testicular steroidogenesis. However, it has recently been observed that the true rate-limiting step is the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where the $P450_{scc}$ enzyme is situated (reviewed in Stocco, 1998). This process is tightly regulated by tropic hormone stimulation, and dependent on *de novo* protein synthesis. Many studies have now identified a hormone-regulated 30 kDa protein as a major modulator of steroidogenesis, that is instrumental in the transport of cholesterol to the inner mitochondrial membrane. This protein has been designated steroidogenic acute regulatory protein (StAR) (Stocco, 1998).

Testosterone synthesis and secretion are the most important and best known functions of the Leydig cells. During fetal life, a testosterone surge is responsible for the differentiation of the male reproductive tract, and during the neonatal period a subsequent surge effectuates "imprinting" of androgen dependent tissues. In the pubertal period, increased testosterone production is responsible for the development of the secondary male sex characteristics, male sexual behavior, and the onset of spermatogenesis. During adult life, testosterone is required for maintenance of the secondary male characteristics, as well as for maintenance of skeletal bone density (Ewing *et al.*, 1980; Tenover, 1994). Mutations in the LH receptor have been identified, and have been shown to result in inactive or constitutively active receptors (reviewed in Themmen *et al.*, 1997). A number of activating mutations have been characterized, where inappropriate G protein activation is associated with mutation in the second or fifth transmembrane segment. This results in precocious puberty in boys carrying a single allele of the mutated gene, while carrier girls or women have no apparent phenotype. Inactivating mutations may occur anywhere in the gene. The complete form of LH receptor insensitivity is characterized by complete male pseudohermaphroditism and Leydig cell hypoplasia. A milder form of this syndrome results in slightly

under-virilized men with a micropenis (Leydig cell hypoplasia type II). The latter syndrome has been associated with a mutation in the seventh transmembrane segment (Themmen *et al.*, 1997).

Histology of the testis

The testis is an oval shaped organ that is encapsulated by a strong fibrous membrane called the tunica albigunia (Kretser and Kerr, 1994 for review). The testicular mesenchyme can be grossly divided into two major compartments: the interstitial space and the seminiferous tubules. The interstitial space contains Leydig cells, macrophages, blood vessels and lymphatic fluid. The outer wall of the seminiferous tubules is formed by peritubular myoid cells and endothelial cells. A basal lamina (or basement

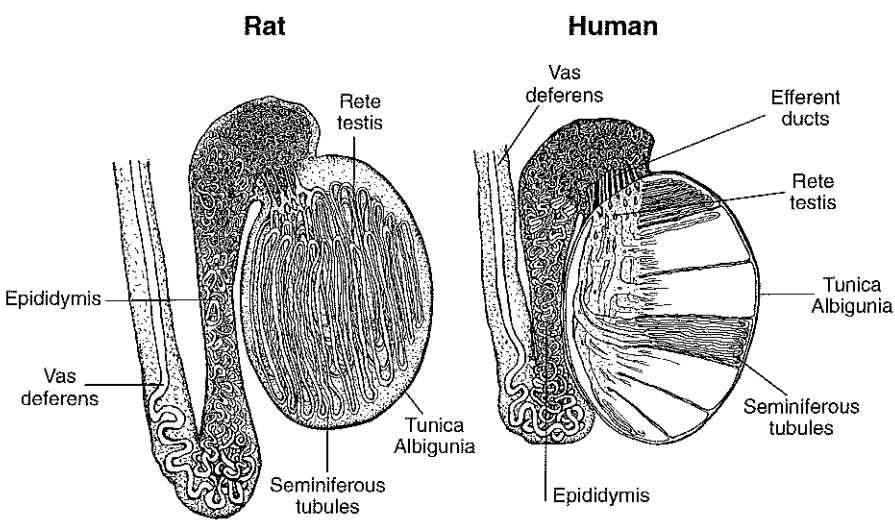

Figure 3. Artistic representation of the rat and human testis and excurrent ducts. The testis of both rat and human consists of a fibrous tunica albuginea, which encases a large number of seminiferous tubuli. The human testis is divided into lobuli, by a number of fibrous partitions within the testis. These partitions are absent in the rat testis. The seminiferous tubuli merge into the rete testis, from where the seminiferous fluid and spermatozoa are transported into the epididymis, through a number of ductuli efferentes. In the rat, the ductuli efferentes quickly unite into a single highly contorted tubule that forms the entire epididymis, and, further distal, the vas deferens. In the human, a more complex tubular network is formed in the proximal epididymis, which conjoins into a single epididymal tubule in the distal caput. This single tubule subsequently forms the distal caput, corpus, and cauda epididymis, and the ductus deferens.

membrane) covers the luminal surface of the tubule. On the basement membrane are situated the germinal stem cells (spermatogonia) and the Sertoli cells. Cytoplasmic extensions of the Sertoli cells form a highly organized structural matrix within which the spermatogonia develop into mature spermatozoa (Sertoli, 1865). Sertoli cells not only provide structural support, but have an important role in the support of biochemical processes of the germinal cells (Russell, 1993). Instrumental for this is the 'blood-testis barrier' or 'Sertoli cell barrier' formed by tight junctional complexes between adjacent Sertoli cells (Dym and Fawcett, 1970; Ross, 1970; Gilula *et al.*, 1976; Russell and Peterson, 1985). This barrier, impenetrable to the larger hydrophilic molecules, divides the tubule into a basal compartment and an adluminal compartment. In the basal compartment reside only the spermatogonia and the early preleptotene spermatocytes. The latter cells, which have entered the prophase of meiosis, start to traverse the barrier to reach the adluminal compartment (Russell, 1980; Russell, 1993). The following meiotic and post-meiotic steps of spermatogenesis all take place in the adluminal compartment. Centrally within the tubule is located the tubular lumen, which is filled with a seminiferous tubular fluid and spermatozoa. All tubuli conjoin in the rete testis, from where the tubular content is directed into the ductuli efferentes and epididymis.

The spermatogenic cycle

As mentioned above, Sertoli cells are the structural and functional 'nurse cells' of the developing germinal cells. When seminiferous tubuli are viewed in cross section through a microscope, one encounters a highly pleiotropic architecture of Sertoli cell-germinal cell complements. These variations are an expression of cyclic changes in phases of development, that have classically been divided into the spermatogenetic stages. In man, six such stages are identified, whereas in the rat the spermatogenic cycle is divided into fourteen stages (Leblond and Clermont, 1952; Clermont, 1963). A given Sertoli cell within a particular stage of the cycle supports a well-defined germ cell complement, encompassing spermatogonia through to elongating spermatids. The germ cell complement (as well as the structural form of the Sertoli cells) changes with each subsequent stage. The germ cell begins its developmental journey at the basement membrane of the tubule, and moves to the adluminal compartment. On moving 'inward', it is closely followed by a

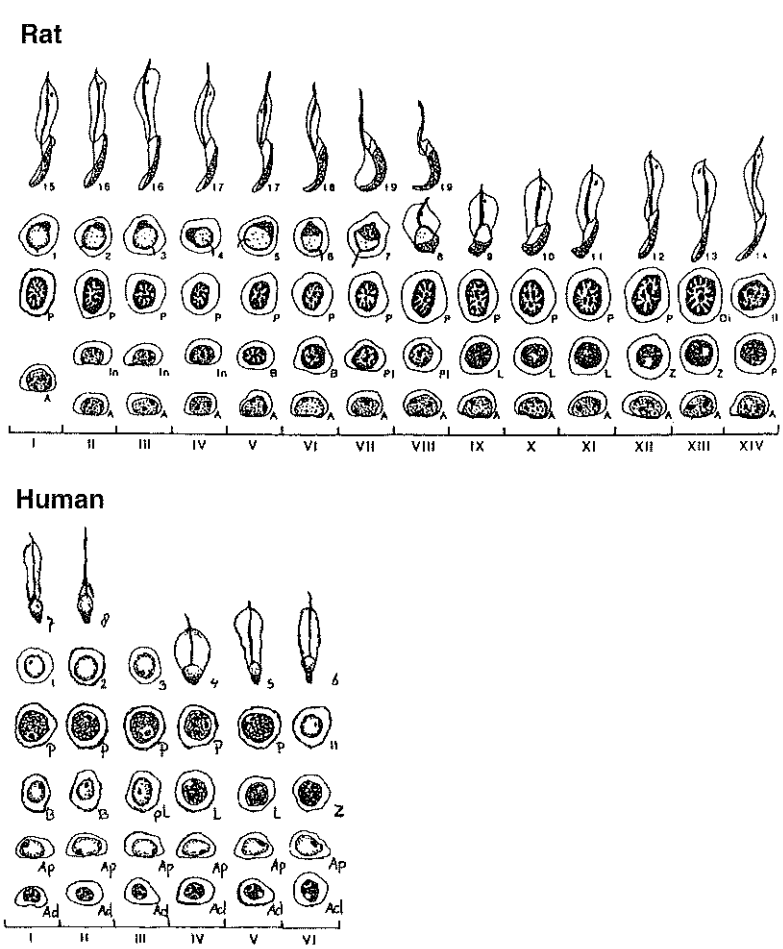

Figure 4. Diagram illustrating the spermatogenic cycle in the rat and in the human. Each vertical column depicts a complement of germ cells associated with a Sertoli cell at a specific stage of the spermatogenic cycle.

The spermatogenic cycle of the rat is subdivided into 14 stages. Abbreviations are: A= type A spermatogonia; In= intermediate type spermatogonia; B= type B spermatogonia; Pl= preleptotene spermatocytes; L= leptotene spermatocytes; Z= zygotene spermatocytes; P= pachytene spermatocytes; Di= diplotene spermatocytes; II= secondary spermatocytes; 1-19= spermatids at their successive steps of spermiogenesis.

The spermatogenic cycle of the human is subdivided into 6 stages. Abbreviations are: Ad= type A 'dark' spermatogonia; Ap= type A 'pale' spermatogomia; B= type B spermatogonia; Pl= preleptotene spermatocytes; L= leptotene spermatocytes; Z= zygotene spermatocytes; P= pachytene spermatocytes; II= secondary spermatocytes; 1-8= spermatids at their successive steps of spermiogenesis.
(Adapted from Sharpe, 1994).

younger germ cell at a specific developmental distance. Thus, for example, at stage VII in the rat, one always finds step 7 and 19 spermatids, mid-pachytene and preleptotene spermatocytes, and A1 spermatogonia, while at stage XIV one invariably finds step 14 spermatids, late diplotene spermatocytes undergoing meiotic divisions, and A3 spermatogonia (Sharpe, 1994 for review).

It must be stressed that the division of the dynamic process of spermatogenesis into stages is arbitrary, and is based for the most part on the development of the acrosome in spermatids (Leblond and Clermont, 1952; Clermont, 1963). Particularly in the rat, pronounced stage specific changes in Sertoli cell activities have been reported (Parvinen, 1993), indicating that, although the spermatogenic cycle is divided into an arbitrary number of stages, this subdivision is helpful to study mechanisms involved in the control of spermatogenesis. The fact that the human spermatogenic cycle is subdivided into less that half as many stages as that of the rat, reflects a less ordered appearance of the spermatogenic epithelium rather that a less intricate developmental process.
An intriguing difference between man and almost all other mammalian species (with the exception of the olive baboon) is the helical organization of the spermatogenic stages within the seminiferous tubuli (Afzelius *et al.*, 1982; Schulze and Rehder, 1984). A cross section of a human tubule will display 3 or more different stages of the spermatogenic cycle in a mosaic fashion, whereas a cross section of a tubule of any other mammal will, as a result of a marked longitudinal organization of the cycle of the spermatogenic epithelium, display only one stage of the cycle, at a given timepoint.

In recent years, many genetic and molecular aspects of spermatogenesis are being studied in mice, taking advantage of transgenic and knockout mouse models. However, spermatogenesis has been studied most extensively in the rat, in particular for the role of hormones and growth factors, and cellular interactions. Therefore the following account applies to the situation in the latter rodent species.
On the basement membrane, at all stages of the spermatogenic cycle, undifferentiated (A) spermatogonia can be found. These are spermatogenic precursor cells that have the ability to proliferate through mitosis. Daughter cells can either remain undifferentiated spermatogonia, ensuring an adequate

precursor population, or they can differentiate into A_1 spermatogonia. From there, differentiation proceeds during a series of mitotic divisions, via the A_2, A_3 and A_4 to the A^{In} (intermediate) and B spermatogonia, increasing the number of spermatogonia. Having reached the B spermatogonia stage, the last mitosis occurs, and the spermatogenic cells enter the meiotic prophase as preleptotene spermatocytes (PL). The preleptotene spermatocytes start to pass across the Sertoli cell tight junctional barrier, to enter the adluminal area of the seminiferous tubuli, now separated from the rest of the body by the Sertoli cell barrier and completely dependent on the Sertoli cells for their biochemical needs. In the preleptotene phase, DNA replication occurs for the last time resulting in diploid (2N) cells with a 4C DNA content. Once in the leptotene phase, the spermatocytes have entered the lengthy prophase of the first meiotic division, encompassing the leptotene (pairing of the homologous chromosomes), zygotene (forming of the synaptonemal complexes), pachytene (crossing over between paternal and maternal chromosomes) and diplotene (desynapsis) spermatocyte phases. Subsequently, the primary spermatocytes undergo the first meiotic division, resulting in haploid secondary spermatocytes (1N, 2C), which rapidly enter the second meiotic division without DNA replication. One diploid 4C-DNA spermatocyte thus forms four haploid 1C-DNA spermatids. Development of the spermatids (spermiogenesis) encompasses a number of *steps*; 1-19 in the rat and 1-8 in man. Within this differentiation process, the round spermatids do not further divide, but start to undergo a startling morphological change. A tail develops from a protrusion in close proximity to the nucleus. At the other end of the nucleus, a granule appears which later spreads itself over one nuclear hemisphere (the acrosome), and subsequently nuclear elongation and condensation occurs. Finally, just prior to the release of the spermatids/spermatozoa from the Sertoli cells (spermiation), practically all of the spermatid cytoplasm is removed and taken up by the Sertoli cells. The resulting spermatozoa are released into the lumen of the seminiferous tubuli, where they are carried to the epididymis with the seminiferous tubular fluid (for review see Sharpe, 94). In the human, the spermatogonia are subdivided into the A_{dark}, A_{pale} and B spermatogonia. The subsequent meiotic development is quite similar to what is found in the rat, but spermiogenesis is divided into only 8 steps. The duration of the complete process of spermatogenesis is 51-53 days in the rat and 64 days in the human, from the formation of differentiating spermatogonia to spermiation.

Sertoli cell-germ cell interactions

Clearly, male germ cell differentiation is a complex process, not only requiring expression of many genes within the germ cells themselves, but also requiring supportive functions of Sertoli cells (Russell, 1993; Griswold, 1995). The most important endocrine regulators of spermatogenesis are androgens and FSH. Receptors for these hormones are present in/on Sertoli cells, but most evidence indicates that germ cells lack these receptors (Grootegoed *et al.*, 1977; Bremner *et al.*, 1994; Chapters 1 and 2). This fact clearly demonstrates the importance of Sertoli cells in spermatogenesis. What then is the role of Sertoli cells during proliferation and differentiation of the germ cells?

The structural matrix which is provided by cytoplasmic extensions of the Sertoli cells, together with the Sertoli cell barrier, is the cellular basis on which germ cell differentiation can proceed. Electron microscopical analysis, of sequential cross sections to reconstruct a three-dimensional model of Sertoli cells, has disclosed that Sertoli cells change shape during the spermatogenic cycle. One group of Sertoli cells (designated group A) have deep crypts that accommodate the germ cells. In the other group (group B), crypts are absent due to the apical movement of the spermatogenic cells (Russell *et al.*, 1983; Weber *et al.*, 1983; Wong and Russell, 1983; Griswold, 1995). These morphological changes correlate well with certain stages of the spermatogenic cycle, and are suggestive of a 'dual mode' of Sertoli cell function: one mode accommodates germ cell developmental stages including meiosis (~stages X-IV of the spermatogenic cycle in the rat), while the other accommodates the spermiation stages (roughly stages V-IX in the rat). In each 'mode', the Sertoli cells secrete a specific set of proteins (Linder *et al.*, 1991).

The role of germ cells in regulation of Sertoli cell function is a subject still under investigation. It is clear, however, that in culture, the activity and morphological characteristics of Sertoli cells are dependent on association with germ cells (Griswold *et al.*, 1989; Wright *et al.*, 1989; Jegou, 1991). The interactions between Sertoli cells and germ cells are, for an important part, paracrine in nature. A large number of possible paracrine regulators have been identified, among which stem cell factor (SCF), Nerve growth factor (NGF), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF/FGF-4), interleukins, transforming growth factors α and β (TGF-α and TGF-β) and other members of the TFG-β family such as activin, inhibin and AMH (reviewed in: Skinner, 1991;

Grootegoed, 1996). The precise role of each of these factors in unknown, although it has been the focus of a considerable amount of research.
One Sertoli cell protein that has been extensively studied is transferrin. Transferrin is an iron transporting protein found in many tissues, including liver and brain, which is also produced and secreted by Sertoli cells. It has been suggested that transferrin is part of an iron shuttle system that transports iron across the tight junctional Sertoli cell barrier to the developing germ cells (Sylvester and Griswold, 1993; Griswold, 1995). Experiments involving transferrin deficient (HP) mice, have shown that spermatogenesis is impaired, and that indeed production of transferrin by Sertoli cells of mice is required for adequate germ cell development (Bernstein, 1987; Huggenvik *et al.*, 1989).
For their metabolic requirements, developing germ cells may also be highly dependent on Sertoli cells. It has been shown that pachytene spermatocytes and round spermatids from rats and hamsters require exogenous lactate or pyruvate for ATP production, apparently because of a relative limitation of the activity of the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase (Grootegoed and den Boer, 1989). The Sertoli cells are, however, metabolically versatile, and can produce lactate and pyruvate from glucose, possibly for use by the germ cells. *In vitro*, spermatocytes and spermatids cannot survive for more than several days when not associated with Sertoli cells, even in the presence of pyruvate and lactate, indicating that other Sertoli cell factors are required (Toebosch *et al.*, 1989; Rassoulzadegan *et al.*, 1993; Kierszenbaum, 1994).

Chromatin structure and remodelling

The DNA of spermatogonia is associated with somatic histones, and is organized in a somatic fashion. Two turns of DNA (160 base pairs) wrap around the four core histones, H2A, H2B, H3 and H4. Together with an associated linker histone (H1), the DNA is further organized into solenoids which are, in turn, supercoiled. This construction allows the 3.3 billion base pair human genome to fit into a cell nucleus measuring less than 10^{-5} M in diameter. During the course of germ cell differentiation, somatic histones are partly replaced by testis-specific histones. In the rat, testis-specific histone 2A and 2B (TH2A and TH2B) are first synthesized in early primary sperma-

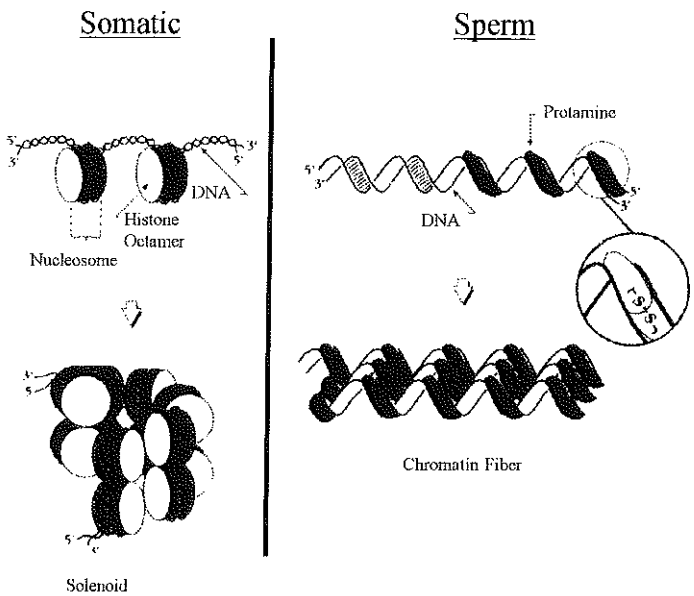

Figure 5. Schematic representation of DNA packaging in somatic cells and spermatozoa. In somatic cells, DNA is wound twice around a histone octamer, forming a nucleosome. Nucleosomes coil further into solenoids. In spermatozoa, highly positively charged protamines bind to DNA lengthwise along the minor groove. This results in a neutral polymer, where the DNA-protamine com-plexes can bind together by van der Waals forces, in a linear fashion. Disulfide bonds between protamines further stabilize the nucleoprotamine complex.

tocytes (peleptotene or pachytene stage) while testis-specific histone 3 (TH3) is present and actively synthesized already in A and B spermatogonia (Brock *et al.*, 1980; Meistrich *et al.*, 1985). H1t is not observed in the germinal cells until the pachytene spermatocyte stage (Meistrich *et al.*, 1985). No testis-specific variant of H4 has yet been reported. It is unclear why testis-specific histone variants have evolved, and their role remains elusive. It has been shown that the thermal stability of chromatin derived from the testis is appreciably less compared to that of liver chromatin. The reduced stability of testicular chromatin may facilitate histone removal during the nuclear histone-to-protamine transition that occurs in spermatids (Grimes, 1986). In man, very little is known about the nuclear protein composition in the early germinal cells, although testis-specific histones have been found in whole testis tissue and in isolated spermatids and spermatozoa.
In the mid spermatid stage (steps 9-12 in the rat), histones are replaced by

testis-specific transition proteins. Prior to this event, the nuclear chromatin appears to undergo decondensation and becomes vulnerable to thermal denaturation (Grimes, 1986). This is in part due to hyperacetylation of H4, and probably a number of additional protein modifications which have not yet been elucidated (Grimes and Smart, 1985; Grimes, 1986). The transition proteins are the major DNA binding proteins found in steps 13-15 spermatids in the rat. Subsequently, the transition proteins are replaced by testis-specific protamines. Protamines are relatively small, highly basic proteins, that are rich in arginine (40-70%), and in mammals frequently contain cysteine residues (18%) (Coelingh *et al.*, 1972; Kistler *et al.*, 1976). Protamines bind to the DNA along the minor groove. This transforms the polyanionic DNA into a neutral polymer, so that the DNA-protamine complex can bind together by Van der Waals forces in a linear fashion, at the molecular level (Ward, 1993). The highly condensed nucleoprotein complex is further stabilized by the formation of disulfide bonds between protamines (Grimes, 1986). This configuration results in a highly compact chromatin arrangement that renders the DNA unavailable for transcription, and results in a high resistance of the chromatin to external harmful influences. Furthermore, the compact arrangement allows for construction of a very small sperm nucleus.

Sperm maturation

When spermatozoa are isolated from the rete testis or the proximal region of the epididymis, they are found to have little or no capacity to fertilize an oocyte. Within the epididymis, spermatozoa undergo a number of subtle, yet profoundly important changes that will enable them to survive in the female genital tract, propel themselves toward, bind to, and fertilize an oocyte (Tournade, 1913; Bedford, 1967; Orgebin-Crist, 1967; Orgebin-Crist, 1969; Eddy and O'Brien, 1994; Yanagimachi, 1994). These changes take time, and while certain characteristics may develop (albeit imperfectly) spontaneously with the passage of time, there exists ample evidence that full maturity can only be attained if the spermatozoon is allowed to migrate down the tortuous duct of the epididymis, and is exposed to the highly regulated varying luminal environment.

Histology of the epididymis

Structurally, the epididymis consists of one long contorted tubule that connects the ductuli efferentes (usually also viewed as part of the epididymis) to the vas deferens. Its length varies between species: in the rat it measures about 6 meters (Jiang *et al.*, 1994), with bigger mammals as a rule having an even longer epididymal duct [up to 50 m in large domestic animals (Bedford, 1994)]. The length of the human epididymis is relatively short, averaging about 6 meters (Bedford, 1994). The rete testis drains into the ductuli efferentes, a collection of tubules lined with tall columnar epithelium. This epithelium consists of ciliated and non-ciliated cells, which on the basis of their appearance and possible function, is characteristic of the division of the efferent ductuli into initial and terminal zones (Reid and Cleland, 1957). The efferent ducts conjoin in the single lumen of the epididymis proper. Based on gross morphological characteristics, the epididymis consists of a caput, a corpus and a cauda. In most species, the caput is relatively modest in size and is joined by a slender corpus to a well-developed cauda. In man, however, the caput is relatively large with a surprisingly small cauda (Bedford, 1994). Microscopically, and on the basis of a number of functional studies, a much more complex series of distinct regions can be identified. For the purpose of this thesis, the subdivision of the various epididymal regions will be confined to: an initial segment, a proximal caput, and a distal caput region, followed by the corpus and the cauda.

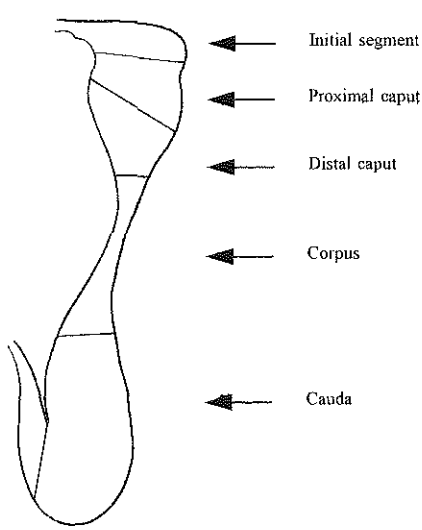

Figure 6. Schematic representation of the rat epididymis. For the purpuse of this thesis, the epididymis is subdivided into the following segments: efferent ducts, initial segment, caput, corpus, cauda, and ductus deferens.

The lining of the epididymal duct is composed of a number of specific cells. Principal cells make up the bulk of the epithelium, accounting for 80% of the cells in the initial segment, where they are tall and columnar, and decline to 67% in the cauda, where they are far less tall and more cuboidal-shaped. Narrow cells are found only in the initial segment and account for 3% of the cell population. Clear cells are found throughout the organ, where they constitute about 5% of cells in the caput to about 7% in the cauda. The basal cells are flat and elongated, and lie close to the luminal surface of the basement membrane. They account for 12% of the cells in the caput increasing to about 21% in the distal portions of the epididymis (Reid and Cleland, 1957; Robaire and Hermo, 1988).

Changes in sperm characteristics during epididymal passage

During the last phase of spermiogenesis, the spermatids loose practically all of their cytoplasm. This results in a marked inability to perform many biochemical processes. The spermatozoon is thus dependent on the epididymis for supporting factors needed for maturation. It has been shown that sperm can modify or degrade carbohydrates and lipids to provide energy (Bedford and Hoskins, 1990). Sperm may need the metabolic energy mainly for their burst of motility after ejaculation. The capacity for motility is gradually acquired within the epididymis through an as yet unidentified process. When comparing different species, it has been found that the capacity to show progressive motility is attained in different parts of the epididymis: the marmoset sperm isolated from the distal caput displays motility, while the rabbit, boar and ram sperm are potentially motile from the distal corpus onwards. Sperm from rat, mouse, hamster and man are capable of motility only when they have progressed into the caudal region (Nishikawa and Wade, 1952; Hoppe, 1975; Holtz and Smidt, 1976; Fournier-Delpech *et al.*, 1977; Moore, 1981; Moore *et al.*, 1983; Orgebin-Crist and Olson, 1984). Interestingly, it has been found that in men suffering from obstruction of the epididymis, even in very proximal regions, spermatozoa from the ductuli efferentes can be motile and fertile (Bedford, 1994). This is in marked contrast to findings in a cohort of men who underwent orchiectomy, where motility was found for the first time in the mid corpus (Bedford *et al.*, 1973) or caudal regions (Moore *et al.*, 1983). This indicates either an intrinsic ability to become motile after a certain period of time, or an interactive

differentiation, induced by the presence of spermatozoa, with the local tubular epithelium. In all studied animals, spermatozoa are immotile while still residing within the epididymis (Hinton *et al.*, 1979). In the rat, this is presumably the result of the presence of a high molecular weight glycoprotein (immobilin), which is synthesized in the principal cells of the caput, and travels down to, and accumulates in the cauda (Usselman *et al.*, 1985; Robaire and Viger, 1995). It has been suggested that immobilin inhibits sperm motility mechanically by creating a highly viscoelastic environment (Robaire and Viger, 1995). In the bovine, however, immobilin appears to be of little importance in inhibition of sperm motility; rather, inhibition of bovine sperm motility is effectuated by the relatively low pH of 5.5 (Carr and Acott, 1984). At an extracellular pH of 7.6, bovine sperm are fully motile.

Next to the attainment of motility, the sperm nucleus undergoes a number of changes, culminating in the formation of a highly compact and stable DNA-protein complex as a result of increased disulfide bonding in between DNA-associated protamine molecules. Caudal rat sperm are thus highly resistant to dissolution by sodium dodecyl sulphate (SDS) (Bedford, 1975; Bedford and Hoskins, 1990). Indeed, it has been suggested that the oocyte cytoplasm is the only known biological medium capable of overcoming the stability of the DNA-nucleoprotein complex (Amann *et al.*, 1993).

One of the most important developmental changes that occur during transit through the epididymis is the profound modification that is seen in the composition of the sperm membranes. Enzymes secreted by epithelial cells have been shown to modify membrane glycoproteins (Hammetstedt and Parks, 1987b; Jones, 1989b; Srivastava and Olson, 1991b; Tulsiani *et al.*, 1993b; Tulsiani *et al.*, 1993b). A number of phospholipids located on the outer acrosomal membrane move through the aqueous space to the inner surface of the overlying plasma membrane, perhaps through activity of a phospholipid transfer enzyme (Amann *et al.*, 1993). A phospholipid 'flippase' may serve as a catalyst for maintaining lipid balance across the sperm cell surface bilayer, and would thus serve as a stabilizing molecule, inhibiting fusion of the sperm membrane with the acrosomal membrane until the acrosome reaction is required (Nolan *et al.*, 1993b; Nolan *et al.*, 1993b). In addition, there is a profound change in the topical regions occupied by specific phospholipids as a result of modification of glyco-proteins, exchange of specific phospholipids, and an increase in the relative amount of cholesterol in the plasma membrane, resulting in decreased fluidity and a

lower membrane stability [as a prerequisite for the acrosome reaction (Nolan *et al.*, 1992)]. An interesting phenomenon is the incor-poration into the sperm plasma membrane of a number of proteins that are secreted by the epididymal epithelium (Vreeburg *et al.*, 1992). The precise role of each of the modified or acquired phospholipids in the plasma mem-brane of spermatozoa remains to be elucidated. However, it is clear that the ability to bind to and fertilize an oocyte is dependent on changes in the sperm plasma membrane during epididymal transit (Jones, 1989).

Epididymal epithelial cell functions

Functionally, the epididymis can be split into three segments: the initial segment where absorption of fluids and ions occurs, a middle segment where sperm maturation takes place, and the caudal section which is suited for sperm storage.

The ductuli efferentes and the initial segment are responsible for absorption of most of the fluid that is expelled from the rete testis. In the efferent ductules, the non-ciliated cells are able to perform fluid and adsorptive endocytosis (Hamilton and Cooper, 1978), while the ciliated cells may have a function mainly in moving the spermatozoa and luminal fluid through the ducts, but may also assist in modifying the luminal environment by endocytosis (Hermo and Morales, 1984; Robaire and Hermo, 1988). In the initial segment, it is primarily the principal cells that are actively involved in fluid absorption. The extraction of water from the epididymal lumen is passive, following active sodium absorption which is dependent on selective sodium channels in the apical membrane. Sodium reabsorption can be inhibited by the Na^+ channel blocker amilioride (Wong and Yeung, 1976; Wong *et al.*, 1978) and is dependent on Na^+-K^+-ATPase activity in the basolateral membrane of the cell. Next to absorption, the epididymis is capable of electrolyte and water transport into the lumen. Secretion is mediated by a Na^+-K^+-$2Cl^-$ symport, and a Na^+-H^+ exchanger and a Cl^- - HCO^{3-} exchanger located in the basolateral membrane of the epithelial cells (Wong, 1988; Wong, 1990). Interestingly, spermatozoa may have an influence on the fluid volume in their own environment; the epithelial cells are known to secrete angiotensin I which is converted into angiotensin II by angiotensin-converting enzyme located on the sperm plasma membrane. Angiotensin II receptors are found on the epididymal epithelial cells, which,

when activated, stimulate fluid secretion (Wong *et al.*, 1990; Wong and Uchendu, 1990).

As mentioned earlier, the epididymis is highly active in synthesis and secretion of proteins. A large number of epididymal proteins have been identified, the function of which is often unknown. Some proteins, however, have been extensively studied, and have been assigned putative roles in functional maturation of spermatozoa.

Studies by Cameo and Blaquier (Cameo and Blaquier, 1976) have elucidated a number of epididymal proteins (B,C,D,E) from cytosolic fractions of rat epididymal epithelium. Proteins B/C were later found to be two products of the same gene, but with a slightly different molecular weight due to three additional amino acid residues at the amino terminus of protein C. Others have also identified proteins B/C in the epididymis of rat and mouse, and have introduced their own nomenclature: proteins B/C are also known as rat epididymal secretory protein I [ESP I] (Jones *et al.*, 1980; Girotti *et al.*, 1992), rat cauda proteins CEF-2,3 (Shabanowitz and Killian, 1987), 18 kD α-lactalbumin-like component (Moore *et al.*, 1990), and mouse epididymal protein protein 10 (Rankin *et al.*, 1992b). Proteins B/C are secreted only in the caput, but are synthesized in both the caput and the corpus regions (Brooks and Higgins, 1980; Moore *et al.*, 1990). The synthesis of proteins B/C is androgen dependent, and is regulated at the level of gene transcription. Indeed a putative androgen response element has been identified upstream from this gene (Girotti *et al.*, 1992). The exact functions of proteins B/C are not completely clear. It has been shown that there exists a similarity with the $\alpha_{2\gamma}$-globulin superfamily of retinoic acid binding proteins (Brooks, 1987). Rankin *et al.* (Rankin *et al.*, 1992a) have shown that the mouse homologue of proteins B/C (MEP 10) does indeed bind retinoic acid with high affinity, indicating that proteins B/C may have a role in the transport of retinoids within the epididymis.

Proteins D/E are also encoded by one gene, and have been identified by a number of investigators: 37 kD glycoprotein (Olson and Hamilton, 1978), specific epididymal protein [SEP] (Kohane *et al.*, 1980), protein IV (Jones *et al.*, 1980), acidic epididymal glycoprotein [AEG] (Lea and French, 1981), sialoprotein (Faye *et al.*, 1980), 32 kD protein (Wong and Tsang, 1982), proteins CEF-12,13 (Shabanowitz and Killian, 1987), and mouse epididymal protein 7 (Rankin *et al.*, 1992b). Amino acid sequence analysis shows similarity with metalloproteins. Interestingly, studies have shown that

spermatozoa may acquire proteins D/E on their plasma membrane during epididymal passage (Brooks and Tiver, 1983). Incubation of rat spermatozoa with rabbit antibodies against proteins D/E caused a large reduction in egg fertilization after artificial insemination (Cameo *et al.*, 1986). Recently Rochwerger *et al.* (1992), have shown the presence of protein D/E recognition sites on the egg surface, and have indicated a putative role for facilitating sperm-oocyte interaction. Indeed, the development of fusability of developing rat oocytes with spermatozoa involves the appearance of protein D/E-binding components on the oocyte surface (Cohen *et al.*, 1996). Other epididymal proteins include clusterin, composed of a 47 kD heavy chain and 34 kD light chain, forming a heterodimeric glycoprotein. It is homologous to the testicular sulphated glycoprotein 2 produced by Sertoli cells. Unlike proteins B/C and D/E, it is not androgen regulated in the initial segment, but an increase in clusterin mRNA was found after orchidectomy in the corpus and cauda (Garrett *et al.*, 1990; Cyr and Robaire, 1992). Spermatozoa are found to associate with clusterin in the caput, but this is followed by dissociation in the cauda (Hermo *et al.*, 1991; Mattmueller and Hinton, 1992).

The epididymis secretes a number of enzymes that may modify and process preexisting sperm plasma membrane glycoproteins. Among them are sialyltransferase, galactosyltransferase, N-acetylglucosidase, and fucosyltransferase, which are present in the caudal fluid (Hamilton, 1980a; Tulsiani *et al.*, 1993a). Glycosidases, such as β-D-galactosidase, may have a role in the removal of galactosyl residues from sperm surface glycoproteins during epididymal transit. Furthermore, α-D mannosidase [also found on sperm membranes and being a putative zona receptor (Cornwall *et al.*, 1991)] is found in luminal fluid (Tulsiani *et al.*, 1993b). This fluid also contains glutathione peroxidase, a 24 kD androgen regulated secretory protein, which is produced in the caput epididymis and associates with spermatozoa. Superoxide dismutase, a powerful antioxidant enzyme, has been detected on the stereocilia of the epididymal principal cells (Perry *et al.*, 1993). Furthermore, a putative sperm-protector enzyme, gamma glutamyl transpeptidase (GGT) plays an essential role in controlling the luminal concentration of glutathione, a potent scavanger of free oxygen radicals which are toxic to spermatozoa (Hinton *et al.*, 1991). Other putative sperm protectors are HE4 and cystatin-related epididymal protein (CRES), which both are similar to protease inhibitors found elsewhere in the body, and may serve to protect the

spermatozoa and/or the epididymal epithelium from proteolytic activity, such as may result from premature release of acrosomal enzymes from some spermatozoa (Kirchhoff *et al.*, 1991; Cornwall *et al.*, 1992).

Aim and Scope of This Thesis

The above paragraphs indicate that male gametogenesis and sperm maturation are fine tuned processes, requiring a large number of regulated molecular interactions. The large amount of effort that has been invested into understanding the intricate control mechanisms of male reproduction, has resulted in the identification of numerous structural and regulatory molecules. The precise role of many of these molecules in normal male reproductive physiology remains to be clarified.

Of prime importance in male reproduction is the action of androgens. In Chapter 2, the role of androgen in spermatogenesis and in sperm maturation is explored. The precise mechanism by which testosterone regulates spermatogenesis is largely unknown. Of major interest is the observation, that the testis, being the site of androgen production, is exposed to, and also requires a much higher level of testosterone for adequate function than do the other androgen dependent organs, such as the prostate and the seminal vesicles.

In Chapter 3, a study is presented, where the action of testosterone, and that of the synthetic androgen methyltrienolone (R1881) is evaluated for male rat testis and accessory sex organs. R1881 is not metabolised and does not bind to androgen binding protein (ABP). This study is based on the premise, that the observed difference in testosterone responsiveness of the testis, compared to other androgen dependent organs, may be caused by a difference in testosterone metabolism, and/or by binding of testosterone to ABP in the testis, possibly making much of the testicular androgen biologically ineffective. In addition, the effect of androgen metabolism and binding of androgen to ABP on the regulation of androgen receptor immunoexpression in the adult rat testis is studied.

In Chapter 4, the immunoexpression of androgen receptor and expression of its mRNA is studied in the rat epididymis. The epididymis is highly androgen dependent in quite a unique fashion, in that it is regulated not only testosterone by from the bloodstream, but also by luminal testosterone,

directly emanating from the testis through the efferent ductules. There is a highly compartmentalized regional variation in epididymal epithelial cell function. While regional differences in androgen receptor immunoexpression have been described for the epididymis of a number of species, little data is available for the rat, a model widely used for male fertility studies. Double staining studies were performed to ascertain whether the cell to cell variation in proteins B/C, a highly androgen dependent protein, correlate with a variation in androgen receptor immunoexpression.

While the above reports are mainly concerned with regulation of processes in laboratory animals, the following Chapters describe observations from studies on infertile human males. Although the diagnostic arsenal within the andrology clinic has grown over the past number of years, many causes of male infertility remain to be elucidated. Knowledge of the cause of a man's infertility is important, not only for providing the patient with a sound diagnosis, but also for adequate counselling purposes if assisted reproductive techniques are considered. Ideally, clarification of the molecular mechanisms of male gametogenic impairment may open the way for a true cure. Additionally, with the health of the offspring in mind, it is important to ascertain what effects suboptimal spermatogenesis and sperm maturation may have on the integrity of the male gamete. In this thesis, two studies are presented that attempt to tackle this problem from different perspectives. Chapter 5 briefly introduces the reader in the genetic and a number of acquired conditions that are related to male infertility. Subsequently, the role of androgen receptor defects in male infertility is scrutinised. In Chapter 6, androgen receptor immunoexpression in testis biopsy samples of a number of infertile males is semiquantitatively assessed. A number of earlier studies have resulted in conflicting data regarding the prevalence of androgen receptor abnormalities in genital fibroblasts of infertile men. As the testis is the target organ for testosterone, where spermatogenesis is concerned, it is reasonable to speculate that putative aberrations of androgen receptor expression in the context of male infertility would be most apparent in that organ. Subsequently, a study is undertaken to assess the integrity of sperm nuclear condensation in subfertile males (Chapter 7). Using an antibody targeting tyrosine hydroxylase, which cross reacts with testis-specific histone 2B (TH2B), we first evaluated the immunoexpression of this major testis-specific histone variant in the various germinal cells within the human testis.

In order to explore the relationship of sperm histone content to semen parameters, we then quantified sperm TH2B content in semen samples from men attending our andrology outpatient clinic, and correlated these results with the routine semen analysis data.
In Chapter 8, the main results and implications of the studies reported in this thesis are discussed.

Part 1

Basic Studies on Spermatogenesis and Sperm Maturation

2

Androgen control of spermatogenesis and sperm maturation

Testicular androgens are necessary for the development and maintenance of male sexual characteristics, including the spermatogenic function of the testis and sperm maturation in the epididymis. In 1930 it was established that removal of the pituitary gland resulted in degeneration of spermatogenesis in the rat. This degeneration was prevented, however, by substitution with a high dose of testosterone (Smith, 1930). Since then, a considerable amount of research has focused on the hormonal regulation of spermatogenesis and sperm maturation, with particular interest in the action of androgens. Surprisingly, the results of this research still leave room for many questions and much discussion. Investigators have yet to elucidate through which mechanisms androgens are involved in initiation and maintenance of spermatogenesis. There is general agreement that the testis requires a much higher level of testosterone for adequate function than the other androgen dependent tissues; however, why this is the case, and exactly what concentration of testosterone is required for spermatogenesis to proceed, is still a matter of debate. What, then, do we know about androgens and male gametogenesis?

The Androgen receptor

Androgens exert their effect on target tissues through interaction with the androgen receptor (AR), a member of the superfamily of ligand-dependent transcription factors. Other members of this family include receptors for all steroid hormones, thyroid hormone, retinoids, vitamin D3, and a number of receptors lacking a known ligand (orphan receptors) (Tsai and O'Malley, 1994). These receptors are similar in structure, and may all have been derived from a single archaic precursor (O'Malley, 1989; Dorit *et al.*, 1990; Amero *et al.*, 1992; Keese and Gibbs, 1992; Laudet *et al.*, 1992). The structure is classically divided into four domains: 1) the N-terminal modulating domain, 2) the DNA binding domain, 3) the hinge region, and 4) the ligand binding domain at the C-terminus.

The single copy gene encoding the AR is located on the X chromosome in all mammalian species, in man at the Xq11-12 locus. The human *AR* cDNA sequence reveals an open reading frame of 2730 nucleotides encoding a protein of 910 amino acids with a calculated molecular mass of 98.5 kDa (Brinkmann *et al.*, 1989; Brown *et al.*, 1989; Kuiper *et al.*, 1989). The information for the protein coding region is separated over eight exons. The

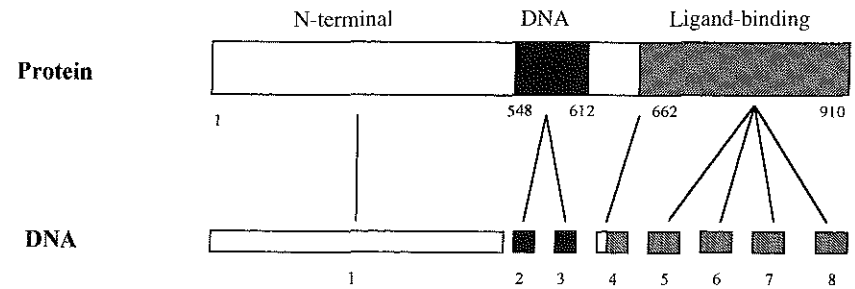

Figure 1. The human androgne receptor is encoded by 8 exons, and has several distinct functional domains: the N-terminal domain, the DNA-binding domain, and the ligand binding domain.

N-terminal modulating domain is encoded by one large exon, while the DNA binding domain is encoded by exons 2 and 3. The hinge region and the ligand binding domain are encoded by the remaining 5 exons. The N-terminal domain of the human AR is characterized by the presence of a number of stretches of amino acid residue repeats (i.e. three polyglutamine stretches, a long polyglycine stretch, a polyproline stretch, and a polyalanine stretch). The functional significance of these homopolymeric stretches is unknown (Faber *et al.*, 1989). The hormone binding domain comprises about 250 amino acid residues. Deletions in this domain result in loss of hormone binding. Interestingly, the complete deletion of the hormone binding domain results in a constitutively active receptor, indicating that the hormone binding domain is an inhibitor of transregulation activity in the absence of hormone (Jenster *et al.*, 1991).

The subcellular location of the androgen receptor is dependent on androgen exposure and other factors. In cell lines where the AR is transiently overexpressed, cellular localization in the absence of hormone was predominantly cytoplasmic in COS-1 cell lines, evenly distributed over cytoplasm and nucleus in CHO and CV-1 cells lines, and predominantly nuclear in HeLa cells. Addition of androgen resulted in a nuclear localization of the AR in all cell lines (Simental *et al.*, 1991; Jenster *et al.*, 1993). *In vivo* immunohistochemical data have shown a nuclear localization of the AR even in androgen depleted tissues (Chang *et al.*, 1989; Husmann *et al.*, 1990; Sar *et al.*, 1990; van der Kwast *et al.*, 1991), although increased cytoplasmic staining of AR has been observed in androgen deprived prostate tissue (Paris *et al.*, 1994).

Upon binding of ligand (testosterone or dihydrotestosterone) to AR in cytoplasm or nucleus, the AR dissociates from heat-shock proteins (hsp90 and hsp70), forms a dimer with a second activated AR molecule, and binds to specific enhancer sequences known as androgen response elements present in the 5' flanking regions of androgen-regulated genes (Riegman *et al.*, 1991). The binding of AR dimers to the androgen response elements are thought to stabilize transcription factors at the promoters of target genes, thereby inducing a high degree of transcription initiation (O'Malley and Tsai, 1992; Jenster, 1998).

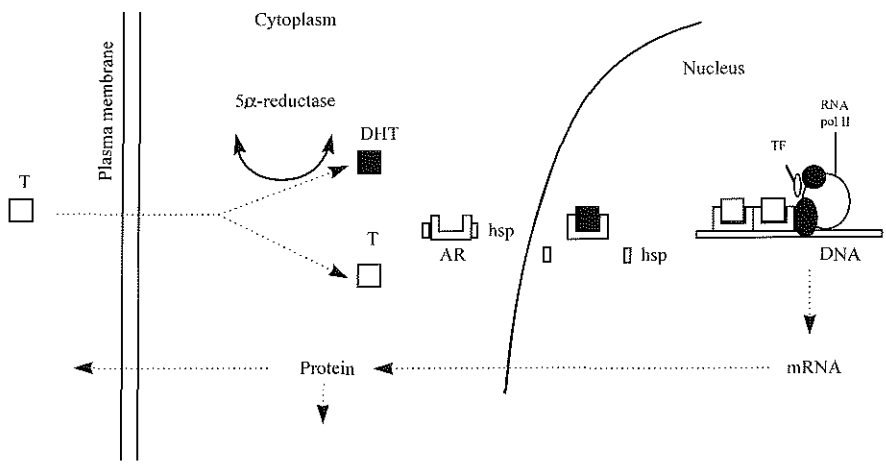

Figure 2. Mechanism of androgen action in a target cell. Testosterone (T) diffuses into the cell cytoplasm where it can be converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Both T and DHT can bind to, and activate, the androgen receptor. Upon binding with ligand, the androgen receptor dissociates from heat shock proteins (hsp), and binds to DNA as a homodimer. TF = transcription factors; RNA pol II = RNA polymerase II.

Expression of the androgen receptor in the testis

The pattern of expression of the androgen receptor has been a focus of research for a considerable amount of time, and has been studied using various methods. Early biochemical studies have demonstrated AR in reproductive, pituitary, and brain tissues of the male rat (Liao and Fang, 1969; Hansson *et al.*, 1975; Kato, 1976; van Doorn *et al.*, 1976; van Doorn and Bruchovsky, 1978; McGinnis *et al.*, 1983; Handa *et al.*, 1986; Handa *et al.*, 1987; Roselli *et al.*, 1989). Autoradiographic studies have indicated a

nuclear localization of AR in a number of tissues (Sar *et al.*, 1970; Sar and Stumpf, 1972). In the mid 1970's androgen receptor was demonstrated in rat seminiferous tubules (Mulder *et al.*, 1974; Mulder *et al.*, 1975). Later, binding studies using isolated cells indicated absence of AR in spermatocytes and spermatids (Grootegoed *et al.*, 1977). The cloning of the genes encoding human, rat, and mouse AR subsequently provided the amino acid sequence of the AR, opening the way to the fabrication of antibodies to synthetic peptides with sequences corresponding to selected regions of the AR (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Trapman *et al.*, 1988; Tilley *et al.*, 1989; van Laar *et al.*, 1989). A number of immunohistochemical studies have indicated a high level of AR expression in the testis, accessory sex organs, excretory ducts, and several other organs (Sar *et al.*, 1990; Takeda *et al.*, 1990; Ruizeveld de Winter *et al.*, 1991; Kimura *et al.*, 1992; Iwamura *et al.*, 1994; Janssen *et al.*, 1994) . The reported immunoexpression patterns of AR within the testis are in agreement with regard to expression of AR in the nuclei of Leydig cells, Sertoli cells, and peritubular myoid cells. In addition, a number of studies reported expression of AR in the (endothelial) cells associated with arterioles (Bergh and Damber, 1992; Sar *et al.*, 1993; Bremner *et al.*, 1994). A controversy has arisen, however, concerning the expression of AR in germinal cells. While most investigators have found no indication of androgen receptor in the nuclei or cytoplasm of developing germ cells, Kimura *et al.* (1993) and Vornberger *et al.* (1994) have reported germ cell AR immunoexpression in human and rat testis, respectively. When reflecting upon the presence of AR in male germ cells going through spermatogenesis, one should perhaps also consider the genetic evidence against functional importance of AR in the germ cells. The common male vole lacks X chromosomes in all spermatogonia and developing germ cells (Griswold, 1995), and it follows that spermatogenesis can proceed without germinal cell androgen receptors. In addition, breeding experiments using chimerae of Tfm (testicular feminization) and normal mice, resulted in Tfm offspring. This implicates that Tfm germ cells, lacking a functional AR, have completed spermatogenesis, probably because they were supported by wild-type, AR positive, somatic cells (Lyon *et al.*, 1975). Lack of androgen receptors in germ cells, however, does not exclude the possibility of a direct androgen action on germ cells not mediated via the AR.

Close scrutiny of AR immunoexpression in Sertoli cells of the rat has disclosed a clear variation in staining intensity at different stages of the

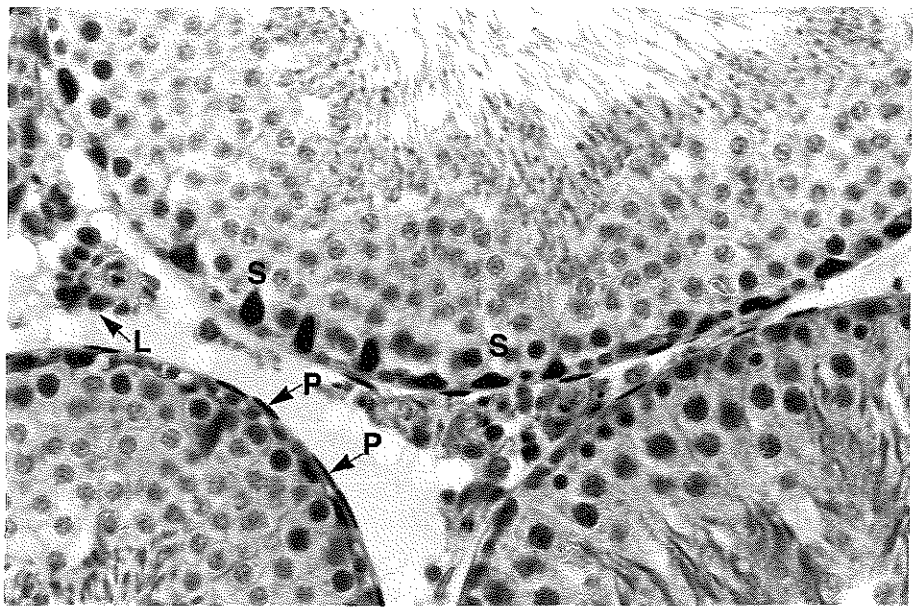

Figure 3. Immunolocalization of androgen receptor (AR) in the testis of an adult rat. S=Sertoli cell, P=peritubular myoid cell, L=Leydig cell. Notice the absence of any AR immunosignal in germinal cells.

spermato-genic cycle (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). Expression was found to be relatively low at stages I-V. Subsequently, there is a rapid rise in the immunosignal, until staining is maximal at stage VII. Thereafter, the AR immunosignal decreases until it is barely detectable at stage IX. The peritubular myoid cells and the Leydig cells also show some variability in AR expression, but this is not related to the stage of the spermatogenic cycle in the neighboring tubule(s). It has recently been shown that the stage-dependent variation in AR immunoexpression in rat Sertoli cells is regulated at the transcriptional level, as AR mRNA *in situ* hybridization studies show an expression pattern which overlaps with that of the protein (Shan *et al.*, 1995).

Androgen receptor expression in the epididymis

Androgens have profound effects on epididymal function. Immunohistochemical analysis of the AR in the epididymis of a number of adult mammalian species, revealed strong expression, most prominently in the

nuclei of the epithelial cells (Tekpetey *et al.*, 1989; Sar *et al.*, 1990; Takeda *et al.*, 1990; Roselli *et al.*, 1991; Ruizeveld de Winter *et al.*, 1991; Iwamura *et al.*, 1994; Janssen *et al.*, 1994; Paris *et al.*, 1994). Both biochemical and immunohistochemical approaches have indicated region-specific variation in AR expression. In the human epididymis, Takeda *et al.* (1990) observed high expression in the proximal portion (caput), decreasing gradually in the corpus region. In the distal portion (cauda) AR immunoexpression was found to be higher than in the corpus (Takeda *et al.*, 1990). A recent report by Ungefroren *et al.* (1997), however, describes low AR mRNA expression in the proximal caput, and high expression in the distal caput and proximal corpus of the human epdidymis. In the above study, these findings were in agreement with the pattern of immunohistochemical AR expression (Ungefroren and Ergun, 1997). The caput and corpus regions of the rhesus monkey epididymis have a more intense AR immunosignal, compared to the ductuli efferentes and the cauda (Roselli *et al.*, 1991). In the ram, AR immunostaining is low in the efferent ducts but very high in the initial segment. Predominant AR immunoexpression was observed in the epithelium of the central caput, central corpus, and cauda of the ram epididymis (Tekpetey *et al.*, 1989). Regional variations in immunoexpression of AR in the epididymis were also detected in the rat: expression was highest in the caput epithelial cells, with lower levels in the corpus and cauda (Sar *et al.*, 1990). AR immunoexpression in the epididymis is regulated by the availability of androgens. Upon androgen withdrawal, the AR immunosignal decreases in all epididymal segments in the rat, and is rapidly restored when testosterone is supplemented (Sar *et al.*, 1990; Paris *et al.*, 1994). The available evidence suggests that epididymal AR expression is dependent on a normal circulating androgen level, but not on testosterone entering the luminal fluid from the testis, although this issue has not been under close scrutiny. At the level of gene transcription, data is relatively scant. Blok *et al.* (1992a) reported a substantial increase in AR mRNA as measured on Northern blot for whole epididymis, 5 days after treatment with the Leydig cell toxin EDS. A pulse treatment with 10 mg testosterone propionate 2 hours prior to killing, did not decrease AR mRNA level. Treatment with testosterone implants (15 cm) for 5 days, however, returned the mRNA level to control value (Blok *et al.*, 1992a).

Androgens: distribution and metabolism in the testis and epididymis

Testicular androgens

Testosterone, by far the most prominent testicular androgen, is synthesized by the Leydig cells upon stimulation by LH from the pituitary gland. Leydig cells do not store testosterone, but the testosterone diffuses rapidly into the interstitial compartment (De Jong *et al.*, 1974; Sharpe, 1994). Subsequently the testosterone may either diffuse into the capillary network from where it enters into the peripheral blood stream, or it is transported into the seminiferous tubules. A large effort has been made in the past decades to elucidate the actual level of testosterone in the testis in relation to the level of testosterone that is required for initiation and maintenance of spermatogenesis. A number of different strategies for measurement of intratesticular testosterone levels have yielded discordant results. Interstitial fluid has been collected using a number of techniques (Sharpe, 1979; Turner *et al.*, 1984), giving an impression of the concentration of testosterone in the extratubular compartment. The obtained values vary widely between individual rats and studies, but fall in the range 60-400 ng/ml (Sharpe, 1994). Micropuncture techniques from rat testis *in situ* have obtained similar results (Comhaire and Vermeulen, 1976). Seminiferous tubular testosterone content has been estimated at 80-90 ng/ml. This value is similar to that found in homogenized rat testis (50-62 ng/ml) (Sharpe, 1994). The circulating T concentration in the rat is in the order of 1-3 ng/ml, about 20-fold lower than the seminiferous tubular testosterone content. A number of studies have measured the testosterone level in testicular venous blood, taken from the vessels that are found on the surface of the testis, containing blood that has just passed through the testicular interstitium. The testosterone concentration in the testicular venous blood appears to be in the range 25-100 ng/ml (Sharpe, 1994). Although there is considerable overlap in the testosterone concentrations found in the interstitial fluid and the testicular venous blood, when measured in the same animal, the testosterone concentration in the interstitial fluid is always two- to three fold higher than that in testicular venous blood (Sharpe, 1994). The blood obtained from the spermatic vein (i.e. spermatic cord) consistently has a testosterone concentration about 25-50% of that found in the testicular veins (Maddocks *et al.*, 1993; Sharpe, 1994). Apparently, as the venous blood travels up the spermatic cord, it is diluted by blood from the testicular artery through the arteriovenous

anastomoses (Wensing and Dijkstra, 1981; Wensing *et al.*, 1981; Hees *et al.*, 1984; Noordhuizen-Stassen *et al.*, 1985). The amount of shunting between the testicular artery and venous plexus is extremely variable, making the spermatic venous blood an unreliable source when one is interested in the testicular testosterone concentration. Peripheral venous blood does give a good indication of Leydig cell function, but provides no insight in the intratesticular testosterone concentration (Maddocks and Sharpe, 1989; Maddocks *et al.*, 1993).

The testosterone concentration observed in the various testicular compartments thus appears to disclose very different values, which indicates that testosterone may not diffuse freely and rapidly from one testicular compartment to another (Sharpe, 1994). However, at this point it is important to note that methodological error may play a large part in the high level of testosterone measured in the interstitial fluid. Normally, the newly produced testosterone diffuses into the interstitial fluid and then into the blood stream. When a testis is removed, the blood vessels are necessarily severed and the circulation is arrested, causing an accumulation of testosterone, still produced by the Leydig cells, in the interstitium. When measuring testicular testosterone concentration in animals with vital Leydig cells, it is important to prevent this accumulation by flash-freezing the testis in liquid nitrogen. The alternative would be to collect interstitial fluid from a testis *in situ*. Sharpe (1994) estimated the testicular testosterone concentration at 25-100 ng/ml, in a review partially dedicated to this issue. Our own data, using flash-frozen homogenized testis reveal an intratesticular concentration of 46 ng/mg, as compared to 1.2 ng/ml in peripheral venous blood (Chapter 3).

Androgens in the epididymis

The epididymis is a unique organ, in that it has a gradient of testosterone along its length, with a very high testosterone level in the initial segment, and much lower levels further distal (Vreeburg, 1975; Turner *et al.*, 1984). Testosterone enters the epididymis with the testicular fluid, and is also supplied via the bloodstream. Studies have shown, however, that DHT [and possibly also 5α-androstan-3α,17β-diol (3α-diol)], rather than testosterone, is vital for adequate function of the epididymis (Lubicz-Nawrocki, 1973; Orgebin-Crist *et al.*, 1976). This is indicated by an *in vivo* experiment where the effect of testosterone and DHT in the presence or absence of an inhibitor

of 5α-reductase in acutely castrated male mice was studied. This study showed that inhibition of 5α-reductase caused a decrease in number of motile spermatozoa, a lower percentage of oocytes fertilized, and a smaller number of blastocysts (Cohen *et al.*, 1981). High-dose testosterone supplementation did not diminish this effect. Rapid washings of epididymal minces and micropucture techniques have indeed indicated a very high level of DHT in the caput epididymis and a somewhat lower level further distal (Vreeburg, 1975; Turner *et al.*, 1984). It can be concluded that, contrary to the situation in the testis, DHT is the most prominent androgen in the adult epididymis.

Androgen binding protein and 5α-reductase in the testis

Androgen binding protein

Androgen binding protein (ABP) may be of some importance when discussing the distribution and action of testicular testosterone. ABP, the rat counterpart of the well known human steroid hormone binding globulin (SHBG), was one of the first Sertoli cell products to be identified and studied (Hansson *et al.*, 1975). ABP is secreted by Sertoli cells upon stimulation by FSH and testosterone, in a bi-directional manner: 20% is released into the bloodstream, probably from the base of the Sertoli cells and 80% is secreted into the seminiferous tubular fluid (Gunsalus *et al.*, 1980). In many species ABP/SHBG is also synthesized in the liver. The fetal rat liver does exhibit ABP synthesis, but the testis is the only observed site of ABP production in the adult (Demyan *et al.*, 1989). Both liver- and testis-derived ABP are encoded by the same gene (Joseph *et al.*, 1988). Loss of germ cells results in a reduction of ABP that is released into the seminiferous tubules, while secretion of ABP at the basal side of Sertoli cells is unaltered (Gunsalus *et al.*, 1985; Morris *et al.*, 1987). As ABP binds testosterone with moderate affinity, and is present at a relatively high concentration in the testis, it was thought that ABP may have a function in storage or buffering of testosterone in the testis. A number of studies attest to this, as treatment of rats with testosterone implants, causing a virtually complete inhibition of testicular testosterone production through inhibition of LH, resulted in the consistent finding that the testicular testosterone level was higher than the serum level (Zirkin *et al.*, 1989). Increasing the implant length caused a sharper increase in the

testicular testosterone level than the serum level (Zirkin *et al.*, 1989). This finding was not dependent on the presence of gonadotropins, as similar results were obtained with hypophysectomized rats (Turner *et al.*, 1985). Another study, using push-pull canulae to obtain interstitial fluid, however, did not support these findings (Maddocks and Setchell, 1989). It was pointed out that the push-pull canulae method of fluid collection may dilute the samples, resulting in an underestimation of the testosterone concentration (Maddocks and Setchell, 1989; Zirkin, 1993). Whether ABP is responsible for the above observations is uncertain. Indeed it has been pointed out by Turner *et al.*, (1984) that, in the intact rat testis, the androgen binding protein concentration is far too low to account for significant retention of androgens. No data are available concerning the possibility that, under conditions of a low intratesticular androgen concentration, ABP may indeed be responsible for the retention of a functional amount of testosterone in the testis. However, a number of observations have been interpreted to indicate that ABP may be of some importance in spermatogenesis. First, the secretion of ABP into the seminiferous tubules is highly regulated. Secretion is maximal at stages VIII-XI in the rat, coinciding with spermiation and a short period thereafter (Ritzen *et al.*, 1982). Second, it has been observed that ABP associates with germ cells of rodent and monkey testis (Bordin and Petra, 1980; Steinberger *et al.*, 1984; David *et al.*, 1985). More recently it was reported that ABP seems to be bound specifically by rat germ cells and endocytosed via a receptor-mediated endocytosis pathway (Gerard *et al.*, 1994). In the rat prostate, which also has ABP receptors at the cell surface, it was shown that ABP binding somehow is coupled to adenylyl cyclase, and that interaction of ABP with its receptor and activation of adenylyl cyclase are modulated by the binding of steroid to the ABP (Hryb *et al.*, 1989; Hryb *et al.*, 1990; Nakhla *et al.*, 1990; Rosner, 1990; Damassa *et al.*, 1991; Rosner *et al.*, 1992). However, as will be discussed in Chapter 3 of this thesis, association of testosterone to ABP in the testis may be of little functional importance for spermatogenesis.

5α-reductase

In androgen dependent tissues of the adult rat, other than the testis, testosterone is first converted into the more potent dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT has a two-fold greater affinity for

the androgen receptor and a dissociation rate one-fifth that of testosterone, resulting in the formation of a more stable receptor-ligand complex (Grino *et al.*, 1990). In the testis, however, the extremely high level of testosterone may lead to the conclusion that testosterone is the major androgen in the testis, abolishing the need for conversion to DHT. There is, on the other hand, ample evidence that conversion of testosterone into DHT does occur in the testis (Matsumoto and Yamada, 1973; Rivarola *et al.*, 1973; Nayfeh *et al.*, 1975; Rivarola *et al.*, 1975), in particular in the prepubertal individual (Van der Molen *et al.*, 1975). In the adult human, the DHT level in spermatic venous blood is much higher than the DHT level in peripheral blood (Pazzagli *et al.*, 1974). In addition, immunohistochemical studies have localized a varying amount of 5α-reductase type 2 in the human testis (Eicheler *et al.*, 1994). Studies in the adult rat have disclosed testicular 5α-reductase activity, albeit at a low level, coinciding with a low level of immunostaining of 5α-reductase type 1 in the testis (Robaire and Viger, 1993; Viger and Robaire, 1995). These reports are in agreement with earlier studies, which concluded that the intratesticular level of DHT is low, but that DHT is not absent (Van der Molen *et al.*, 1975; Turner *et al.*, 1984). At a functional level, it was found that treatment of beagle dogs with finasteride (a potent 5α-reductase inhibitor) does not affect spermatogenesis (Juniewicz *et al.*, 1993). Treatment of rats with testosterone implants, however, disclosed a clear role for 5α-reductase when the intratesticular concentration is low (O'Donnell *et al.*, 1996), but a functional role for 5α-reductase was no longer apparent when testosterone implants were larger than 6 cm. Individuals with a 5α-reductase type 2 deficiency are often infertile, but there is little evidence that this is a result of a testicular deficiency of the enzyme in adulthood. Clearly a 5α-reductase type 2 deficiency during development adversely affects testis descent, resulting in impaired spermatogenesis. In addition, this condition causes developmental and functional abnormalities of the highly DHT-dependent accessory sex organs, resulting in a rudimentary prostate and underdeveloped seminal vesicles with consequently highly viscous semen with a very small volume. Nevertheless, paternity has been reported in men with a 5α-reductase type 2 deficiency (Katz *et al.*, 1997). Based upon the available evidence, it is not likely that conversion of testosterone to DHT is of major importance for the process of spermatogenesis, at least in the rat. Additional data on the role of 5α-reductase in the rat testis, and its possible requirement for spermatogenesis is further discussed in Chapters 3 and 8.

5α-reductase and ABP in the epididymis

The expression of 5α-reductase in the epididymis has now been extensively documented. It is well known that 5α-reductase is present in a gradient along the length of the epididymis (Vreeburg, 1975; Turner *et al.*, 1984). There appears to be a nuclear fraction and microsomal fraction of enzyme activity (Robaire *et al.*, 1981; Scheer and Robaire, 1983). The activity of the enzyme associated with the nuclear fraction is highest in the proximal regions of the epididymis, namely in the initial segment and the caput, and declines further distally. The microsomal fraction of 5α-reductase activity is lower and found throughout the epididymis (Robaire *et al.*, 1981). The regulation of enzyme activity in the two fractions is differentially regulated, as orchiectomy and testosterone supplementation will restore the microsomal 5α-reductase activity, but not the nuclear activity. Any treatment that results in the cessation of flow of testicular fluid through the initial segment of the epididymis results in an inability to restore the normal nuclear 5α-reductase level, no matter how much androgen is supplemented. The appearance of the nuclear fraction of 5α-reductase activity does not follow the appearance and pattern of testosterone production by the testis during development (which is the case for the microsomal fraction), nor of the appearance of spermatozoa. Furthermore, the developmental pattern of nuclear 5α-reductase is identical to that of ABP. Taken together, the above-described observations have led to the suggestion that ABP may be of critical importance in regulation of the nuclear 5α-reductase activity (Robaire and Viger, 1995). In contrast, all data indicate that the microsomal fraction of 5α-reductase activity is solely regulated by the serum testosterone level.

After the cloning of two cDNAs, representing two different genes, encoding for the two 5α-reductase isoenzymes, named type 1 and 2 based on their chronological order of identification (Andersson *et al.*, 1989; Jenkins *et al.*, 1991; Labrie *et al.*, 1992; Normington and Russell, 1992), the expression and regulation of expression of the mRNAs of the two isoenzymes could be investigated. Northern blot analysis showed that the 5α-reductase type 1 mRNA is expressed at a high level in the initial segment of the epididymis (3- to 7-fold higher compared to the further distal regions), and reveals a pattern that is identical to the pattern of expression of the nuclear 5α-reductase activity (Robaire *et al.*, 1981; Viger and Robaire, 1991; Robaire and Viger, 1995). Orchiectomy resulted in a decrease in type 1 mRNA in all segments, but most dramatically in the initial segment. High-dose testosterone

supplementation resulted in restoration of type 1 mRNA expression throughout the epididymis, with exception of the initial segment. Unilateral orchiectomy or efferent duct ligation resulted in decrease of type 1 mRNA in the initial segment only. During development, changes in the type 1 mRNA level were observed only in the initial segment: a decrease to 50% between days 7 and 21 post partum, and then a 5-fold rise until day 56, after which the level remains constant. The observed variation in 5α-reductase type 1 mRNA is well correlated with enzyme activity. Northern blot analysis of 5α-reductase type 2 indicated a high level of expression in the caput epididymis, but not in the initial segment. During development, no increase was seen in type 2 mRNA expression. In fact, all epididymal regions show no change in type 2 mRNA from birth to adulthood (Viger and Robaire, 1994; Robaire and Viger, 1995). In the adult rat, unilateral castration or efferent duct ligation results in a marked increase in type 2 mRNA in the initial segment of the epididymis. Clearly the mRNAs of the 5α-reductase type1 and type 2 isoenzymes are differentially regulated, and the data suggest that it is primarily the type 1 mRNA expression pattern that correlates best with enzyme activity, while the mRNA encoding the type 2 isoenzyme appears to be poorly translated in the epididymis (Robaire and Viger, 1995; Viger and Robaire, 1996).

Testicular effects of androgen deprivation

The germ cell complement

The early observation that depletion of androgens, although causing severe qualitative impairment of spermatogenesis, does not influence the duration of the spermatogenic cycle or the duration or number of spermatogenic stages (Clermont and Harvey, 1965), is at the basis of many later studies. A number of studies in the rat have shown that, upon withdrawal of androgens, the earliest morphological changes in the seminiferous epithelium are seen at stages VII and VIII (Russell and Clermont, 1977; Sharpe *et al.*, 1990; Sun *et al.*, 1990; Sharpe *et al.*, 1992; Kerr *et al.*, 1993; Sinha Hikim and Swerdloff, 1993). These changes consist of the appearance of degenerating germ cells. Interestingly, when time passes after androgen withdrawal, the number of degenerating germ cells at several stages following stages VI-VIII increase, while virtually no degenerating germ cells are seen in the stages preceding

stage VII. An explanation for this observation is that, as a consequence of the low androgen level, certain developmental events have not occurred during stages VII and VIII, initiating impaired germ cell development and their degeneration at later stages of spermatogenesis (Sharpe, 1994). The morphological data thus support a stage-specific temporal window of androgen action during spermatogenesis, namely at stages VII and VIII in the rat.

Which germ cell types are affected most by androgen depletion, and which developmental processes are compromised? A study using intact rats treated with low-dose testosterone for a 13 week period, reported a decrease in the number of spermatogonia to 57%, in the conversion of spermatogonia to spermatocytes to 85%, and in the conversion of round to elongating spermatids to 19% (Sun *et al.*, 1990). Only the latter effect was largely prevented by high-dose, rather than low-dose, testosterone treatment (Sun *et al.*, 1990). Sustenance of spermatogenesis, particularly the conversion of spermatocytes to round spermatids, was less effective in hypophysectomized rats using the same testosterone treatments, indicating that possibly (an)other pituitary factor(s) may be of importance.

Another study, using a GnRH antagonist, showed a marked and rapid (after 1 week of treatment) reduction of preleptotene and pachytene spermatocytes and step 7 and step 19 spermatids at stage VII (Sinha Hikim and Swerdloff, 1993). After 4 weeks of treatment, the conversion of A1 spermatogonia to preleptotene spermatocytes was suppressed to 79%. This finding was attributed to loss of preleptotene spermatocytes, since no degenerating spermatogonia were encountered, and full restoration of the number of preleptotene spermatocytes was attained after 1 week resupplementation with testosterone (Sinha Hikim and Swerdloff, 1993). When GnRH antagonist treatment was continued during transition from preleptotene to pachytene, 55% cell loss was encountered. The development from pachytene spermatocytes to step 7 (round) spermatids showed a 15% cell death. The step 7 spermatids failed completely to progress through spermiation. Clearly, in this study, androgen deprivation results in pronounced loss of germ cells during the meiotic and spermiogenic stages of germ cell development. Quantitative results obtained using hypophysectomy were identical to those obtained using a GnRH antagonist (Sinha Hikim and Swerdloff, 1993).

An important effect of testosterone on spermiogenesis was also observed in a study where rats were treated with low-dose testosterone and estradiol (TEst)

for 11 weeks to suppress spermatogenesis, after which the rats received high-dose testosterone supplementation which resulted in a testicular testosterone concentration 7-12% of control (O'Donnell *et al.*, 1994). Round spermatid production at stages I-VII was suppressed to 29-35% of control in TEst treated rats, but remained unchanged after the high-dose testosterone treatment. At stage VIII, however, round spermatid production was reduced to 5% of control in TEst treated rats, and increased to 27% after 4 days of high-dose testosterone supplementation. Moreover, the conversion of round spermatids to elongating spermatids at stages VII and VIII was reduced to 16% of control in TEst treated rats, but fully returned to normal after 4 days of high-dose testosterone supplementation. Thus, in this study, spermiogenesis was fully restored when the testicular testosterone concentration was only 7-12% of control.

The above reports indicate that testosterone withdrawal acts primarily at stages VII and VIII of the rat spermatogenic cycle. The hourly production of round spermatids is impaired by androgen depletion -possibly as a result of germ cell depletion (degeneration or detachment from the Sertoli cells). Spermiogenesis (i.e. conversion of the available round spermatids) is also impaired, but is restored fully in situations where the testicular testosterone level is about 10% of normal (classically viewed as restoration of qualitatively normal spermatogenesis; see Sharpe, 1994). Interestingly, quantitative restoration of spermatogenesis, requiring the restoration of normal numbers of spermatogonia, spermatocytes and spermatids, appears to depend on a much higher level of testosterone. Additional experiments to reinitiate normal spermatogenesis after TEst treatment using hCG to restore Leydig cell function, did not succeed in restoring normal spermatogonial numbers despite obtaining a testicular testosterone level comparable to control (Meachem *et al.*, 1997).

A clear dose-dependent effect of high-dose testosterone supplementation on the quantity of spermatozoa produced in the androgen-deprived rat, has been reported on numerous occasions (Sun *et al.*, 1989; for review see Sharpe, 1994) and has resulted in speculation on why testis tissue seems to dependent on such a high level of testosterone for adequate function. This issue is further elaborated on in Chapters 3 and 8.

Protein synthesis and secretion

A number of interesting observations have been made concerning the regulation of protein secretion by Sertoli cells. In the normal rat, there is a two-fold increase in the overall level of protein secretion by the seminiferous epithelium at stages V-VIII, compared to the other stages (Sharpe *et al.*, 1992). The secretion of a number of the major proteins is 'constitutive', that is, remains the same in the presence or absence of a high androgen level (McKinnell and Sharpe, 1995). The secretion of one Sertoli cell protein and six proteins found to be secreted by germ cells, however, was highly regulated by androgens at stages VI-VIII of the spermatogenic cycle. These results indicate that not the synthesis, but rather the secretion of a number of proteins in the testis is androgen-regulated (McKinnell and Sharpe, 1995). The mechanism of action of androgens on protein secretion, however, remains elusive.

Gene expression

Although testis function is clearly highly androgen dependent, the identification of androgen regulated genes in the testis has proven difficult. Only recently, a transcription factor has been identified, that may be directly regulated by androgen. Using a subtraction hybridization technique, the *Pem* cDNA clone was isolated in a screen for developmentally regulated mouse genes (MacLeod *et al.*, 1990; Wayne and Wilkinson, 1998). *Pem* encodes a protein containing a homeodomain. In the adult mouse and rat, the gene is expressed selectively in reproductive tissues. The *Pem* gene has two promoters, a male specific (P_p) proximal promoter, that is responsible for its transcription in the testis and the epididymis, and a female specific (P_d) distal promoter, that is responsible for transcription in the ovary and the placenta (Maiti *et al.*, 1996; Wayne and Wilkinson, 1998). In the male, Pem protein is specifically expressed in Sertoli cells and the proximal cauda of the rat epididymis (Lindsey and Wilkinson, 1996). As Pem is a member of a large family of homeodomain transcription factors, and, in the male, is regulated by androgen, the evidence points to a putative role for Pem in androgen-regulated gene expression in testis and epididymis (Wayne and Wilkinson, 1998). Identification of down-stream genes which are regulated by Pem, is a subject of current research.

Epididymal effects of androgen deprivation

The epithelium

It has been known for many years that removal of the testis causes extensive atrophy of the epididymis (Benoit, 1926). Castration experiments have revealed that epididymis weight cannot be restored by androgen supplementation. This appears to be due to the large weight fraction that is attributable to the luminal fluid and spermatozoa, making organ weight an unreliable parameter by which to monitor epididymal function (Karkun *et al.*, 1974; Brooks, 1979). Microscopic studies have elucidated substantial changes in the epididymal epithelium upon deprivation of androgens. Most of these changes could be reversed, however, when the normal circulating level of testosterone was restored (Fawcett and Hoffer, 1979), although a decrease in duct diameter and an increase in epithelial height are observed during long term interruption of the inflow of testicular fluid. Holland *et al.* (1992) noticed obvious changes in the morphology of the epithelium in the distal corpus region of the rat epididymis, when the efferent ducts were ligated for a period of 8 months. The efferent ducts and the initial segment of the epididymis, however, appear to be differentially regulated. A number of studies have clearly shown that efferent duct ligation or castration causes widespread regression of the epididymal epithelium, and marked inhibition of protein synthesis and secretion, that cannot be reversed by high-dose testosterone supplementation (Fawcett and Hoffer, 1979; Holland *et al.*, 1992). It has been postulated that other testicular factors are necessary for the normal function of the proximal portions of the epididymis (Fawcett and Hoffer, 1979). The identity of these factors is as yet unknown. However, it has been postulated that ABP may have a significant role, especially in the transcription regulation of the 5α-reductase type 1 enzyme (Robaire and Viger, 1995), as previously outlined.

In addition, it has been reported that the intermediary energy metabolism of the epididymal tissue is dependent on lipid oxidation in the presence of androgens, but changes to full carbohydrate dependence when androgens are withdrawn (Brooks, 1981). Furthermore, the transport of ions across the epididymal epithelium, and the systems responsible for the transport of inositol and carnitine across the membrane of the epithelial cells, are androgen dependent (Bohmer *et al.*, 1977; Wong and Yeung, 1977; Brooks, 1980; Yeung *et al.*, 1980; Pholpramool *et al.*, 1982).

Gene expression

In a review by Cornwall and Hann (95), eight genes are described which are solely or partially regulated by androgens (genes encoding proteins B/C, proteins D/E, AEPI, GPX, 5α-reductase type 1, GGT, SGP2, and NGF) (Cornwall and Hann, 1995). The mRNA levels of proteins B/C and D/E, and GPX fall dramatically following androgen withdrawal, and recover to normal levels following testosterone supplementation. The SGP-2 mRNA level increases in the corpus and cauda regions, with little change in the initial segment and caput. Testosterone supplementation lowers the SGP-2 mRNA level in both the corpus and cauda. EAPI and 5α-reductase type 1 are both regulated by androgens and an as yet unidentified testicular factor. EAPI mRNA level is restored only to 70% of the precastrate level following high-dose testosterone replacement. Putative androgen response elements have been identified upstream of the GPX (Ghyselinck *et al.*, 1993) and protein B/C (Girotti *et al.*, 1992) genes. Studies on the effect of androgen supplementation on expression of GPX mRNA, indicate a role for androgens in stabilization of this mRNA (Ghyselinck *et al.*, 1993; Cornwall and Hann, 1995).

Experimental models and pitfalls

A logical approach to the question of how androgens support spermatogenesis, is the observation of molecular and cellular changes which occur when androgens are withdrawn from the testes. A number of methods have been devised to effectuate such a withdrawal, including: hypophysectomy; chemical suppression of pituitary LH and FSH secretion through treatment with GnRH antagonists or agonists; treatment with a low dose of testosterone with or without estradiol; treatment with ethane dimethane sulphonate (EDS), which eliminates all mature Leydig cells in the rat testis. The different models, although all resulting in a very low intratesticular testosterone level, interact at different levels within the hypothalamus-pituitary-testis axis, and have a variety of additional side effects:

Hypophysectomy: Hypophysectomy results in the complete loss of all pituitary hormones, thus also inducing a complete absence of LH (and thereby loss of Leydig cell stimulation) and FSH (known to interact synergistically with testosterone). An advantage of this model is that the effect of testosterone can

be studied in the complete absence of FSH activity. A serious drawback, however, is that hypophysectomy causes a number of major changes as a result of the loss of the other pituitary hormones, which clouds the obtained data.

GnRH antagonists and agonists: Treatment with GnRH agonists results in a surge of LH and FSH, followed by a fall to undetectable levels, whereas GnRH antagonists evoke a direct fall in LH and FSH levels. Treatment with GnRH analogues, but also *immunization against GnRH* can be employed to study hormonal control of spermatogenesis. However, there is a problem when this strategy is used in the rat, because testosterone supplementation results in release of FSH through a GnRH-independent effect of testosterone on the pituitary (Rea *et al.*, 1986; Bhasin *et al.*, 1987).

Low dose testosterone: Treatment with low dose testosterone, either by repeated injection of testosterone ester or by implantation of a silastic tube containing hormone crystals, results in suppression of serum LH, in turn resulting in a low intratesticular testosterone level. Testicular testosterone is suppressed to about 3% of the control level in the rat. When estradiol is added to this regimen, the intratesticular testosterone level drops even further, through a direct effect of estradiol on Leydig cell steroidogenesis (Tsai-Morris *et al.*, 1986). In this model, the secretion of other non-gonadotropin pituitary hormones is unaffected. The secretion of FSH, however, is inhibited, resulting in a serum FSH level of about 60% of control, and this difference might well be of importance in situations where the testicular testosterone level is low.

Ethane dimethane sulphonate (EDS): EDS is an alkylating agent that results in the destruction of Leydig cells in adult rats through an unknown mechanism, within about 36 hours after intraperitoneal injection. Testosterone suppression is highly effective for a period of up to at least 2 weeks, after which time Leydig cells start to regenerate if serum LH levels are not suppressed. EDS has a destructive effect on the integrity of the epididymis (Cooper and Jackson, 1973), whereas spermatogenesis can be fully maintained when testosterone is supplemented (Sharpe *et al.*, 1990).

What is quite clear from close scrutiny of these models, is that it is very

difficult to influence the secretion of one hormone while leaving the serum and testicular levels of other hormones unchanged. It should be stressed that when studying the effects of modulation of testosterone, the FSH level must be well controlled. Numerous studies attest to the fact that FSH and testosterone have synergistic effects in the initiation, maintenance, or reinitiation of spermatogenesis (Kerr *et al.*, 1992). In studies where testicular testosterone is suppressed by low dose testosterone implants or injection, the serum FSH level falls to about 60% of the normal value (i.e. Sun *et al.*, 1989). In our opinion, 60% of normal is a significant decline which may have important implications. Upon increasing the supplemented dose of testosterone, the FSH level will rise to control levels through an as yet unidentified mechanism. This rise in FSH may well flatter the effect of testosterone supplementation.

A second problem is, that it is practically impossible to completely abolish the secretion of testosterone by testicular Leydig cells (Sharpe, 1994). When LH secretion is inhibited by administration of a GnRH agonist or hypophysectomy, Leydig cells continue to secrete a small amount of testosterone, resulting in a testicular testosterone level roughly equal to the serum testosterone level of control rats (Sharpe, 1994). The implications of a very low level of testosterone in the testis are uncertain, but it has been shown that when EDS is administered to a hypophysectomized rat, a major additional loss of germ cells is observed, with a subsequent testis weight loss of 50%, compared to hypophysectomized controls (Kerr *et al.*, 1992). In addition, it has been reported that in rats, where testicular testosterone production is suppressed to about 3% of control values by treatment with low dose testosterone and oestradiol, the observed regression in spermatogenesis was clearly amplified by the co-administration of flutamide, an androgen antagonist (Meachem *et al.*, 1997). Similar observations were made by us, during a pilot study using a combination of GnRH antagonist (Org30267) and EDS *versus* each of the two treatments separately, in rats. Daily sperm production, as measured by the number of homogenization resistant spermatids and testis weight, were appreciably lower in the combined treatment group.

Finally, it should be duly noted that Sertoli cell function is highly regulated by germinal cells, and undoubtedly by a number of paracrine factors to which they are exposed in the testis. Anything longer than short-term androgen deprivation will cause loss of germ cells from the spermatogenic epithelium.

Many of the reported changes in Sertoli cell function found after androgen deprivation, were actually the result of the loss of a normal germ cell complement (McKinnell and Sharpe, 1992; Sharpe *et al.*, 1993). Experiments using *in vitro* Sertoli cell models (which traditionally employ prepubertal Sertoli cells which are differentially regulated from adult Sertoli cells) even in the presence of a limited germ cell complement, will not show normal Sertoli cell function. The above flaws in the various models that are routinely used in a large number of studies should be kept in mind when reviewing the data in the literature.

3

Comparison of the response of rat testis and accessory sex organs to treatment with testosterone and the synthetic androgen methyltrienolone (R1881)

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Published in: Journal of Andrology 1997; 18:51-61

Abstract

We have studied the ability of the synthetic androgen methyltrienolone (R1881) to maintain testis and accessory organ weights, as compared to the effect of testosterone propionate (TP). In contrast to TP, R1881 is not metabolized and does not significantly bind to androgen binding protein (ABP). Thirty-six rats were treated with ethane dimethane sulphonate (EDS) and GnRH antagonist (Org30267) to abolish all testicular androgen production, and recombinant human FSH (rec-hFSH, Org32489) was administered to ensure adequate FSH levels. Of these rats, five groups of 4 rats were treated daily with 0, 50, 100, 200, and 400 μg TP s.c., and four groups of 4 rats were treated daily with 150, 300, 600, and 1200 μg R1881 s.c.. One control group of 4 rats received vehicle injections only. EDS treatment, followed by GnRH antagonist and rec-hFSH treatment for 17 days, significantly reduced testis, prostate, and seminal vesicle weights ($p<0.001$, $p<0.01$, $p<0.001$, respectively). Simultaneous treatment with androgens prevented this organ weight decrease, in a dose dependent manner. In all TP-treated animals, relative weights (% of control) of the accessory sex organs were significantly higher than the relative testis weights ($p<0.001$). However, there was no difference in relative weights between testis and accessory sex organs in the R1881-treated animals. In another series of experiments, we investigated the effect of treatment with Finasteride, a 5α-reductase inhibitor, on testis and accessory sex organ weights in rats treated with EDS and TP. Treatment with EDS, TP (300 μg/day) and Finasteride (40 mg/kg/day) did not alter testis weight as compared to the effect of treatment with EDS and TP alone. Prostate and seminal vesicles weights were, however, markedly reduced (significantly different from rats treated with EDS and TP alone; $p<0.01$ and $p<0.05$ respectively). Immunohistochemical analysis of androgen receptor (AR) expression in the testis revealed that testicular AR immunoexpression is androgen dependent, and that FSH alone is not able to maintain AR immunoexpression. Furthermore, the stage dependent pattern of AR immunoexpression in Sertoli cell nuclei, during the spermatogenic cycle, is identical in all TP- and R1881-treated rats. It is concluded that testis, prostate, and seminal vesicle are equally stimulated when the androgen receptor in these tissues is exposed to the same intracellular concentration of free androgen and that the low 5α-reductase activity in the testis plays a critical role in the differential response of the testis and the accessory sex organs to T. Furthermore, stage dependent AR immunoexpression in Sertoli cells does occur in the absence of testicular androgen production, and is not due to androgen metabolism or local differences in androgen concentration.

Introduction

In mammals, spermatogenesis is dependent upon a number of endocrine, paracrine and autocrine regulators (Sharpe, 1994). The role of testosterone (T), in particular the testicular concentration of T needed for qualitatively and quantitatively normal spermatogenesis, has been the subject of a considerable amount of research (for review, see Sharpe, 1994). Various studies have clearly established that the physiological concentration of T in the testis is more than an order of magnitude higher than that in serum (Comhaire and Vermeulen, 1976; Maddocks and Sharpe, 1989; Sharpe, 1994). Furthermore, it has been shown that, while qualitatively normal spermatogenesis can be maintained at relatively low levels of T (Cunningham, R. and Huckins, 1979; Sun *et al.*, 1989; Santulli *et al.*, 1990), quantitatively normal spermatogenesis is dependent on a testicular level that far exceeds the physiological serum T concentration (Berndtson *et al.*, 1974; Blok *et al.*, 1992; Sharpe, 1994). These data indicate that the testis is less responsive to circulating T than other androgen responsive organs, such as prostate or seminal vesicle. Several differences exist between the testis and the accessory sex organs that may account for this discrepancy. For example, it has been shown that 5α-reductase activity, which converts T into the more potent androgen dihydrotestosterone (DHT), is considerably lower in testis compared to accessory sex organs (Van der Molen *et al.*, 1975; Turner *et al.*, 1984). Furthermore, the testis contains a high amount of androgen binding protein (ABP) (French and Ritzen, 1973; Sharpe, 1988), which may effectively prevent a large fraction of testicular androgens from interacting with the androgen receptor in testicular androgen responsive cells. It is not known what concentration of testicular T is free and biologically active.

The present study was undertaken to investigate whether the synthetic androgen methyltrienolone (R1881), which is not metabolized and which has very little affinity for ABP (Bonne and Raynaud, 1976; Kirchhoff *et al.*, 1979; Levinson and Decker, 1985; Carlson and Katzenellenbogen, 1990), might be equally effective in maintaining testis weight compared to maintenance of the accessory sex organ weights. We compared the effects of various doses of testosterone propionate (TP) and R1881 on the relative weights of testis, seminal vesicles, and prostate. Rats were first treated with ethane dimethane sulfonate (EDS) to eliminate mature Leydig cells, thereby abolishing testicular T production (Kerr *et al.*, 1985; Morris *et al.*, 1986). These animals also received daily injection of a GnRH antagonist (Org-

30276; Organon, The Netherlands) to prevent regeneration and maturation of remaining immature Leydig cells by suppressing serum LH (Molenaar *et al.*, 1986). Previous studies in rats have shown that treatment with a GnRH antagonist decreases serum FSH levels significantly (Rea *et al.*, 1986; Bhasin *et al.*, 1987). This effect, however, is partially reversed by subsequent treatment with testosterone, through an undefined mechanism (Rea *et al.*, 1986; Bhasin *et al.*, 1987). To ensure that our data reflect variations in androgen response only, adequate FSH stimulation for each treatment group was obtained by twice-daily injection of recombinant human FSH (rec-hFSH, [Org32489; Organon, The Netherlands]).

In a subsequent series of experiments, we studied the effect of Finasteride, a 5α-reductase inhibitor, on the maintenance of testis and accessory sex organ weights in rats treated with EDS and 300 μg/day TP. In rats treated with EDS and such a high dose of TP, sufficient endogenous serum FSH is present, and serum LH is sufficiently suppressed to inhibit Leydig cell repopulation (unpublished results). Therefore, GnRHa treatment and rec-hFSH supplementation was deemed not necessary in this series of experiments.

Androgen action is mediated by the androgen receptor (AR). Immunohistochemical studies on the cellular localization of the AR in rat testis have obtained conflicting results. In a study by Vornberger *et al.* (1994), immunohistochemical expression of AR was found in the nuclei of Sertoli cells, Leydig cells, peritubular myoid cells, smooth muscle cells forming the walls of blood vessels, and in the cytoplasm of step IX elongating spermatids. Bremner *et al.* (1994) have also demonstrated AR immunoexpression in the nuclei of Sertoli cells, Leydig cells, peritubular myoid cells, and cells associated with arterioles, but not in germinal cells at any stage of their development, which would be in agreement with biochemical data concerning the absence of androgen binding in spermatocytes and spermatids (Grootegoed *et al.*, 1977). The reports by Vornberger *et al.* (1994) and Bremner *et al.* (1994) describe a stage dependent immunoexpression of AR in the nuclei of Sertoli cells during the spermatogenic cycle, with expression increasing at stages II through VII and decreasing rapidly at early stage VIII to nearly undetectable levels at stages IX through XIV. Putative local differences in testosterone concentration, caused by changes in the production and secretion of ABP by Sertoli cells during the spermatogenic cycle (Ritzen *et al.*, 1982) might be involved in the stage dependent expression of AR in Sertoli cells. Therefore we also investigated the testicular

pattern and intensity of AR immunoexpression in rats treated with R1881, compared to rats treated with TP and control rats.

Materials and Methods

Animals

Young adult male Wistar rats (body weights 300-350 g) from our own breeding stock were divided in groups of 4 or 5 animals, such that the mean body weight of each group was virtually identical. The animals were housed at 22 C, with a light cycle of 14 h light and 10 h dark. Water and rat diet was available *ad libitum*.

Experimental design

EXPERIMENT 1: One group of 4 rats was injected twice daily for 17 days with vehicle (control group). The remaining 36 animals received one injection of ethane dimethane sulphonate (EDS) [75 mg/kg body weight in DMSO/H_2O 1:3 (v/v), i.p.] on day 0, followed by daily injection of a GnRH antagonist (Org30276; Organon, Oss, The Netherlands) [350 µg/100 g body weight in 0.1 ml saline, s.c.] and twice daily injection of rec-hFSH (Org32489; Organon, Oss, The Netherlands) [2 IU in 0.1 ml saline, s.c.,]. One group of 4 animals received no further treatment. The eight remaining groups were substituted daily with varying amounts of TP (50, 100, 200, and 400 µg/day) or R1881 (150, 300, 600, and 1200 µg/day). The androgens were injected s.c. in 0.1 ml of corn oil. The last injections were given in the evening of day 16. Twelve hours later, blood was collected from the retro-orbital plexus under ether anesthesia. The animals were subsequently killed by cervical dislocation. One testis was removed immediately and flash frozen in liquid nitrogen, for determination of testicular T levels. The other testis was weighed and placed in Bouin's fixative, after perforation of the tunica albuginea, for histological and immunohistochemical analysis. The ventral prostate and the seminal vesicles were dissected, the secretory fluid was expressed from the seminal vesicles, and the organs were weighed (the two seminal vesicles combined).

EXPERIMENT 2: In order to study the effect of 5α-reductase inhibition on the weights of the testis and accessory organs, a group of 5 rats was treated once with EDS, and then daily with 300 μg TP and Finasteride (40 mg/kg body weight/day in 0.2 ml corn oil, s.c.). This dose of TP (300 μg/day) is suboptimal for testis function but results in supra-physiological prostate and seminal vesicle weights. For comparison a dose response-curve was made by injection of 4 groups of 4 rats on day 0 with EDS (75 mg/kg body weight in DMSO/H_2O 1:3 (v/v) i.p.), followed by daily injection with 50, 100, 200, or 400 μg TP (in 0.1 ml corn oil). Treatment lasted for 17 days, after which the rats were killed by cervical dislocation. The testes, the prostate and the seminal vesicles were removed and weighed as described above.

Hormone assays

Testicular and serum T levels were determined in duplicate samples by RIA (Verjans *et al.*, 1973). The sensitivity of the assay was 50 pg/ml, with intra-assay and inter-assay coefficients of variation of 10% and 15%, respectively. Serum LH and FSH levels were estimated by RIA as previously described (Van Cappellen *et al.*, 1995). Intra-assay variations were 4% (FSH) and 5% (LH). Cross reactivity of rec-hFSH with rat FSH was previously found to be 2.6% by weight in this assay (Van Cappellen *et al.*, 1995). Serum rec-hFSH concentrations were determined using a two-site FSH time resolved fluoroimmunoassay (Delfina; Pharmacia, Woerden, The Netherlands) for human serum. Rat FSH was previously found to be undetected or below the detection limit of this assay (Van Cappellen *et al.*, 1995). The detection limit of the assay applied for rat serum is 0.7 pg/ml. The intra-assay variation is 4.3%.

Immunohistochemistry

The primary antibody used (SP197) is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the first 20 N-terminal amino acid residues of the human, rat and mouse androgen receptor. Immunostaining results obtained with this antibody, in the rat testis, were found to be identical (results not shown) to those obtained with a number of other polyclonal antibodies targeting the N-terminal region of the AR (Sar *et al.*, 1990; Takeda *et al.*, 1990; Bremner *et al.*, 1994).

Testicular tissue was left immersed in Bouin's fixative for 2 days, after which it was stored in 70% ethanol. The tissue was embedded in paraffin, and 10 mm sections were prepared. The sections were mounted on slides coated with 3-aminopropyltrietoxysilane (Sigma Chemical Company, St. Louis, MO), and kept at 60 C overnight. The tissues were dewaxed in xylene, and endogenous peroxidase was blocked with a 20 min incubation in 3% H_2O_2 in methanol. An antigen retrieval step was performed by heating the sections in 0.01M citric acid monohydrate (Merck, Darmstadt, Germany) (pH 6.0) in a microwave oven at 700W (4 x 5 min). The tissues were washed in PBS and placed in a Sequenza immunostainer (Shandon, Zeist, The Netherlands). Non-specific antibody binding was blocked with normal goat serum (Dako, Glostrup, Denmark), diluted 1:10 in 5% (w/v) BSA in PBS (pH 7.4). The tissues were incubated at 4 C overnight with the primary antibody, diluted 1:2000 in 5% BSA (w/v) in PBS. Immunostaining was performed using biotinylated goat anti-rabbit immunoglobulin (Dako) for 30 min, streptavidin-peroxidase (Dako) for 30 min, and metal-enhanced diaminobenzidine (Pierce, U.S.A.) for 7 min. The sections were counterstained for 15 sec with Mayer's hematoxylin, and viewed with a Zeiss Axioskop 20 light microscope at magnifications 100x and 400x. Control sections were incubated with SP197 (1:2000) preincubated with an excess of peptide against which the antibody was raised (5 mg/ml), or pre-immune serum diluted to the same extent as the primary antibody.

Statistics

The Students t-test for unpaired samples was used when comparing the organ weights of a treatment group with control values. Organ weights of all rats treated with either TP or R1881 were compared using Wilcoxon's signed rank test for paired samples.

Results

Hormone levels

In rats, combined treatment with EDS, GnRH antagonist, and rec-hFSH (indicated with: EDS/GnRHa/rec-hFSH) lowered testicular T levels to 1.6 ±0.9 ng/ml (mean ±SEM) and serum T levels to under detection limits (Table 1). Supplementation of increasing doses of TP increased testicular T levels, up to a level of 11.5 ±4.4 ng/g in rats treated with 400 µg per day of TP. This level is still significantly lower than the control level (46.5 ±9.7 ng/g; $p<0.05$). The serum T level was comparable to the control level in rats treated with 50-100 µg TP per day, but was significantly higher than the control level when 400 µg TP per day was administered ($p<0.05$). The testicular T level was 0.3 ±0.4 ng/g in rats treated with R1881. In these animals, serum T was invariably undetectable.

In all EDS/GnRHa/rec-hFSH rats, endogenous LH and FSH levels were significantly reduced ($p<0.05$ and $p<0.01$ respectively; Table 2). The serum LH level remained low, irrespective of subsequent treatment, while the

Table 1. Testicular and serum testosterone levels.

	Testicular testosterone ng/g	Serum testosterone ng/ml
Control	46.5 (±9.7)[a]	1.2 (±0.24)
EDS/GnRHa/rec-hFSH:		
No androgen substitution	1.6 (±0.9)[c]	ND[c]
+TP 50 µg/day	3.4 (±1.5)[c]	0.9 (±0.2)
+TP 100 µg/day	7.0 (±3.3)[c]	1.6 (±0.8)
+TP 200 µg/day	6.5 (±4.5)[c]	2.5 (±1.0)
+TP 400 µg/day	11.5 (±4.4)[b]	6.1 (±1.4)[b]
+R1881 150 µg/day	0.3 (±0.3)[c]	ND[c]
+R1881 300 µg/day	0.3 (±0.2)[c]	ND[c]
+R1881 600 µg/day	1.7 (±1.7)[c]	ND[c]
+R1881 1200 µg/day	0.3 (±0.4)[c]	ND[c]

ND = not detectable.
[a] Mean ± SEM
[b] Significantly different from control ($p<0.05$)
[c] Significantly different from control ($p<0.01$).

Table 2. Serum LH, FSH and rec-hFSH levels.

	LH ng/ml	FSH ng/ml	rechFSH ng/ml
Control	0.63 (±0.16)[a]	10.3 (±0.5)	ND
EDS/GnRHa/rec-hFSH:			
No androgen substitution	0.05 (±0.03)[b]	3.4 (±0.2)[c]	4.7 (±0.7)
+TP 50 µg/day	0.05 (±0.03)[b]	9.2 (±0.9)	3.7 (±1.2)
+TP 100 µg/day	0.03 (±0.03)[b]	7.7 (±1.2)	4.7 (±0.8)
+TP 200 µg/day	0.07 (±0.03)[b]	10.9 (±1.7)	5.8 (±1.6)
+TP 400 µg/day	0.05 (±0.03)[b]	12.5 (±1.0)	5.5 (±0.9)
+R1881 150 µg/day	ND[c]	8.4 (±0.6)	7.3 (±1.3)
+R1881 300 µg/day	0.05 (±0.03)[b]	8.9 (±1.3)	4.2 (±0.5)
+R1881 600 µg/day	0.03 (±0.03)[b]	10.6 (±0.5)	4.6 (±0.3)
+R1881 1200 µg/day	ND[b]	11.6 (±1.2)	5.1 (±0.6)

ND = not detectable.
[a] Mean ± SEM
[b] Significantly different from control ($p<0.05$)
[c] Significantly different from control ($p<0.01$).

endogenous FSH level returned to control value in all rats treated with TP or R1881. The rec-hFSH levels was comparable in all groups receiving rec-hFSH supplementation, irrespective of the androgen level.

Weights of testis and accessory sex organs

For Experiment 1, organ weights are shown as percentage of control value, per dose of TP (Figure 1a) or R1881 (Figure 1b). Testis, seminal vesicles, and prostate weights in 4 control animals were 1.433 ±0.035 g, 0.268 ±0.015 g, and 0.389 ±0.033 g (mean ±SEM), respectively. In EDS/GnRHa/rec-hFSH rats, the weights of the testis, prostate and, seminal vesicles were significantly reduced, to 0.555 ±0.012 g ($p<0.001$), 0.090 ±0.004 g ($p<0.01$), and 0.126 ±0.006 g ($p<0.001$), respectively. Increasing daily doses of TP resulted in more effective maintenance of testis weight, which, however, was still somewhat lower than control value ($p<0.05$) in rats treated with 400 µg TP per day. Higher daily doses of R1881 also resulted in more effective

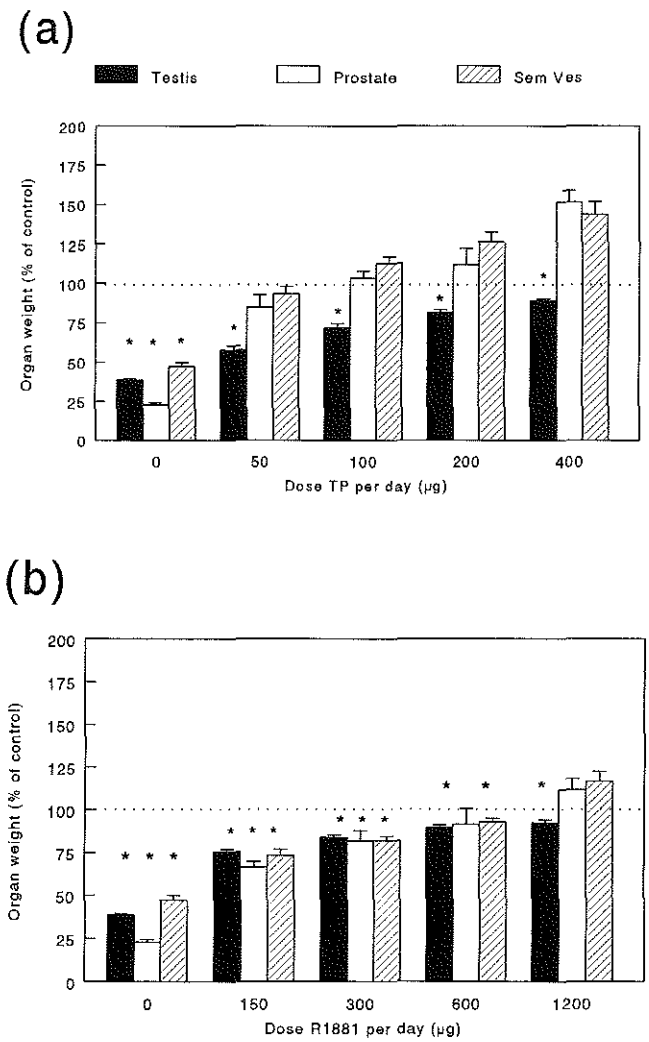


Figure 1. Maintenance of tissue weights by different androgens. The weights (shown here as percentage of control) of testis, seminal vesicles, and prostate were measured for rats treated with EDS/GnRHa/rec-hFSH, and with different daily doses of TP (a) or R1881 (b) for 17 days. Values are means ±SEM. Asterisk (*) denotes a value significantly lower than control.

maintenance of testis weight, which reached a plateau somewhat below control level ($p<0.05$) at 600-1200 µg R1881 per day.
In EDS/GnRHa/rec-hFSH rats treated with 50-400 µg TP per day, the relative weights (% of control) of the accessory sex organs were significantly higher than that of the testis ($p<0.001$; Fig 1a). Prostate and seminal vesicles weights were fully maintained at 50-100 µg TP per day, a dose at which the mean relative testis weight was only 58%. Prostate and seminal vesicles were found to grow well above control values in rats treated with more than 100 µg of TP per day, reaching 151% ($p<0.01$) and 143% ($p<0.001$) of control weight, respectively, at 400 µg TP per day.
In EDS/GnRHa/rec-hFSH animals treated with R1881, no difference in relative testis weight compared to relative accessory sex organ weights was observed. At a dose of 1200 µg R1881 per day, however, the relative prostate and seminal vesicles weights increased well above 100%, whereas relative testis weight was maintained at 93% (Fig 1b).
For Experiment 2, organ weights are shown in Figure 2, as percentage of control values per treatment group. Testis, prostate and seminal vesicles weights in the control animals were 1.35 ±0.06 g, 0.399 ±0.028g, and 0.237 ±0.005g (mean ± SEM), respectively. In rats treated with EDS and 100-400 µg/day TP, the relative weights of the accessory sex organs were well above control value and significantly greater than that of the testis ($p<0.01$). In rats treated with 300 µg/day TP and Finasteride, testis weight was 82% of control value, which is in line with the extrapolated value for rats treated with EDS and 300 µg/day TP alone (Figure 2). The relative weights of the prostate and the seminal vesicles were, however, dramatically reduced to 80% and 99.6% of control. These latter weights of the prostate and the seminal vesicles correspond to values found in EDS-treated rats supplemented with 50 and 75 µg TP/day, respectively.

Immunohistochemistry

Immunohistochemical studies of androgen receptor expression in testicular tissue of control rats, revealed immunoexpression of AR in nuclei of Sertoli cells, peritubular myoid cells, interstitial cells, and cells associated with arterioles (Fig. 3a). Sertoli cell AR immunoexpression is dependent on the stage of the spermatogenic cycle, showing faint expression at stages I-II, which slowly increases in intensity until expression is at its maximum at stage

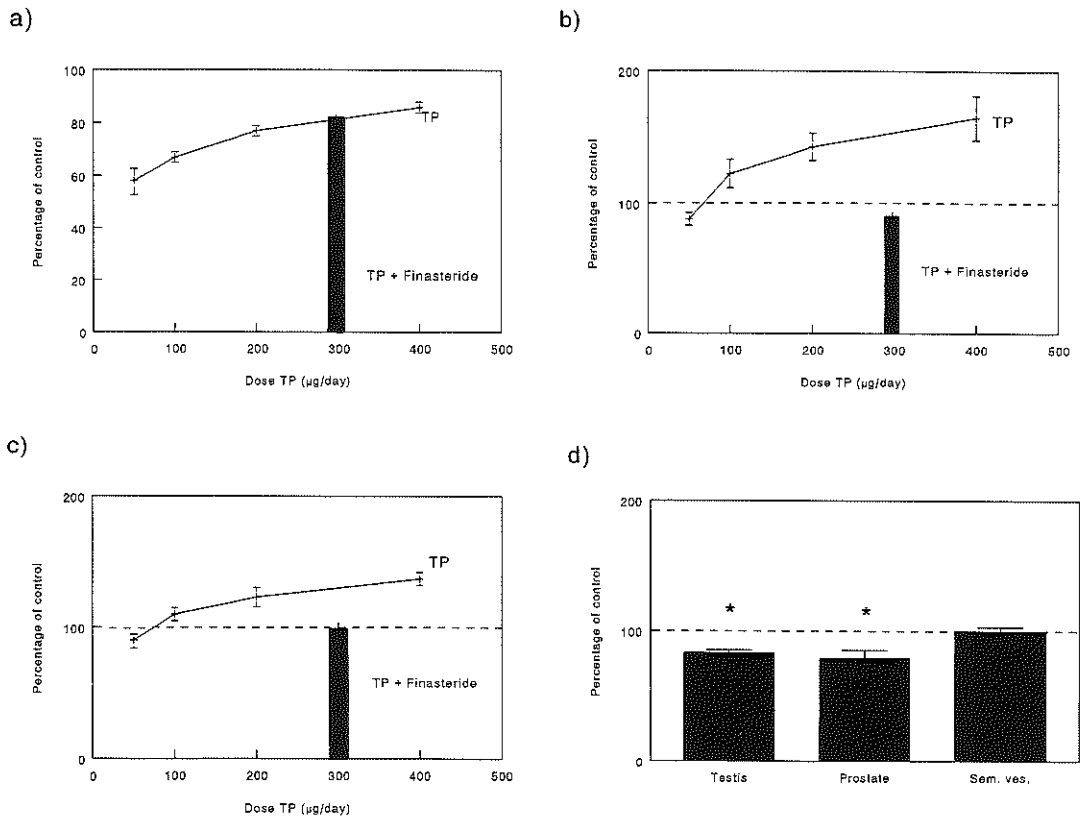
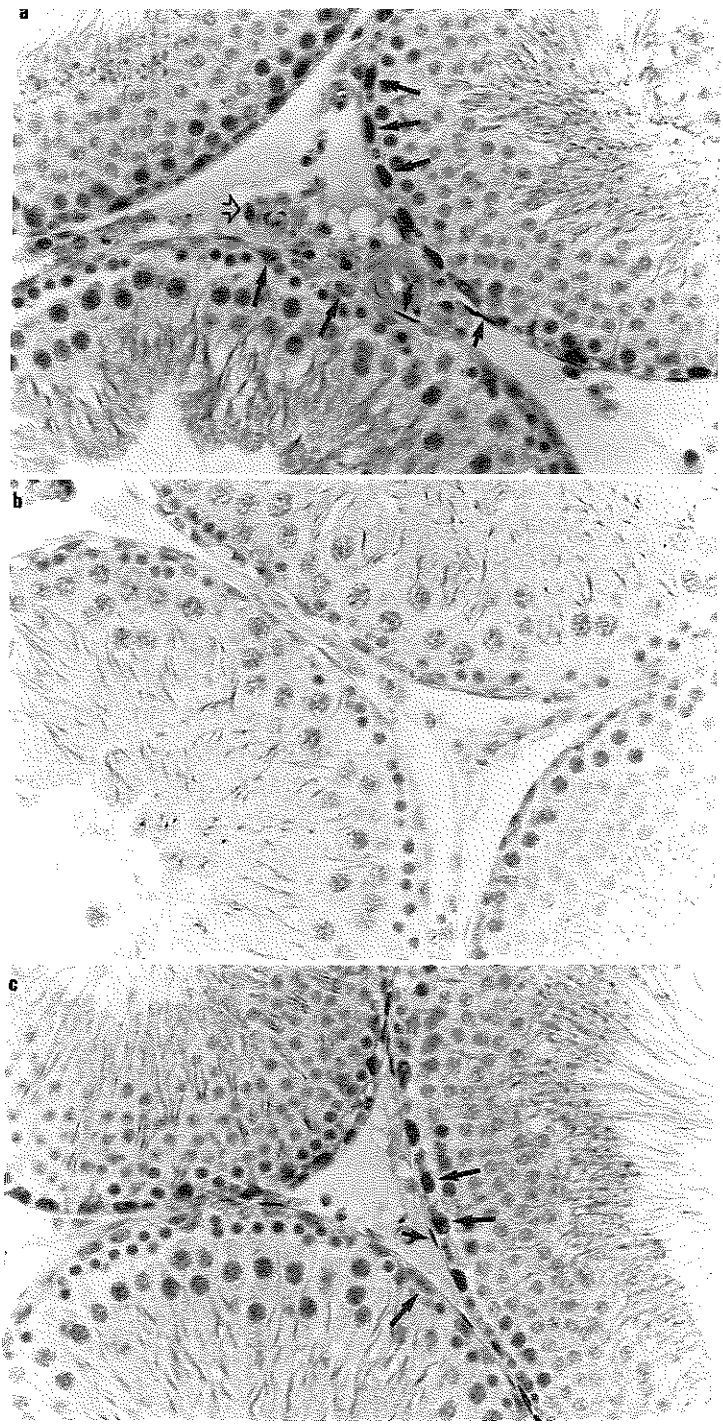


Figure 2. Effect of Finasteride on maintenance of tissue weight by testosterone propionate. Dose-response curves of the weights of testis (a), prostate (b), and seminal vesicles (c) in rats treated with EDS and TP are shown. The weights of testis, prostate, and seminal vesicles of rats treated with EDS, TP (300 µg/day) and Finasteride (40 mg/kg/day) are depicted in these graphs by bars. Relative organ weights for rats treated with EDS, TP and Finasteride are shown in (d). All graphs show organ weights as percentage of control. Treatment lasted for 17 days. Values are means ±SEM. Asterisk (*) denotes a value significantly lower than control.

VII. In the transition from stage VII to VIII, AR immunoexpression rapidly decreases, such that at stage VIII the expression is virtually undetectable. From stages VIII through XIII, AR immunoexpression in Sertoli cells remains relatively low. The peritubular myoid cells, Leydig cells and periarteriolar cells did not display a stage dependent immunoexpression of AR. Leydig cells, however, showed a clear, apparently random, variation in AR immunoexpression; such a phenomenon was much less pronounced, though still discernable, in peritubular and periarteriolar cells. These results are in agreement with published data (Bremner *et al.*, 1994).

In rats treated with EDS/GnRHa/rec-hFSH, spermatogenesis was severely disrupted, with relatively small diameter tubuli that were devoid of elongating and condensing spermatids. Mature Leydig cells were absent, while other interstitial cells, presumably macrophages and immature Leydig cells, were still present. In the testis of EDS/GnRHa/rec-hFSH rats, AR immunoexpression was virtually undetectable in all cell types (Fig. 3e). In EDS/GnRHa/rec-hFSH rats supplemented with 50 μg TP per day, spermatogenesis was qualitatively intact but quantitatively severely impaired, with a relatively small number of advanced spermatids per tubule. AR immunoexpression was faint, but revealed essentially the same stage dependent pattern in Sertoli cells, when compared to control testis tissue (data not shown). The peritubular myoid cells and periarteriolar cells also displayed faint AR immunostaining. No AR immunoexpression was detected in the Leydig cell depleted interstitium. Testicular tissue from EDS/GnRHa/rec-hFSH rats treated with 400 μg TP/day showed essentially the same pattern and extent of AR immunoexpression as testis from control rats (with the exception of Leydig cells, which are absent due to the EDS treatment; Fig 3c). The testis from EDS/GnRHa/rec-hFSH rats treated with 150 μg R1881/day contained many elongated spermatids, and also a pattern of AR immunoexpression that was essentially similar to control rats. Upon supplementation with a high dose of R1881 (1200 μg/day) the pattern of AR immunoexpression in Sertoli cells was still comparable to control rats: strong expression at stage VII and virtually undetectable staining at stage VIII (Fig. 3d).


a
b
c

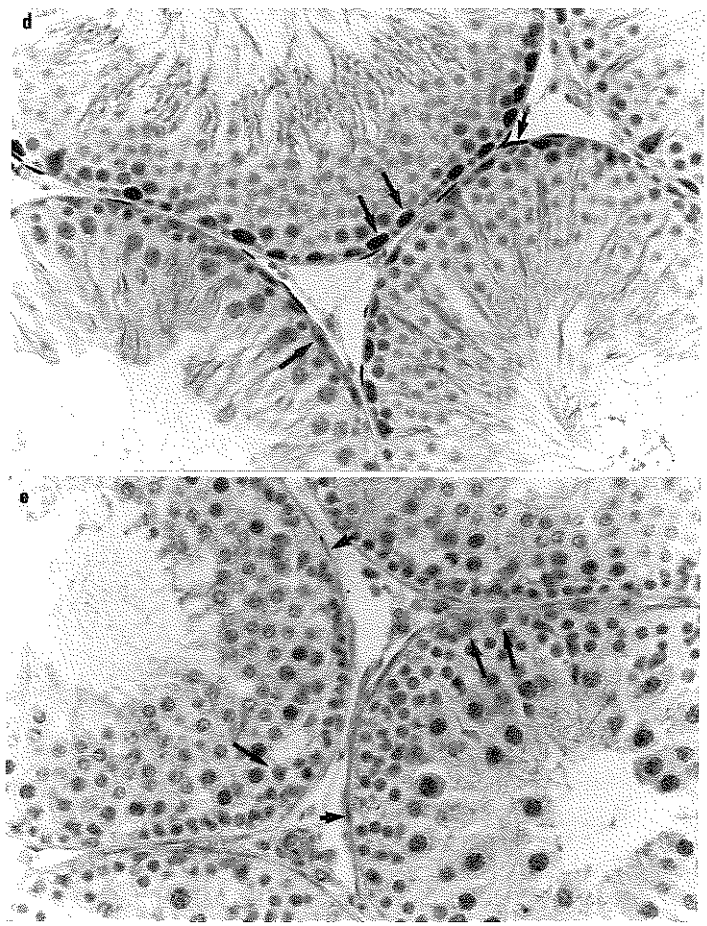


Figure 3. Immunohistochemical detection of androgen receptor in rat testis. Testes were fixed in Bouin's solution and embedded in paraffin, and immunostained with SP 197.
(a) Normal rat testis with positive staining in Sertoli cells (slender arrows), peritubular myoid cells (thick arrows), and interstitial cells (open arrows). Note the difference in the intensity of Sertoli cell AR immunoexpression at stage VII compared with the adjoining tubules.
(b) Normal rat testis stained with SP197 pre-incubated with an excess of peptide ligand.
(c) and (d) Testis of a rat treated with EDS/GnRHa/rec-hFSH, and 400 μg TP or 1200 μg R1881 per day, respectively (note the similar stage dependent pattern of AR immunoexpres-sion in the Sertoli cells of both specimens).
(e) Testis of a rat treated with EDS/GnR-Ha/rec-hFSH, but without androgen replacement. Original magnification: 400x.

Discussion

The present study demonstrates, that, in the presence of FSH, a given dose of the synthetic androgen methyltrienolone (R1881) can maintain testis weight equally well as that of the prostate and the seminal vesicles (though never quite reaching control level, presumably because of the destruction of all mature Leydig cells). This finding is highly interesting, since all previous data have indicated that maintenance of testicular weight, upon elimination of testicular testosterone production, requires a circulating androgen level much higher than needed to maintain accessory sex organ weights (for review, see: Sharpe, 1994). All evidence suggests that spermatogenesis requires a relatively high level of testicular androgens, as compared to the circulating level of androgens that can evoke a normal response in other androgen dependent tissues (Berndtson *et al.*, 1974; Cunningham, R. and Huckins, 1979; Sun *et al.*, 1989; Santulli *et al.*, 1990; Blok *et al.*, 1992; Sharpe, 1994). Indeed, our experiments confirm that supplementation with a dose of TP that maintains normal accessory organ weights, leads to a low testis weight. A relatively normal testis weight can only be attained by doses of TP that are almost an order of magnitude higher than those needed for normal accessory organ weights.
It is well known that R1881 also binds to the progesterone receptor (Carlson and Katzenellenbogen, 1990). In order to elucidate if this binding might play a role in the present experiment, several EDS-treated rats were supplemented with either TP, or TP and progesterone, for a period of 17 days. It was found that the combined administration of TP and progesterone had no additional stimulatory effect on testis weight or on the other androgen sensitive organs (data not shown).

Presuming that all androgens exert their effect through the known nuclear androgen receptor, several explanations, not mutually exclusive, can be given for the fact that a given dose of R1881 is as effective in the testis as it is in the accessory sex organs, while TP supplementation results in large relative differences.
The first of these explanations involves the fact that the prostate and the seminal vesicles have high levels of 5α-reductase, which converts T into the more potent 5α-dihydrotestosterone (DHT) (Buric *et al.*, 1972). In the testis, the rate of conversion of T into DHT is low, though probably not absent (Van der Molen *et al.*, 1975; Turner *et al.*, 1984). The formation of

DHT in the accessory sex organs undoubtedly contributes to the difference in relative organ weights in the TP-treated rats. It has been clearly established that DHT has a two-fold greater affinity for the androgen receptor and a dissociation rate one-fifth that of testosterone, resulting in formation of a more stable ligand-receptor complex (Grino *et al.*, 1990). This results in a potency which, *in vitro*, is approximately 10-fold greater than testosterone. Based upon the assumption that a difference in 5α-reductase activity between testis and accessory sex organs contributes to the differential response to testosterone, one might expect that DHT would maintain testicular weight at a lower dose. This, in fact, is the case (Chen *et al.*, 1994). However, the DHT level needed to maintain qualitatively normal spermatogenesis is still high (about 50% of that of testosterone) when the hormone level is estimated in seminiferous tubule fluid. This might be due to rapid intracellular conversion of DHT to 3α-androstanediol in Sertoli cells, making DHT unavailable for interaction with the AR. In contrast to T and DHT, R1881 is not metabolized in androgen sensitive tissues (Bonne and Raynaud, 1976; Levinson and Decker, 1985).

In the present study, the contribution of conversion of T to DHT in the differential response of accessory sex organs and testis to T was investigated. In rats depleted of Leydig cells and treated with TP, testis weight was not affected by the additional treatment with Finasteride, indicating that even at a low intratesticular T level, conversion of T to DHT does not play a significant role in maintaining spermatogenesis. This is in agreement with a number of observations that the testis has little 5α-reductase activity (Van der Molen *et al.*, 1975; Turner *et al.*, 1984) and that Finasteride has no effect on spermatogenesis in beagle dogs (Juniewicz *et al.*, 1993). The weights of both the prostate and the seminal vesicles, however, were dramatically influenced by the concomitant treatment with Finasteride. Relative prostate weights were no longer significantly different from that of testis, while the relative weight of the seminal vesicles, though obviously affected by Finasteride treatment, was still significantly higher than that of the other two organs ($p<0.05$; Wilcoxon signed rank test for matched pairs). The differential response of prostate and seminal vesicles to treatment with Finasteride might be due to residual 5α-reductase activity in the seminal vesicles.

It is thus apparent that, judging by relative organ weight, the conversion of T to DHT in the accessory sex organs accounts for most, if not all, of the differential response to T of testis compared to accessory sex organs.

In the rat testis, androgen binding protein (ABP) binds T with moderate affinity (French and Ritzen, 1973; Kirchhoff *et al.*, 1979). The production of ABP by Sertoli cells is stimulated by androgens and FSH (Danzo *et al.*, 1990). A clear function of this protein as a modulator of androgen action in the testis, nor a role for ABP in spermatogenesis have been established, although it has been sugested that ABP binds to, and is internalized by, the germ cells (Gerard *et al.*, 1994). Production and secretion of ABP into seminiferous tubule fluid and transport into the epididymis might diminish free testosterone levels in Sertoli cells, such that ABP actually acts as a local inhibitor of androgen action. Since AR immunoexpression is androgen dependent, one might suggest that T is made less available to the AR in Sertoli cells at stages VIII-XI, through binding to ABP, which at those stages is produced at the highest rate (Ritzen *et al.*, 1982). R1881 administration would not result in variable intracellular availability since this androgen is known to bind to ABP with very low affinity, so that a putative regulatory function of ABP is bypassed in this model (Kirchhoff *et al.*, 1979; Carlson and Katzenellenbogen, 1990).

Our data confirm the findings of Bremner *et al.* (1994) that no AR immunoexpression is detected in spermatogenic cells as was reported by Vornberger *et al.* (1994). In testis of EDS/GnRHa/rec-hFSH rats, immunoexpression is essentially undetectable in all cell types, confirming that AR immunoexpression is androgen dependent (Bremner *et al.*, 1994), and indicating that FSH alone is not able to maintain AR immunoexpression. Our results indicate that testicular concentration of testosterone, or its metabolites, does not influence the temporal pattern of AR immunoexpression in Sertoli cells: the present immunohistochemical data show a defined pattern of stage dependent immunoexpression of AR in Sertoli cell nuclei during the spermatogenic cycle, after administration of either testosterone or R1881. In addition, even in the presence of a low testosterone concentration, the stage dependent AR staining in Sertoli cells during the spermatogenic cycle was invariably present. These data are consistent with the recent evidence that, in intact rats, the cyclic variation in AR expression in Sertoli cells is regulated at the level of gene transcription (Shan *et al.*, 1995). The loss of immunodetectable AR, in the absence of androgens, most likely results from decreased stability of the receptor (Syms *et al.*, 1985; Zhou *et al.*, 1995).

In conclusion, when a sufficient level of R1881 is given to maintain accessory sex organ weight, testis weight is also maintained. In addition, treatment with

Finasteride virtually abolishes the difference in response to testosterone between testis and accessory sex organs, suggesting that extra-testicular conversion of T to DHT is of critical importance in the observed difference in responsiveness to testosterone of testis compared to accessory sex organs. Furthermore, the stage dependent pattern of AR immunoexpression in Sertoli cells during the spermatogenic cycle is not dependent on the concentration of testosterone or its metabolites, nor of binding of androgens to ABP.

4

Androgen receptor expression and androgen-dependent protein expression in the rat epididymis

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Submitted for publication

Abstract

The epididymis is a highly specialized organ that is actively involved in the maturation of spermatozoa. This organ can be subdivided into a number of morphologically distinct regions that show well-defined differences in structure and cellular composition, gene expression, and protein production. The function of the epididymis is highly dependent on testosterone, which reaches the target cells either via the blood stream, or directly from the testes via the ductuli efferentes. Within the epididymal target cells, testosterone is metabolized into the more potent 5α-dihydrotestosterone, which then interacts with the androgen receptor (AR). Using an antibody targeting the first 20 N-terminal amino acid residues of the rat AR (SP197), we studied the immunoexpression of AR in the different regions of the epididymis of rats, under various conditions. Rats were either castrated, or had their ductuli efferentes ligated for periods of 7 to 28 days. Castrated rats were supplemented with 100, 25, 6.25, and 1.56 μg of testosterone propionate (TP) per day. In addition to AR immunoexpression, we investigated the co-immunoexpression of the highly androgen dependent epididymal protein (proteins B/C), and androgen receptor in the above organs.

AR immunoexpression was observed in the nuclei of principal cells, clear cells, and basal cells, but not in halo cells. The cells in the ductuli efferentes showed only light staining, while in the rest of the principal cell nuclei AR immunostaining was intense. Interestingly, we found a marked variation in intensity of AR immunoexpression between morphologically indistinguishable principal cells within a single tubular cross section. This phenomenon was most pronounced in the caput. In situ hybridization experiments showed a regional variation in AR mRNA expression. The AR mRNA level in the principal cells of the initial segment was high, while the AR mRNA level in the principal cells from the caput onwards was markedly lower. Clear differences in the AR mRNA level were also observed in neighbouring, morphologically similar principal cells. Observations on serial 8 mm sections, alternately immunostained for AR protein and subjected to in situ hybridization to detect AR mRNA, suggest a correlation between AR protein and mRNA expression, indicating that the cell-to-cell variation in AR expression as observed in a single tubular cross-section may be regulated at the transcriptional level. In castrated rats, AR immunoexpression in the entire epididymis decreased to a very low level. In rats with ligated ductuli efferentes, the intensity of AR immunostaining was not consistently different from that of control rats. In castrated rats supplemented with various doses of

TP, a daily dose of 6.25 μg TP was sufficient to induce an AR immunoexpression of normal intensity, indicating that 10% of the normal serum level of T is sufficient to maintain epididymal AR immunoexpression. Double staining experiments with SP197 and with antibodies targeting proteins B/C, showed that in cells with a low or absent AR immunoexpression a normal immunoexpression of proteins B/C can occur. This suggests that in these cells the intensity of AR immunoexpression is no measure for androgen mediated activity. An alternative explanation might be, that epididyal cells show a cyclic cellular activity, with a considerable time lag between the disappearance of the androgen receptor and the production of androgen dependent proteins.

Introduction

When, in mammalian species, spermatozoa leave the testis, they have not yet acquired full maturity. A number of developmental processes, such as attainment of the capacity for motility, and incorporation of a number of vital proteins into the plasma membranes, are dependent on passage through the epididymis (Orgebin-Crist, 1967; Bedford, 1975; Orgebin-Crist and Olson, 1984; Jones, 1989; Amann *et al.*, 1993). Subsequently, spermatozoa are stored in the cauda of the epididymis, in an environment that is intricately controlled to optimize sperm survival. To perform this complicated task, the epididymal epithelium relies on a small number of different cell types: the principal cell, the clear cell, the basal cell, and the halo cell (Reid and Cleland, 1957). The principal cells outnumber the others, and are clearly involved in protein synthesis and secretion into the epididymal lumen. The morphology of the principal cells varies along the length of the epididymal lumen: principal cells are distinctly tall columnar in the initial segment and caput, but become progressively less tall further distally, such that in the cauda they are more cuboidal shaped. These differences in morphology are associated with differences in function, as is clearly demonstrated by regional variations in gene expression and tubular fluid composition (Holland and Orgebin-Crist, 1988; Holland *et al.*, 1992; Hinton and Palladino, 1995). The ductuli efferentes and the initial segment are involved in resorption of a large amount of fluid through active sodium absorption, thereby creating a high spermatocryt value (Jones and Jurd, 1987; Wong, 1990). More distally, the epithelium of the epididymis is a major site of protein production and release into the lumen (Holland and Orgebin-Crist, 1988; Vreeburg *et al.*, 1990; Holland *et al.*, 1992; Hinton and Palladino, 1995). In addition to regional variation in principal cell function, there is ample evidence that within specific areas, neighbouring principal cells are morphologically indistinguishable, but differ greatly in expression of specific proteins (Rankin *et al.*, 1992; Robaire and Viger, 1995). Very little is known about the factors which regulate gene expression in the epididymis on a regional scale, and about local variation in cell function.

It has long been recognized that epididymal function is highly dependent on testicular androgens that enter the epididymis both through the ductuli efferentes and via the peripheral circulation (Benoit, 1926; Brooks, 1981). The influx of testosterone from the ductuli efferentes causes a gradient with very high T levels in the initial segment and proximal caput, and a relatively low level in the cauda (Vreeburg, 1975; Turner *et al.*, 1984). Within the

epithelial cells, testosterone is metabolized into the more potent 5α-dihydrotestosterone (DHT) which binds to the androgen receptor (Robaire and Viger, 1995). The androgen receptor is then activated and interacts with specific enhancer sequences, known as androgen response elements, thereby regulating the expression of specific genes (Carson-Jurica *et al.*, 1990). Although a number of studies have focused on androgen receptor expression in the epididymis of the ram (Tekpetey *et al.*, 1989), the goat (Goyal *et al.*, 1998), the rhesus macaque (Roselli *et al.*, 1991), and the human (Ungefroren and Ergun, 1997), no detailed data is available on the expression and regulation of the AR in the epididymis of the rat. In ram, goat, rhesus macaque and human, differences in regional expression of the AR have been found; however, the implications of these findings remain unclear.

Using a polyclonal antibody (SP197) targeting the first 20 N-terminal amino acid residues of the AR (Van Roijen *et al.*, 1997), we performed a number of experiments to study the regulation of AR immunoexpression in the rat epididymis under a number of conditions, and searched for regional and local variations in AR immunoexpression. In addition, we performed in situ hybridization for AR mRNA, to ascertain if variations in AR immunoexpression coincide with expression patterns of AR mRNA. Furthermore, we correlated the observed differences in AR immunoexpression with the immunoexpression of highly androgen dependent proteins: proteins B and C (Brooks and Higgins, 1980). Proteins B/C [also known as EBP1 & EBP2 (Ong and Chytil, 1988; Newcomer and Ong, 1990), ESP 1(Girotti *et al.*, 1992), and E-RABP (Newcomer, 1993; Newcomer *et al.*, 1993)] are members of the lipocalin superfamily (Girotti *et al.*, 1992). Mouse epididymal protein 10 (MEP 10) is the mouse homologue of proteins B/C, with 86% sequence similarity (Rankin *et al.*, 1992b). MEP 10, recently renamed murine epididymal retinoic acid binding protein [mE-RABP] (Lareyre *et al.*, 1998), binds active retinoids (cis and all-trans retinoic acid, but not retinol) with high ligand specificity (Rankin *et al.*, 1992a), and is intricately regulated by androgens (Lareyre *et al.*, 1998). In the present study, antibodies raised against MEP 10, known to specifically cross react with proteins B/C (Rankin *et al.*, 1992; Lareyre *et al.*, 1998), were used to study the differential expression of protein B/C in rat epididymis, in relation to AR immunoexpression.

Materials and Methods

Animals

Three- to four-month-old male Wistar rats from our own breeding stock were selected. All animals weighed 300-350 g. Water and food was available ad libitum. The rats were maintained on a 12L:12D cycle.

Experimental design and surgical procedures

Fourteen rats were divided into two groups of 6, and one (control) group of 2 animals. One group was castrated, and in one group ductuli efferentes were ligated. Castration, and ligation of the ductuli efferentes were performed through the abdominal route. All surgical procedures occurred under ether anesthesia. Two rats per group were killed by cervical dislocation 7, 14, and 28 days after surgery. The two control rats were sacrificed on day 28, together with the last group of treated animals. Epididymides were removed, cleared of surrounding fatty tissue, and divided into a proximal segment, which included the initial segment, the caput and the proximal corpus, and a distal segment, comprising the distal corpus and the cauda. The organs were either flash frozen in liquid nitrogen for evaluation on Western blot, or placed in Bouin's fluid for 24 hours. Subsequently, the Bouin-fixed tissues were thoroughly washed and stored in 70% ethanol.
In an additional experiment, four groups of 2 rats were castrated as described above. After 3 weeks, they were supplemented by injection of 100 µg, 25 µg, 6.25 µg, and 1.56 µg TP per day (s.c. in 0.1 ml vegetable oil) for 2 weeks. All animals were killed by cervical dislocation under ether anesthesia. The epididymides were fixed in Bouin's solution for 24 hours, for subsequent immunohistochemical analysis.

Antibodies

For detection of AR immunoexpression we used SP197, a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the first 20 N-terminal amino acid residues of the rat AR (Van Roijen *et al.*, 1997). The antibody targeting MEP10 is a rabbit polyclonal raised against mouse epididymal protein 10, as described previously (Rankin *et al.*, 1992a; Rankin *et al.*, 1992a). Anti-MEP 10 antibodies are known to specifically cross react

with rat proteins B/C (Rankin *et al.*, 1992b). In all sections SP197, was stained with metal-enhanced diaminobenzidine (DAB), staining positive nuclei brown, and antibodies targeting proteins B/C were stained with fuscine, resulting in a red colored cell cytoplasm.

Western blot

The proximal and distal portions of the epididymides, and seminal vesicles were pulverized under liquid nitrogen. The resulting powder was suspended in 1 ml high salt buffer (0.5M NaCl, 40 mM Tris, 10% (v/v) glycerol, 1 mM EDTA, pH 8.5) in the presence of inhibitors of proteolytic breakdown (0.6 mM bacitracin and 0.6 mM PMSF). Immunoprecipitation and western blot were performed as previously described (Van Laar *et al.*, 1989). Equal amounts of protein were loaded on each lane.

Immunohistochemistry

The tissues were embedded in paraffin, and 8 mm sections were prepared. The sections were mounted on slides coated with 3-aminopropyltrietoxy-silane (Sigma, St. Louis, MO, USA), and kept at 60 °C overnight. The tissues were dewaxed in xylene, and endogenous peroxidase was blocked with a 20 minute incubation in 3% (v/v) H_2O_2 in methanol. For SP197, an antigen retrieval step was performed by heating the sections in 0.01M sodium citrate buffer (pH 6.0) in a microwave oven at 700 W (4 x 5 minutes). No antigen retrieval was necessary for staining with anti-MEP10 antibodies. Non-specific antibody binding was blocked with normal goat serum (Dako, Glostrup, Denmark), diluted 1:10 in 5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS; pH 7.4). The tissues were placed in a Sequenza immunostainer (Shandon, The Netherlands), and incubated at 4C overnight for AR, or for 4 h at room temperature for proteins B/C, with the primary antibodies diluted 1:4000 and 1:200, respectively, in 5% (w/v) BSA in PBS. Immunostaining was performed using biotinylated goat anti rabbit immunoglobulin (Dako, The Netherlands) for 30 min, streptavidin-peroxidase or streptavidin-alkaline phosphatase (Dako, The Netherlands) for 30 min, and metal-enhanced DAB or fuscine (Pierce, USA) for 7 min where appropriate. The sections were counterstained with Mayer's hematoxylin, and viewed with a Zeiss Axioscop microscope. In sections that were stained

with both antibodies, the procedure for proteins B/C staining was completed first, followed by the protocol for AR immunostaining.

In situ hybridization

An EcoRI fragment containing bp 2778-3329 of the mouse AR cDNA was cut from a vector named pmAR^{o} (Faber *et al.*, 1989), and cloned in pBluescript KS (Stratagene, Westburg, Leusden, The Netherlands) using standard molecular biology techniques (Sambrook *et al.*, 1989). This construct was used to generate digoxigenin-labeled sense and anti-sense RNA probes. A labeling mixture containing digoxigenin-UTP (Boehringer Mannheim, Almere, The Netherlands) was incubated with AR DNA template and T7 or T3 RNA polymerase (Stratagene) (to prepare sense or antisense AR RNA respectively) according to the instructions provided by the manufacturer of the labeling mixture. The resulting RNA probe was dissolved in 20 ml sterile H_2O and analyzed using agarose-gel electrophoresis and ethium bromide staining for rough estimation of yield of full length transcripts. Different dilutions of digoxigenin-labeled RNA were spotted on dot blots, to determine the relative amount of detectable probe using the DIG nucleic acid detection kit (Boehringer Mannheim). One in vitro transcription reaction (using 1 mg of template DNA) yielded enough probe to prepare 10 ml hybridization mixture.
Tissue fixation, embedding and slide preparation methods were as described for immunohistochemistry. The in situ hybridization procedure was performed as described by (Millar *et al.*, 1993). The (pre)hybridization mixture contained 50% (v/v) deionized formamide, 4x SSC, 1x Denhardt's solution, 125 mg/ml denaturated herring sperm DNA, and 125 mg/ml yeast tRNA. Sections were prehybridized for 2 h at 55C in moist chambers. After removal of the prehybridization mixture (through blotting on tissue) 140 ml preheated (5 minutes at 85C) hybridization mixture (containing 1 ml sense or antisense RNA probe/500 ml hybridization mixture) was applied on each (prewarmed) slide containing several tissue sections. Subsequently, a coverslip was applied and the slides were incubated overnight at 55C in a moist chamber. Posthybridization washes, treatment with RNase and detection of digoxigenin were performed (Millar *et al.* 1993). The sections were counter-stained using Mayer's hematoxylin.

Results

Western blot

Immunoprecipitation and Western blot resulted in detection of a single 116 kD band in the caput and cauda epididymis and in the seminal vesicles of control rats (Figure 1). Castration induced a marked reduction of AR signal, which was apparent in both the caput and cauda epididymis of rats killed 7 days after surgery. In rats with ligated ductuli efferentes we found no change in AR expression in the caput and cauda of the epididymis, compared to control (Figure 1).

Immunolocalization of androgen receptors

Androgen receptor immunoexpression was detected in the nuclei of principal cells, clear cells and basal cells, but not in halo cells. The peritubular (myoid) cells stained positively for AR, as did a number of interstitial cells. In intact rats, there was little regional variation in the intensity of AR immuno-expression in the nuclei of the principal cells along the length of the

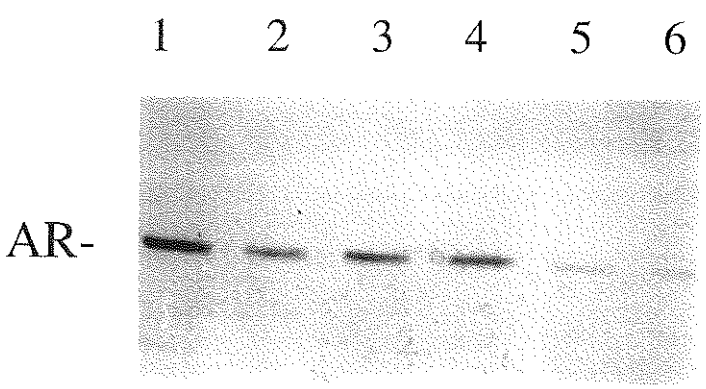

Figure 1. Immunodetection of androgen receptors with SP197, using SDS-PAGE and Western blot. Panel A, depicts the AR immunosignal in rat epididymides 14 days after various treatments. The AR immunosignal in rat epididymides is shown, 7 days after various treatments. Lanes 1 and 2 correspond to the proximal and distal portions of control epididymis respectively. Lanes 3 and 4 correspond to the proximal and distal portion of epididymis with ligated ductuli efferentes, respectively. Lanes 5 and 6 correspond to the proximimal and distal portions, respectively, of epididymides of castrated rats. Note the weak immunoexpression of androgen receptor in the epididymis of castrated rats.

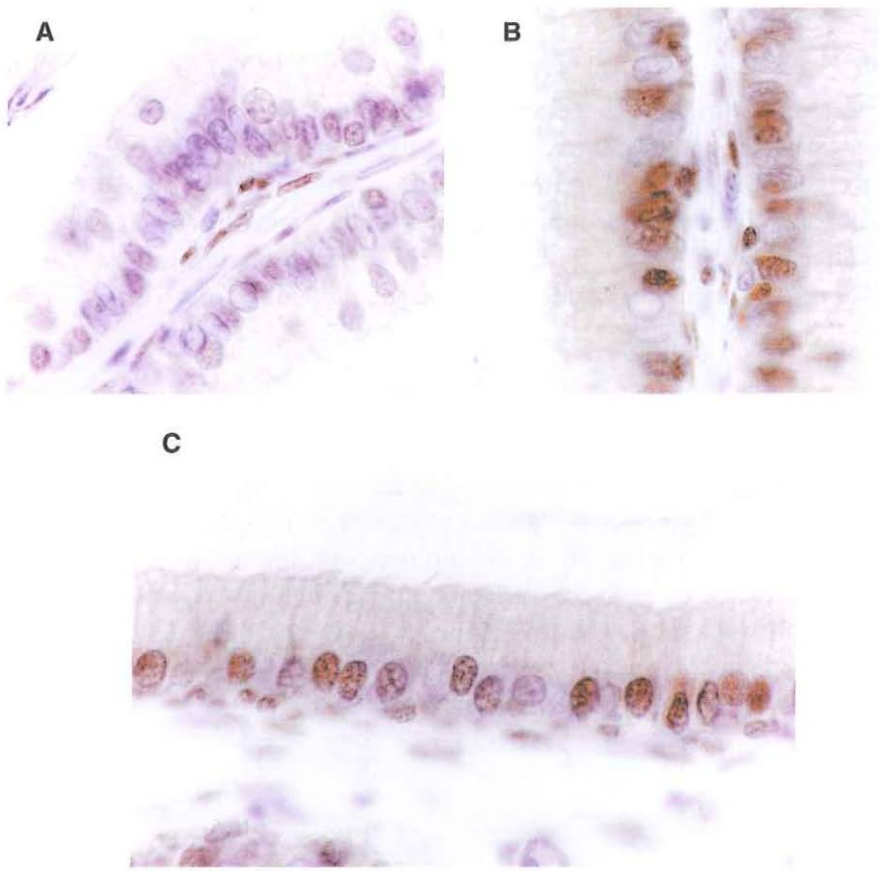

Figure 2. Immunolocalization of androgen receptor in rat epididymal tissue. Bouin's fixed rat epdidymis was stained with a polyclonal antibody specific for the androgen receptor (SP197). Positive (brown) staining is observed in the nuclei of principal cells, peritubular cells, and some interstitial cells. Halo cells (not shown on these photographs) are negative. The nuclei of principal cells of the ductuli efferentes (A) stain much less instensely that the nuclei of the initial segment (B) or caput epididymis (C). Note the clear heterogeneity in AR immunoexpression of adjacent, morphologically identical, principal cells, particularly in the initial segment and caput regions.

epididymis (Figure 2). However, the epithelial cells of the ductuli efferentes stained weakly for AR, while, in the same section, the peritubular cell nuclei were intensely stained (Figure 2). In the initial segment, the caput, and the cauda, AR immunoexpression is clearly much more pronounced, with clear staining of the nuclei of principal cells. In these segments, some cytoplasmic staining is also observed; however, the same cytoplasmic staining is found in

tissues incubated with pre-immune serum, and is thus considered non-specific (data not shown). In castrated rats, the immunoexpression of AR in the nuclei of all principal cells was strongly reduced. We found no clear difference in AR immunoexpression in the epididymis of rats that were castrated for periods of 7, 14 and 28 days. Ligation of the ductuli efferentes did not alter the immunoexpression of AR in the principal cells throughout the length of the epididymis (data not shown).

We observed a pronounced variation of AR immunoexpression in adjacent principal cells within a given cross section of a tubule (Figure 2). Although this variation was observed in all segments, it is clearly most apparent in principal cells of the proximal part of the epididymis. In all epididymal segments, the heavily stained principal cells far outnumber the lightly or unstained cells. In the caput the proportion of unstained principal cell nuclei seemed higher than elsewhere.

In castrated rats supplemented with TP, a daily dose of 6.25 µg was sufficient to reinduce the normal intensity of the nuclear AR immunoexpression in all epididymal epithelial cells. In these tissues, local variability in AR immunoexpression was observed in the principal cells, comparable to intact rats.

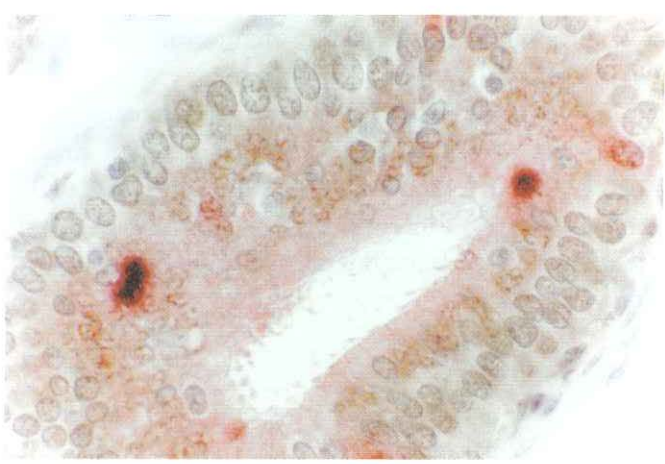

Figure 3. Photomicrograph of double-immunostaining with SP197 and an antibody targeting proteins B/C in the epididymis of a castrated rat, treated with 1.56 µg TP per day. Androgen receptor stain brown (DAB), and proteins B/C stain red (fuscine). Notice the very weak staining of nuclear androgen receptor in the principal cells, while an intense red colouring depicting a high level of proteins B/C, can be discerned in at leats two principal cells, indicating that proteins B/C can be synthesized in some principal cells under the influence of low testosterone levels that are not able to maintain normal AR expression in the principal cells. Notice also the pseudostratified appearance of the epididymal epithelium; a well-known result of subptimal androgen stimulation.

Immunolocalization of proteins B/C

In the intact rat, very faint immunoexpression of proteins B/C was observed in the cytoplasm of the principle cells of the ductuli efferentes and initial segment of the epididymis. In the proximal caput, a mosaic pattern of expression was observed with very intense staining of cytoplasm and nucleus of a number of principal cells, while other principal cells were not stained at all. Intensely stained principal cells were found throughout the caput, corpus, and cauda; however the relative number of cells with intense cytoplasmic proteins B/C immunostaining was highest in the caput. The extent of cytoplasmic staining in the principal cells was variable in that some cells showed light staining near the nucleus, some were intensely stained throughout the cell, and other cells had limited staining only at the apical end of the cytoplasm.

All principal cells from the distal caput onward had intense staining along their apical membrane including the stereocilia. In the distal corpus and cauda, the cytoplasm of the clear cells was strongly immunostained.

Castration had a dramatic effect on proteins B/C expression: in the cytoplasm of virtually all principal cells, proteins B/C immunoexpression was no longer apparent 7 days after treatment. Proteins B/C staining of the apical membrane and stereocilia was appreciably decreased, but was still visible at day 14 after castration. Ligation of the ductuli efferentes did not alter the intensity and pattern of proteins B/C immunoexpression.

Proteins B/C immunoexpression was effectively reinduced in the principal cells of castrated rats treated with 1,56 µg TP per day, in a pattern and intensity similar to that of intact rats. Interestingly, the dose of TP needed to maintain proteins B/C expression in the rat epididymis (1,56 µg TP/day) was lower than that needed to maintain normal androgen receptor expression (6,25 µg TP/day) (Figure 3).

Double-staining

Almost all proteins B/C positive principal cells stained intensely positive for AR. However, a few positive cells showed a very low or absent AR immunoexpression (Figure 4). This phenomenon was apparent throughout the epididymis; however, most proteins B/C positive cells with very low AR immunoexpression were found in the caput.

In situ hybridization of AR mRNA

AR mRNA was observed in the principal cells throughout the length of the epididymis. A regional variation in abundance of AR mRNA was clearly apparent: an intense signal was observed in the initial segment, while in the caput, corpus and cauda, AR mRNA was less intensely expressed (Figure 5). Expression was most apparent in the cytoplasm, in the perinuclear region of the cells (Figure 6 and 7). We also observed a clear heterogeneity in AR mRNA expression in adjacent principal cells within the same tubular cross section (Figure 6). Some principal cells express high levels of AR mRNA while neighbouring, morphologically indistinguishable, principal cells, stain only very lightly. This heterogeneity in AR mRNA expression is most apparent in the initial segment and the proximal caput.
In order to elucidate if the observed cell-to-cell variation in AR immunoexpression may be regulated at the transciptional level, we obtained a series of 8 μm thin slices of control epididymis, and alternately performed immunohistochemistry and in situ hybridization. Subsequently, in areas where, in alternate sections, a single principal cell nucleus was suspected, the expression of AR mRNA and AR immunoexpression was compared. This strategy resulted in evidence that the local variation of AR immunoexpression in epididymal principal cells was regulated at the level of mRNA expression (Figure 7).

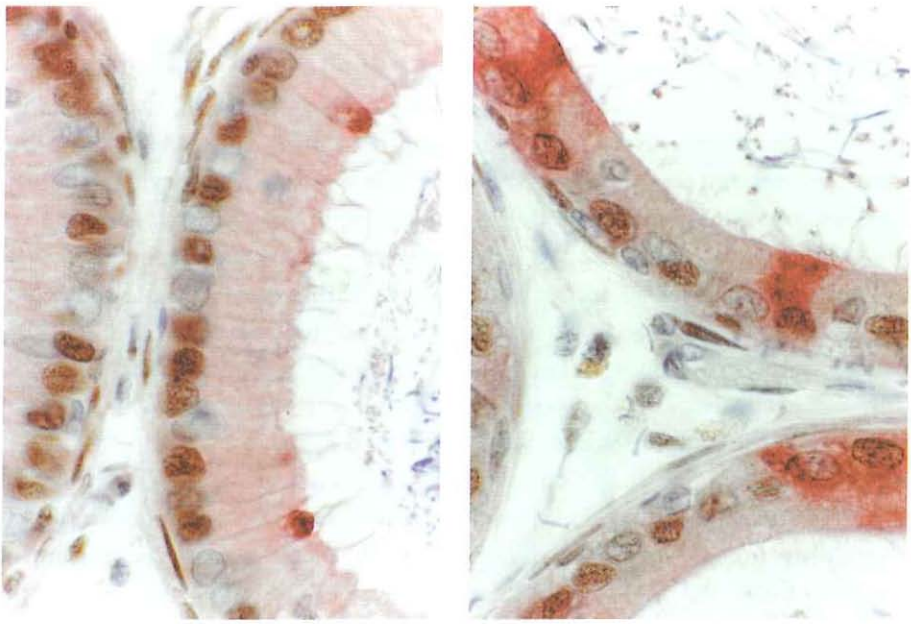

Figure 4. Photomicrographs of immunohistochemical double-staining studies of androgen receptor (stained brown) and proteins B/C (stained red) in the rat epdidymis. Both panels show a high expression of proteins B/C in principal cells with high and very low levels of nuclear AR immunoexpression, and *vice versa*. No consistent correlation between AR expression and proteins B/C expression could be discerned in the principal cells of the rat epididymis.

Figure 5. Photomicrograph of AR mRNA expression in the untreated rat epididymis, using *in situ* hybridization. Note the intense staining in the initial segment of the epididymis.

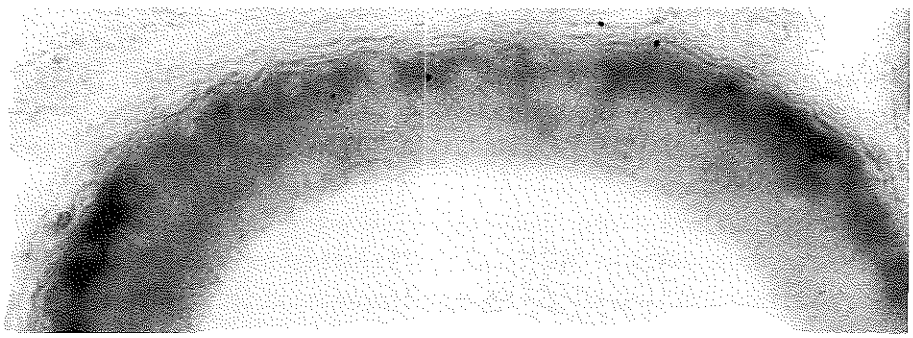

Figure 6. Photomicrograph of AR mRNA expression in a portion of a ductule from the caput of the rat epididymis. Using in situ hybridization, AR mRNA was visualized in control rat epididymis (see Materials and Methods). Notice the perinuclear staining of AR mRNA, and the marked cell to cell variation in ARm RNA expression.

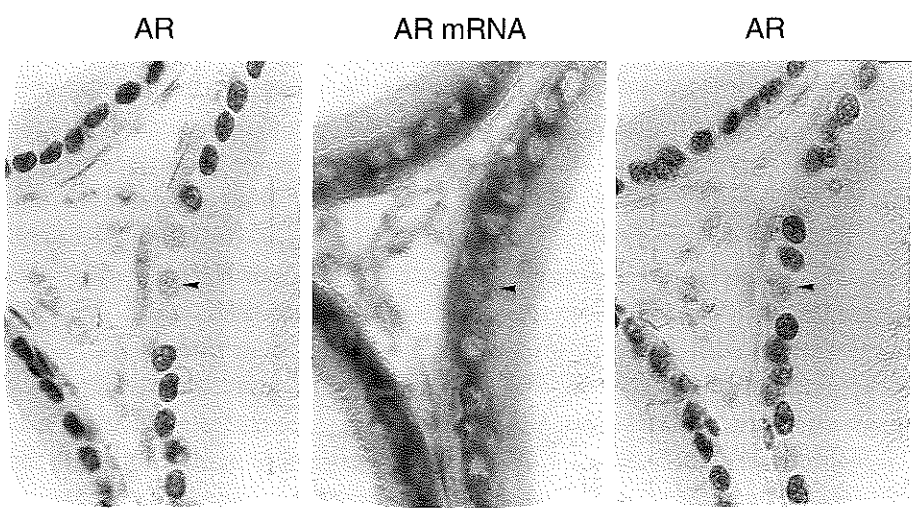

Figure 7. Serial 8 μm sections, alternately showing AR immunoexpression and AR mRNA expression. As an average principal cell nucleus measures 10 μm in diameter, slices of the same nucleus will inevitably appear on consecutive sections. These serial micrographs provide evidence that principal cells, showing low levels of AR immunostaining, also have low levels of AR mRNA expression, indicating that the cell-to-cell heterogeneity in AR protein expression may be regulated at the transcriptional level.

Discussion

In the present study, we have characterized the pattern of androgen receptor immunoexpression in the rat epididymis, and correlated these findings with the pattern of AR mRNA expression and the cellular production of androgen dependent proteins B/C.

Previous studies have shown nuclear AR immunoexpression in the epithelial cells of the rat epididymis (Sar *et al.*, 1990; Paris *et al.*, 1994). None have reported regional variation in AR immunoexpression in the various regions of this organ. In the ram, goat, rhesus macaque, and human, clear regionally defined variations in AR expression in the epididymis have been found (Tekpetey *et al.*, 1989; Roselli *et al.*, 1991; Ungefroren and Ergun, 1997; Goyal *et al.*, 1998). In accordance with a number of reports, we observed strong AR immunoexpression in the nuclei of principal cells, basal cells, peritubular myoid cells and a number of interstitial cells, but not in halo cells. We found little region-specific variation in AR immunoexpression in the nuclei of the principal cells, although the nuclei of the epithelial cells of the efferent ducts were clearly less intensely stained compared to the rest of the epididymis. In castrated rats, nuclear AR immunoexpression in the epithelial and interstitial cells is clearly reduced in all segments. When TP is supplemented at a dose of 6.25 μg/day, AR immunoexpression is comparable to control epididymis. Epididymides of rats with ligated ductuli efferentes for periods of 7 to 28 days show no decrease in, or change in pattern of, AR immunoexpression, indicating that testicular factors other than testosterone do not regulate epididymal AR immunoexpression in rats. Although testosterone reaches the epithelial cells via the blood stream and through the lumen of the ductuli efferentes, 10% of the normal serum level of testosterone is sufficient to maintain normal AR immunoexpression.

A previous study suggested that androgen withdrawal by castration or treatment with a GnRH-antagonist results in a decrease in AR immunoexpression, with the remaining AR staining discernible in the nuclei of the principal cells (Paris *et al.*, 1994), indicating that the remaining AR is still in the tightly bound nuclear form. We confirm the marked reduction in AR immunoexpression in all segments of the epididymis when rats were castrated, but found most of the residual AR immunostaining in the cytoplasm of the principal cells, with only a few AR-positive nuclei in the proximal caput (results not shown). The regulation and distribution of AR immunoexpression by androgen-AR interactions in the epididymis thus seem

similar to those observed in the testis (Bremner *et al.*, 1994; Van Roijen *et al.*, 1997), the rat prostate (Blok *et al.*, 1992a), and the seminal vesicles (Paris *et al.*, 1994). Interestingly, androgen withdrawal causes no change in AR mRNA in the testis (Blok *et al.*, 1992b), and marked AR mRNA up-regulation in the prostate (Quarmby *et al.*, 1990a; Blok *et al.*, 1992a) and epididymis (Blok *et al.*, 1992a), indicating that AR expression is regulated at the level of mRNA translation and/or that AR degradation is enhanced in the absence of androgens. It has indeed been shown that binding of androgen to the AR results in a more stable complex with a much longer half-life than an unliganded AR molecule (Zhou *et al.*, 1995).

A surprising finding was the distinct variation of AR immunoexpression in the nuclei of morphologically identical adjacent principal cells throughout the epididymis, but especially in the distal caput and proximal corpus. Heterogeneity in AR immunoexpression has previously been reported in the rat and human testis (Bremner *et al.*, 1994; Vornberger *et al.*, 1994; Van Roijen *et al.*, 1997), in human hepatocytes (Hinchliffe *et al.*, 1996), and in prostate and ovarian tumor tissue (Chadha *et al.*, 1993; Ruizeveld de Winter *et al.*, 1994).
In the rat, spermatogenesis can be subdivided into fourteen specific stages (Leblond and Clermont, 1952). At stages VI and VII, AR immunoexpression in Sertoli cells is high, while at stages VIII through XIV and I through V, AR immunoexpression is appreciably lower (Bremner *et al.*, 1994). It is thought that AR immunoexpression in the Sertoli cells is highest in those stages that are most dependent on androgens (Sharpe, 1994). The stage dependent AR expression is not regulated by the local level of available androgen, but by the level of AR mRNA expression in Sertoli cells at the respective stages (Shan *et al.*, 1995; Van Roijen *et al.*, 1997).
We subsequently performed in situ hybridization studies to clarify the pattern of AR mRNA expression in the normal rat epididymis on a regional and local scale. AR mRNA levels are highest in the initial segment, and appreciably lower further distal from the testis. The transition from high AR mRNA expression to low AR mRNA expression is rapid, and probably does not reflect a change due to local variation in the androgen level. The level of AR mRNA expression in adjacent, morphologically identical, principal cells can vary considerably. In order to assess if cells with high androgen receptor immunoexpression also contain a high level of AR mRNA, serial 8 mm

sections were alternately stained for AR protein and AR mRNA. As shown in Figure 7, our results indicate that indeed cells with high AR immunoexpression show a high cellular AR mRNA content, indicating that, in epididymal epithelial cells, AR expression may be regulated at the transcriptional level.

Epididymal cell protein production is highly dependent on androgens (Brooks and Higgins, 1980; Holland *et al.*, 1992). As AR immunoexpression may putatively be an indication of androgen mediated cell activity, we attempted to correlate AR immunoexpression in principal cells with the expression of a highly androgen dependent gene. Proteins B and C are two splice variant products of a gene, expression of which is highly androgen dependent (Brooks *et al.*, 1986). Surprisingly, as shown in Figure 4, double staining experiments revealed that some cells had a high expression of proteins B/C, but not of AR.

Another tissue that shows a highly heterogeneous pattern of nuclear androgen receptor immunoexpression, is prostatic adenocarcinoma (Sadi *et al.*, 1991; Van der Kwast *et al.*, 1996). Because of the high inter- and intra-individual variation in the relative number of AR positive cells in this malignant tissue, many studies have been performed to elucidate if the level of AR expression in prostate carcinoma has prognostic significance. The results have, so far, been inconclusive (Sadi *et al.*, 1991; Prins *et al.*, 1998). Prostatic epithelial cells produce a highly androgen dependent protein, prostate-specific antigen (PSA). In prostate carcinoma, the immunoexpression of PSA is also heterogeneous. Interestingly, in a double staining study, no correlation was found in the cellular immunoexpression of AR and PSA (Ruizeveld de Winter *et al.*, 1994). Some PSA positive cells were found to express high levels of AR, while others were AR negative.

The above observations could mean that the intensity of AR immunoexpression is not be related to androgen driven cell activity, and may thus be caused by other factors. It is interesting to note that the supplemental dose of testosterone propionate needed to maintain normal proteins B/C expression (1.56 µg/day) was lower than that needed to maintain normal nuclear AR immunoexpression (6.25 µg/day). One could speculate that normal transcription some androgen dependent genes in the epididymis can be maintained by a low level of androgen receptor, not detectable by immunohistochemistry. Alternatively, the activity of the principal cells may not be

constant, but may vary in a cyclic fashion. This would result in cells that are 'active' with a high level of AR immunoexpression and cells with low AR immunoexpression. To some extent this hypothesis parallels the situation in the testis, where Sertoli cells express high levels of AR at stages where androgens are thought to exert most of their effect (Sharpe, 1994). The lack of an obvious correlation between AR immunoexpression in principal cells and the expression of proteins B/C is at first sight not consistent with the above train of thought. However, the disappearance of the AR might precede the reduction in protein by an appreciable time period.

In summary, AR immunoexpression is found in the nuclei of principal cells, clear cells and basal cells throughout the length of the epididymis. The epithelial cells of the ductuli efferentes stain weakly for AR; however, the AR immunoexpression in the nuclei of the epithelial cells further distal stain more intensely. In situ hybridization studies of AR mRNA in these tissues showed a regional variation in AR mRNA expression; the mRNA level was clearly high in the initial segment of the epididymis compared to the rest of the caput, corpus and cauda. A marked cell-to-cell variation in AR immunoexpression and AR mRNA was found within a single tubular cross section. Double-staining experiments using antibodies targeting the AR and the androgen regulated epididymal proteins B/C, revealed no correlation between the immunoexpression of proteins B/C and the AR. The observation that epididymal epithelial cells expressing an androgen dependent protein may show no AR immunoexpression indicates that expression of proteins B/C may be maintained by a low level of androgen receptor, not detectable by immunohistochemistry. The current data are, however, also consistent with a putative cyclic variation in epididymal epithelial cell activity, where the expression and disappearance of AR precedes the expression and disappearance of proteins B/C. No information is currently available concerning the function of individual epididymal epithelial cells over a period of time. Further research is needed to fully comprehend the androgen regulation of epididymal epithelial cell function.

Part 2

Towards Clinical Andrology

5

Spermatogenic disorders

Introduction

The focus of the previous chapters is on the normal physiology of male reproductive function, mainly as studied in the rat. When any part of the intricate chain of events leading to male reproductive capacity breaks down, reproductive dysfunction or infertility may ensue. Infertility may follow from dysfunction of the testis, or may be the result of defective transport and/or maturation of the spermatozoa. Furthermore, deposition of semen into the vagina requires the interplay of a number of physiological processes, and dysfunction of any one of these processes may result in impotence, premature- or anejaculation, or retrograde ejaculation.
In a number of patients, developmental aberrations or genetic defects can be identified as the cause of their infertility. In others, their subfertility can be attributed to external influences (heat, infection, chemicals) or disease, as in testicular malignancy. Often, however, no cause for a man's subfertile condition can be found.
The following chapters in this thesis deal with some of the issues involved in disruption of human spermatogenesis, including the testicular expression pattern of the androgen receptor in the testis (Chapter 6), and patterns of expression of sperm nuclear proteins during spermatogenesis and in spermatozoa (Chapter 7). These studies were performed using semen samples and testis biopsies from male partners in infertile unions.
In this chapter, the basis of spermatogenic disruption is briefly explored. Specific attention is directed towards possible genetic mechanisms behind testis dysfunction, as much of the present research regarding male infertility is focussed on this area.

Male factor infertility: the magnitude of the problem

World-wide, 60-80 million couples are unable to conceive a child (Diczfalusy and Crosignani, 1996). Although infertility poses no direct threat to physical health, it can be disastrous for the psychological and social well-being of those involved. It was traditionally the women who were singled out as the cause of a barren marriage, but it is now clear that male factor infertility plays a major role. In a joint analysis by the World Health Organisation (WHO) and the World Bank, it is estimated that, globally, reproductive ill health accounts for 12% of the overall burden of disease in the male (Diczfalusy and Crosignani, 1996). Among subfertile couples, defined as not having conceived after 1 year of unprotected coitus, about 50% of the male partners have subnormal

semen parameters according to WHO criteria (The ESHRE Capri Workshop Group, 1994). The increasing awareness of the role of male reproductive (dys)function in infertile couples, has resulted in a large number of clinical and more fundamental studies to elucidate the mechanisms of male reproductive disease.

In a large cohort of subfertile couples, semen abnormalities or other reproductive dysfunctions could be identified in 51.6% of the male partners (Farley and Belsey, 1988). In 51.2% of these men, no cause for their subfertility could be identified (idiopathic male infertility). A later study performed in a European center with a special interest in male reproductive endocrinology revealed 31.7% idiopathic male infertility (Behre *et al.*, 1994). Although the population of the two studies are probably quite different, it is evident that in a large percentage of subfertile males the underlying pathology cannot be identified. At our Andrology Clinic, we found that about 25-30% of subfertile men present with unexplained semen abnormalities (Pierik *et al.*, submitted for publication). Nevertheless, in males where a clear underlying anomaly is found, the influence of this condition upon reproductive function is not always clear. Varicocele, a condition where the venous pampiniform plexus, carrying blood from the testis, is dilated with dysfunctional valves, causing backflow of blood from the body to the testis, resulting in a higher scrotal temperature (Lerchl *et al.*, 1993; Wright *et al.*, 1997), is found in approximately 30% of subfertile men (Aafjes and van der Vijver, 1985; Marks *et al.*, 1986; Pierik *et al.*, unpublished results). Varicocele is also found in about 15-20% of fertile men (Nieschlag *et al.*, 1997). One must be weary of identifying a diagnosed varicocele as the cause of a man's infertile condition (Hargreave, 1993).

Chromosomal abnormalities in subfertile males

Male infertility may result from chromosomal abnormalities or (multi) gene defects. The genetic analysis of infertile men has, until relatively recently, mostly been restricted to karyotyping of lymphocytes or spermatozoa (Zuffardi and Tiepolo, 1982a; Bourouillou *et al.*, 1985a; Martin *et al.*, 1988a; Martin *et al.*, 1991a; Vogt, 1995a). These studies have shown a clearly increased incidence of chromosome aneuploidy and chromosome structure anomalies in subfertile men, especially in men with severe oligoasthenoteratozoospermia. Autosomal translocations are found 10 times more frequently in

infertile men compared to fertile men, with concomitantly an increased risk of spontaneous abortion of pregnancies fathered by these men (Vogt, 1995a). Similarly the frequency of pericentric chromosome inversions of chromosomes 1,2,5,6,9 and 10 is also increased. The transfer of so-called Robertsonian translocations may increase the risk of mental retardation in the offspring (Lange *et al.*, 1990a; Vogt, 1995a). A recent review article pooling and comparing the results of 7 of the largest and most relevant studies, comprising a total of 7,876 infertile men, versus the data for 94,465 newborn infants, revealed a 5.1% incidence of chromosomal abnormalities in infertile men: 3.8% involved sex chromosomes (see below) and 1.3% the autosomes. In newborn infants, the frequency of chromosome abnormality was 0.38%: 0.14% involved sex chromosomes and 0.25% autosome anomalies (Van Assche *et al.*, 1996). The comparison of a number of studies using different cut-off values for inclusion of oligozoospermic males, indicates that an increase in the severity of spermatogenic dysfunction is concomitant with an increase in chromosome abnormalities. Three studies using $20x10^6$ spermatozoa per ml as the cut-off value, obtained and average of 2.4% chromosomal abnormalities, compared to 6.6% for two other studies using a cut-off value of $10x10^6$ sperm per ml (Van Assche *et al.*, 1996). A further increase in chromosomal abnormality is observed in azoospermic individuals. In six studies of azoospermic men, an average of 13.7% chromosomal abnormalities was found (Van Assche *et al.*, 1996). The ratio between autosome and sex chromosome aberrations is different in azoospermic and oligozoospermic men: in the azoospermic men, sex chromosome abnormalities predominate with an incidence of 12.6%, while in the oligozoospermic group autosome anomalies are most frequent with an incidence of 3% (Van Assche *et al.*, 1996). Thus, early observations indicating an inverse correlation between the incidence of chromosomal abnormalities and sperm count (Kjessler, 1966) are confirmed by many recent findings.

Sex chromosome aneuploidies are a relatively frequent finding in infertile men. Most frequent among these, is Klinefelter's syndrome which has an incidence of about 1/500 in the male population and 7-13/100 in azoospermic males. Ninety percent of men with Klinefelter's syndrome have a 47,XXY karyotype, with the other patients having either a larger number of X chromosomes or some form of mosaicism (mostly 46,XY/47,XXY). Practically all patients with Klinefelter's syndrome are infertile, with azoospermia and small firm testes. These testes show sclerosis and hyalini-

zation of the seminiferous tubules with absence of spermatogenesis (Vogt, 1997a).

Somewhat more rare is the occurrence of men with a 47,XYY karyotype (1/750). These men are not always infertile. In pachytene spermatocytes of patients with a 47,XYY karyotype, an increased rate of YY bivalents are observed. This suggests disturbance of the XY sex body formation during the male meiotic prophase, and possibly explains the infertility in most of these men (Mak and Jarvi, 1996a; Vogt, 1997a).

A very rare chromosomal aberration is the 45,XO male where the distal part of the long arm of the Y chromosome is lost, while the rest (including the *SRY* locus) is translocated to an autosome, usually the short arm of one of the chromosomes 13, 15 or 22 (Vogt, 1997a).

A group of microdeletions of the long arm of the Y chromosome, with subsequent azoospermia or severe oligozoospermia, has led to a search for loci that contain genes that are essential for spermatogenesis. On Y_q11 a region is now known to contain at least three loci that are involved in spermatogenesis, AZFa, AZFb and AZFc (AZF referring to azoospermia factor). Men with a deletion of AZFa have a Sertoli-cell-only syndrome; tubules contain only Sertoli cells, and no germinal cells at any stage of development can be found. Testis histology of men with an AZFb deletion is different, with a spermatogenic arrest at the spermatocyte stage, at the prophase of meiosis. In histological sections of testes from men with a deletion of AZFc, a pleitropic effect is found, with some tubules containing only Sertoli cells while other tubules contain germinal cells reaching various stages of development including (small numbers of) mature spermatozoa. The latter suggests a defect in post-meiotic maturation with variable penetrance (Vogt, 1997a). Genes located within the AZF regions will be discussed below.

Genetic defects causing infertility as one of a number of pleitropic effects

Although relatively rare in the andrology clinic, a large number of genetically determined clinical syndromes result in male infertility through a number of very different mechanisms. Generally, the syndromes can be grouped into: a) those causing spermatogenic defects, and b) those that have normal spermatogenesis, but result in anatomical or functional defects causing a disability to deliver sperm to the female genital tract (Mak and Jarvi, 1996a; Vogt, 1997a). In Table 1, a summary of the known syndromes resulting from

a genetic defect and causing male infertility is displayed along with the Mendelian Inheritance in Man (MIM) reference number, obtained at http://www3.ucbi.ulm.nih.gov/Omim/. It is not our intent to extensively review all the mentioned aberrations; however, in prelude to Chapter 6, genetic defects resulting in impaired androgen receptor function will briefly be discussed.

Androgen receptor defects

Initially recognised in 1937 by Petterson and Bonnier, dysfunction of the androgen receptor in a 46,XY individual results in the development of an external female phenotype with gynecomastia, but absent axilarry and pubic hair, and an internal phenotype with undescended testes, absence of uterus and ovaries, and a blind ending vagina. This condition was referred to as testicular feminization (Tfm) but is now more commonly known as complete androgen insensitivity syndrome (CAIS). As the gene coding for the androgen receptor resides on the X chromosome, the disorder is inherited in an X-linked recessive fashion. The frequency of occurrence of complete or incomplete AIS is about 1/60,000 male births (Griffin and Wilson, 1989). Mutations resulting in either complete or partial androgen resistance syndromes have been identified in all exons (Brinkmann *et al.*, 1995; Brinkmann *et al.*, 1996). Mutations in exon 1, usually result in premature stop codons causing a highly truncated non functioning androgen receptor, and hence in complete androgen resistance. To date, only three missense mutations have been identified in exon 1 resulting in either a reduced translational efficiency or a slightly increased hormone dissociation rate (Choong *et al.*, 1996; Gottlieb *et al.*, 1997). Many mutations leading to a variety of phenotypes have been found in the hormone binding domain, the hinge region and the DNA-binding domain of the androgen receptor. Androgen resistance can be complete, with an invariable female psychosexual identity. Partial androgen resistance is associated with an extremely variable phenotype, including a predominant female external phenotype or individuals with ambiguous genitalia.

A mild form of androgen receptor dysfunction, known as Reifenstein syndrome, displays a predominantly male phenotype with penoscrotal hypospadias, cryptorchidism and gynecomastia. The psychosexual identity of the latter patients vary, but is usually male.

Interestingly, some AR mutations do not result in a specific phenotype, since two individuals with the same mutation in the androgen receptor gene may well differ considerably in the degree of masculinization (Batch *et al.*, 1993; Imasaki *et al.*, 1994; Evans *et al.*, 1997). No clear explanation has as yet been offered for this phenomenon, although receptor activity is probably influenced by other factors such as androgen levels and co-factor expression. Furthermore the length of the polymorphic polyglutamine stretch in the NH_2 terminal domain of the AR may also have a modulatory function on receptor activity (Jenster *et al.*, 1994).

In addition to the -mild- Reifenstein syndrome, evidence exists that an even milder form of androgen resistance may exist in the form of infertile, otherwise fully androgenized, males. In 1979 Aimann *et al.* presented three unrelated infertile men with high serum LH and testosterone levels (Aiman *et al.*, 1979). In all three men, genital skin fibroblasts contained a diminished amount of androgen receptor, indicating a mild form of androgen insensitivity syndrome. Subsequently, a quantitative analysis of androgen receptor from the scrotal skin of a number of infertile men with oligo-zoospermia and azoospermia was compared to fertile controls (Aiman and Griffin, 1982). Using the lowest value found in normospermic males as a cut-off, the above investigators found a staggering 40% incidence of androgen receptor deficiency in idiopathic azoospermic or oligozoospermic males. A later study (Morrow *et al.*, 1987) indicated a 19% incidence of androgen receptor defects in fully androgenized men with idiopathic oligozoospermia, largely corroborating the findings of Aimann and Griffin (1982). In a small group of infertile men, however, Eil *et al.* (1985) found no indication for a quantitative androgen receptor anomaly using pubic skin fibroblasts (Eil *et al.*, 1985). Bouchard *et al.* (1986) also found no evidence for a quantitative androgen receptor defect in genital skin fibroblasts of a group of 24 men with severe oligozoospermia (Bouchard *et al.*, 1986). The above-mentioned studies are clearly contradictory, and a serious flaw appears to be the lack of study pertaining to the molecular basis of the putative androgen receptor impairment. Molecular techniques were utilized in a report by Akin *et al.* (1991) who found one patient in a group of seven infertile males with a deletion of exon 4 (Akin *et al.*, 1991). It is questionable, however, whether this relatively large deletion would result in such a mild phenotype, as much smaller deletions in exon 4 have resulted in complete or partial androgen resistance. Yong *et al.* (1994) identified a mutation in exon 5 of the androgen

receptor of a man with oligozoospermia (Yong *et al.*, 1994). Whether the mutations found in these selected individuals are indeed cause of infertility remains to be elucidated. Interestingly, Tut *et al.* (1997) found a strong association between the risk of impaired spermatogenesis and the length of the polyglutamine stretch-encoding sequence in exon 1 of the androgen receptor (Tut *et al.*, 1997). Men with 28 or more glutamine residues had more than a 4-fold increased risk of spermatogenic failure, while men with 23 glutamine residues or less had a relative risk of 0.5 for hypospermatogenesis. In addition, the more severe the oligozoospermia, the higher the proportion of patients with a long polyglutamine stretch (Tut *et al.*, 1997).

Several of the reports described above, clearly indicate that a quantitative or qualitative defect in the androgen receptor may play a role in the pathogenesis of infertility in otherwise fully androgenized males. Much remains unclear, however. It may well be that a mild defect in the androgen receptor results in spermatogenic failure, because spermatogenesis requires a high testicular testosterone level, and 5α-reductase is largely absent in the testis (see Chapter 3). Also, one may postulate the existence of testis-specific co-factors which fail to function properly in case of certain AR mutations. Although the testis is the relevant target organ, in case of infertility related to subtle dysfunction of the androgen receptor, all of the above reports have studied either genital skin or pubic skin fibroblasts. In Chapter 6, we have studied the expression of androgen receptor in the testis of infertile men using immunohistochemistry. An immunohistochemical investigation of the androgen receptor will generate quantitative rather than functional data. However, expression of the androgen receptor may in many instances be dependent on its function. Based on rat studies (Chapter 2), it can be postulated that an androgen receptor defect, particularly in the hormone-binding region, however subtle, might result in decreased stability of the receptor protein. In the studies described by Aimann and Griffin (1982) and Morrow *et al.* (1987), clear quantitative differences in androgen receptor expression in fibroblasts were noted in fertile men, as compared to a number of infertile men. Possibly such a difference would more pronounced when testis tissue is investigated.

Defects in "spermatogenesis genes"

As explained in Chapter 1, spermatogenesis is a unique process, encompassing mitotic divisions, meiotic prophase and divisions, and spermiogenesis. This series of events, as well as many of the proteins and enzymes required for this process, are regulated by a number of genes expressed only in the testis. Mutation or deletion of any one of these genes may result in decreased fertility without any other phenotypic manifestation. At the present time, a considerable number of these genes have been found, but many more remain to be identified. In addition, with the exception of genes within the AZFa/b/c loci, none are known to contribute significantly to the male infertility case-load. There is a large effort to elucidate the genetic control of spermatogenesis, with particular consideration for testis-specific genes. Most of the knowledge so far has been attained with the use of mouse models, which involve knockout of both alleles of one or more genes. Endeavours to find mutations in the homologous human genes in the infertile male population, however, have been tediously unfruitful. Evidently, human male infertility may involve multigene defects.

Testis-specific genes

Spermatogenesis is highly dependent on the presence of a Y chromosome, and regions have now been identified that contain genes that are essential for male fertility. First and foremost, the *SRY* gene is located on the short arm of the Y chromosome very near the pseudo-autosomal region, and the timely expression of this gene in the early embryo is essential for testis determination and subsequent development of the male phenotype (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Koopman *et al.*, 1991). The three regions identified on Y_q11 (AZFa/b/c; see above) contain a number of genes that appear to play a vital role in spermatogenesis (Vogt *et al.*, 1996a; Vogt, 1997a). AZFa contains the *Drosophila fat facets*-related Y gene (*DFFRY*) which has an X chromosomal homologue (*DFFRX*) (Jones *et al.*, 1996). *DFFRX* encodes a deubiquinitination enzyme, which releases the ubiquitin peptide chain from ubiquitinated proteins. The precise role of *DFFRY* in spermatogenesis has yet to be established. AZFb contains the multicopy gene *RBM*, encoding a protein with a ribonucleic acid binding motif (Vogt *et al.*, 1996). *RBM* is expressed in the nuclei of pre-meiotic germ cells, and the encoded protein is probably involved in metabolism of newly synthesised RNA at that stage of

spermatogenesis (Najmabadi *et al.*, 1996a; Elliot *et al.*, 1997a; Vogt, 1997a). No mutations in *RBM* genes have yet been found in relation to male infertility, and the AZFb region may contain other genes, which also play a role in spermatogenesis. AZFc contains genes of the *DAZ* (*deleted in azoospermia*) family (Vogt *et al.*, 1996). Like *RBM*, *DAZ* is a multicopy gene encoding RNA binding protein, and its protein product has been found in late spermatids and in sperm tails (Vogt *et al.*, 1996; Habermann *et al.*, 1998). The function of DAZ, however, is not known. Some men with deletions in the AZFc region can produce mature spermatozoa and procreate (Terada and Hatakeyama, 1991b; Vogt, 1997b). In the mouse, the *DAZ*-related autosomal gene *Dazla* is essential for both male and female gametogenesis (Ruggiu *et al.*, 1997).

During the meiotic prophase, the X and Y chromosomes condense and become transcriptionally inactive, forming the so-called sex body (Monesi, 1965). Although most, if not all, of the Y-chromosomal genes are reactivated in spermatids, this is not the case for many X-chromosomal genes (Eddy *et al.*, 1993; Hendriksen *et al.*, 1995; Grootegoed, 1996). Interestingly, enzymes encoded by a number of genes on the X chromosome [*Pgk-1* (phosphoglycerate kinase) and *Pdha-1* (E1α-subunit of pyruvate dehydrogenase)] and taking part in the energy-yielding machinery of somatic cell types, appear to be replaced during spermatogenesis by specific isoenzymes encoded by autosomal genes (Kramer, 1981; Takakubo and Dahl, 1992; Hendriksen *et al.*, 1997). These autosomal genes (*Pgk-2 and Pdha-2)* are intronless retroposons which have originated from reverse transcribed mRNA, and which are functional both in mouse and human (Boer *et al.*, 1987; McCarrey and Thomas, 1987; Dahl *et al.*, 1990). For the mouse, a similar switch from *G6pd*-1 to *G6pd*-2 (glucose 6-phosphate dehydrogenase 1 and 2, respectively) has been reported (Hendriksen *et al.*, 1997). A number of other genes (glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase subunits and, cytochrome c), situated on autosomes, are also silenced during the meiotic prophase and their gene products are substituted for by germ cell-specific activation of alternate autosomal genes (see Grootegoed, 1996; Eddy, 1998). A mutation in any of the genes encoding for these testis-specific isoenzymes may result in male infertility without any other phenotypic abnormality, but no such mutation has yet been found in the human infertile male population.

Interaction of ligand-activated gonadotropin receptors with G protein, which

modulates adenylyl cyclase activity, results in an increase in the intracellular level of cAMP. Regulation of transcription of certain genes is under the influence of cAMP, through the binding of activated cAMP-response element (CRE)-binding proteins with CRE-elements associated with promoter regions of these genes (Monaco and Sassone-Corsi, 1998). A number of CRE-binding proteins have been identified, including transcription activators (CREB, CREMτ and ATF-1) and transcription repressors [i.e. interleukin-1β-converting enzyme repressor (ICER)]. In the adult testis, a high level of CREMτ (a testis-specific *CREM* splice variant) has been identified (Monaco and Sassone-Corsi, 1998). Western blot and immunohistochemical studies have located the expression of the CREMτ protein specifically in round spermatids (stages VII-VIII in the mouse and stages I-III in man), although *CREM* transcripts are identifiable as early as the pachytene spermatocyte stage, implicating a translational control mechanism (Monaco and Sassone-Corsi, 1998; Weinbauer *et al.*, 1998). The importance of CREMτ in spermatogenesis is illustrated by a knockout mouse model, where female mice homozygous for the inactivated gene are phenotyically normal, but heterozygous males have a clear deficiency in spermatogenesis. Male knockout mice are infertile showing developmental arrest of spermatogenesis at the early spermatid stage, without any other somatic phenotypic abnormality (Blendy *et al.*, 1996; Nantel *et al.*, 1996). The relevance of CREM in human spermatogenesis is suggested by studies showing reduced immunoexpression of CREM protein in round spermatids of male infertility patients with round spermatid maturation arrest (Weinbauer *et al.*, 1998).

Among the post-meiotic germ cell-specific processes, is the condensation of the sperm nucleus. This condensation involves the removal of nuclear histones, which are first replaced by transition proteins and later by protamines, resulting in a highly compact nucleoprotein complex (see Chapter 1). This histone-to-protamine transition requires considerable protein synthetic capabilities along with prodigiously efficient processing and breakdown of proteins. The molecular mechanisms for removal of histones and transition proteins are largely unknown. Undoubtedly the signal to remove and degrade the histones and later the transition proteins is preceded by some form of posttranslational modification, such as the known hyper-acetylation of H4 (Meistrich *et al.*, 1992). One other form of modification that may be involved in the removal and degradation of spermatid nuclear proteins is

Clinical syndrome	Chromosomal Position	MIM* Ref. Number
Alpha thalassemia (ATRX)	Xq13	301040
AMH resistance I	19p13.3-p13.2	261550
AMH resistance II	12q13	261550
Androgen resistance syndromes	Xq12	300068
Bardett-Biedl Syndrome	16q21	209900
Campomelic dysplasia	17q24.3-q25.1	211790
Cystic fibrosis	7q31.2	219700
Congenital absence of the vas deferens	7q31.2	277180
Corticol steroid deficiency	9q33	184757
Dystrophia myotonica	17q13.2-q13.3	160900
Dose sensitive sex (DSS)	Xp21	300200
BPES syndrome	3q22-q23	110100
Fanconi syndrome A (FACA)	16q24.3	227650
Fanconi syndrome B (FACB)		
Fanconi syndrome C (FACC)	9q22.3	227646
Fanconi syndrome D (FACD)	3p	
Fanconi syndrome E (FACE)		600901
FSH receptor dysfunction		136435
Fragile X syndrome	Xq27.3	309550
Garlin syndrome		119500
Hanhart syndrome		262600
Hutchinson-Gilford-Progeria syndrome	176670	
Kallman 1	Xp22.3	308700
Kartagener syndrome		244400
Male pseudohermaphroditism	2p23	264600
Leopard syndrome		151100
LH receptor dysfunction		152790
Nonne-Milroy-Meige syndrome		153000
Noonan syndrome	12q22	163950
Opitz syndrome	22q11.2	145410
Prader-Willi syndrome	15q11	176270
Prune belly syndrome		100100
Rieger syndrome Type 2	13q14	601499
Robinow syndrome		180700
Rothmund-Thomson syndrome	8	268400
Russell-Silver syndrome	17q25	180860
Sex reversal (Y-linked)	Yp11.2	480000
Stein-Leventhal syndrome		184700
Werner syndrome	8p12-p11.2	308700
Wilms tumors	11p	194080
Young syndrome		279000

* Mendelian Inheritance in Man

ubiquitination. Ubiquitin is a highly conserved 76 amino acid protein that can be covalently attached to proteins through a process requiring a number of steps and enzymes, including ubiquitin-activating (E1) enzymes (such as Ube1x and Ube1y), ubiquitin-conjugating (E2) enzymes (such as mHR6A), and ligating (E3) enzymes (Scheffner *et al.*, 1995). Ubiquitination of proteins is involved in protein (re)folding, in protein degradation, and in the endocytotic pathway of integral membrane proteins. Poly-ubiquitinated proteins are degraded by 26S proteasomes (Varshavsky, 1997). Most likely, protein ubiquitination is of much importance for protein degradation in spermiogenesis.

The testis clearly is a specialised organ, also when it comes to the ubiquitin pathway of protein metabolism. A number of ubiquitin-conjugating enzymes have increased, or even exclusive, expression in the testis. These include HR6A and HR6B, $E2_{17kb}$, hUBC9, and UbcH-ben (see Baarends *et al.*, 1998). In addition, microdeletion of a region of the Y chromosome (AZFa) involving *DFFRY*, a gene encoding putative deubiquitination enzyme, results in male infertility (Jones *et al.*, 1996; Vogt, 1997).

The importance of ubiquitination of testicular proteins in spermatogenesis was recently illustrated using a knockout mouse model in which the ubiquitin-conjugating enzyme *m*HR6B was inactivated (Roest *et al.*, 1996; Grootegoed *et al.*, 1998). The *mHR6B* gene is one of the mouse homologs of *RAD6*, found in *S. cerevisiae* (budding yeast) where its gene product is involved in a number of processes including postreplication DNA repair and sporulation (Lawrence, 1994). *RAD6* null mutants exhibit cell cycle perturbance, temperature-sensitive growth, inability to sporulate, and increased retrotransposition (Lawrence, 1994). RAD6 has been shown to mono- and polyubiquitinate histone 2A and histone 2B *in vitro* (Jentsch *et al.*, 1987). The yeast RAD6 and mammalian HR6B proteins are highly conserved, sharing about 70% amino acid sequence identity. One hundred percent homology is observed between mouse mHR6B and the human homologue hHR6B, suggesting a highly conserved and evolutionary important functional activity (Koken *et al.*, 1991; Koken *et al.*, 1996). The male mice that were homozygous for the inactivated *mHR6B* gene had a normal phenotype, with apparently no increased sensitivity to mutagens (possibly because of a functional redundancy of mHR6B and mHR6A proteins). However, the *mHR6B* knockout males appeared to be infertile. Upon closer scrutiny, spermatogenesis appeared to progress normally during

the beginning of the first wave of germ cell development, until the germ cells arrived at the post-meiotic stages when the spermatid nuclei should start to condense. At that point, a profound derailment of spermatogenesis starts to occur, with variable loss of the germinal epithelial architecture. Although all *mHR6B* knockout males were found to be infertile, the epididymides of most of these mice contained a small number of morphologically abnormal sperm. Two-dimensional Western blot analysis has shown that mouse elongating spermatids and spermatozoa containing a relatively low mHR6A protein level relative to that of mHR6B. This has not been found in any other mouse tissue. As a result, knockout of *mHR6B* will result in a more than 50% reduction of total mHR6A+mHR6B protein, in spermatids.
Screening of men with unexplained infertility for *hHR6B* mutations at our Andrology Clinic,thus far yielded no abnormalities (Roest *et al.* unpublished results).
It is well known that about 15% of the basic nuclear protein in human sperm are histones (Tanphaichitr *et al.*, 1978), possibly indicating an inherent imperfection of histone-to-protamine transition during human spermatogenesis. We have tried to identify human testis-specific histone 2B, using a monoclonal antibody raised against tyrosine hydroxylase, which was previously shown to crossreact with rat TH2B (Unni *et al.*, 1995). Subsequently, TH2B content in the spermatozoa of a number of infertile men was estimated, to elucidate if indeed the amount of residual histone in spermatozoa might represent a measure of some form of spermatogenic derailment (Chapter 7).

Non-genetic causes of male infertility

Endocrine disruption/stress

The hypothalamus-pituitary-testis axis is of paramount importance for the development, initiation and maintenance of spermatogenesis (see Chapter 2). The hormones that are secreted by the fetal testis (anti-Müllerian hormone and testosterone) regulate the development of the male excretory ducts, the involution of the female duct system, and are probably important factors in testis descent. Furthermore, testosterone and its reduced metabolite dihydrotestosterone are essential for the differentiation of the male external genitalia and other sex characteristics (Hunter, 1995). Any external hormonal

influence that disrupts this homeostasis will inevitably affect the male reproductive system. Over the last number of years, a great deal of concern has been raised with respect to the possible decline of human sperm counts and dysregulation of reproductive organ development and function (Carlsen *et al.*, 1992; Auger *et al.*, 1995). Although the debate whether the analysis is accurate is far from over, in particular for sperm counts, theories have emerged that may explain the possible dysregulation of sex differentiation and reproductive function (Sharpe and Skakkebaek, 1993). Of primary importance in these theories is the almost ubiquitous presence of estrogenic compounds in the environment. Whether the source is in plants (phytoestrogens), in dairy milk, or from estrogenic chemicals, estrogenic compounds may have a profound effect on the male fetus. Exogenous estrogenic compounds can be more active than endogenous estrogens, due to low metabolism and low protein binding. The fetal pituitary is highly sensitive to oestrogen, and stimulation will cause a decline in LH and FSH secretion. The decline in FSH may exert a negative effect on Sertoli cell development and the secretion of anti-Müllerian hormone, while the lower level of LH will result in lower testosterone secretion (Sharpe and Skakkebaek, 1993). Both these influences may cause impaired development of the testes and accessory sex organs. A lower fetal testosterone level may also be of importance in the pathogenesis of hypospadia. Indeed, male children who were exposed *in utero* to the synthetic oestrogen diethylstilbestrol (DES) appeared to have a higher incidence of cryptorchidism and hypospadias, and also decreased semen volume and sperm counts (Stillman, 1982), although this is not supported by all publications (Wilcox *et al.*, 1995). In addition, a higher maternal oestrogen level appeared to be linked to the incidence of cryptorchidism (Depue, 1984).

In adult life, men may come into contact with a number of chemicals that have a hormonal or anti-hormonal action, such as *p,p'*-DDE, a metabolite of DDT, which is a potent androgen receptor antagonist (Kelce *et al.*, 1995). The most frequent source of exogenous hormone action in young adult males, however, is the use of anabolic steroids to enhance physical appearance and performance. Anabolic steroids are potent inhibitors of pituitary LH and FSH secretion, resulting in a dramatic fall in testicular testosterone production and inhibition of Sertoli cell function. Spermatogenesis is, as a result, negatively affected (Lloyd *et al.*, 1996). Cessation of the use of anabolic steroids will result in spontaneous restoration of fertility (Lloyd *et al.*, 1996; Gazvani *et al.*, 1997).

Stress as a result of overtraining in men suppresses the hypothalamus-pituitary-gonadal axis, causing a drop in serum testosterone level, and a decrease in sperm concentration, together with an increment of serum cortisol and prolactin (Dohle *et al.*, 1998; Weber, 1998). In addition to physical stress, psychological stress as a result of psychiatric disorders, death sentence or combat stress have also been shown to be detrimental to male reproductive function (Amelar *et al.*, 1977; Moghissi and Wallach, 1983). It has been shown that during exposure to stressful conditions, an overactivity of the hypothalamic pituitary adrenal axis results in an increase in corticotropin-releasing factor (CRF), pro-opiomelanocortin (POMC), adrenocorticotropin (ACTH) and β-endorphin. Stress-induced hypogonadism may result from suppression of gonadotropin-releasing hormone (GnRH) by increased release of POMC, which in turn may result from a higher CRF level (Rivier and Rivest, 1991). The role of prolactin in stress-induced hypogonadism is unclear, although prolactin has been shown to be a potent upregulator of CRF secretion (Weber and Calogero, 1991; Calogero *et al.*, 1993).

Non-hormonal toxins

As our knowledge of male reproductive dysfunction broadens, so does the list of chemicals that are known to be detrimental to male reproductive health (Chatterjee and Ralph, 1998). Environmental agents include a long list of pesticides, herbicides, rodenticides, fungicides, fumigants and sterilants. Food contaminants, industrial chemicals, and other factors including tobacco smoke, alcohol, and radiation, have been implicated. Furthermore, a large number of antineoplastic agents are also highly toxic to the male germinal epithelium. A number of drugs that primarily act on the nervous system such as anaesthetic gases, anti Parkinson drugs, appetite suppressants, opioids, neuroleptics, tranquillizers and anti-adrenergic drugs have been shown to cause damage to male reproductive capacity. Other drugs, including antibiotics, diuretics, and gout-suppressants, are also included in the list. It is not our intention to provide a comprehensive overview of all male gonadotoxic agents in this thesis; the reader is referred to reviews on the subject (Schrader, 1992; Brinkworth and Handelsman, 1997; Chatterjee and Ralph, 1998). Whether the use certain medications or exposure to gonadotoxic chemicals actually contributes significantly to the male infertility case-load, is presently not certain.

Trauma to the testis and infection

Disruption of the anatomic and histological integrity of the male reproductive system, is a situation that is often encountered in the andrology clinic. Torsion of the testis results in an acute hypoxic state of the testis, that will cause irreversible damage to the seminiferous epithelium within hours of onset, if not immediately surgically corrected (Turner and Brown, 1993; Carroll *et al.*, 1997; Rajfer, 1998). Invasion of micro-organisms into the testis not only causes an acute inflammatory reaction which by itself is detrimental to spermatogenesis, but may eventually result in disruption of the integrity of the testicular structure. In particular, disruption of the Sertoli cell barrier within the seminiferous tubuli (as caused by trauma or inflammation due to the mumps), but also damage of the excretory ducts (as in vasectomy and vasectomy reversal), by any traumatic cause, will bring the immune system of the male in contact with germ cell antigens. Many antigens of meiotic and post-meiotic germ cells are not found on any somatic cell type or on pre-meiotic germ cells. Because these antigens are first expressed during puberty, the immune system recognises developing germ cells as foreign. Exposure of germ cell antigens to the immune system may thus cause an immune response, and, subsequently, the production of anti-sperm antibodies. The association of anti-sperm antibodies with the sperm membrane may result in impaired cervical mucus penetration, premature acrosome reaction, impairment of zona binding, or fertilisation failure. (Turek and Lipshultz, 1994; Sigman and Howards, 1998). An inflammatory reaction within any part of the male reproductive tract will also result in local accumulation of a large number of white blood cells. In particular the polymorphic neutrophils generate large amounts of reactive oxygen species (ROS) that are detrimental to the integrity of spermatozoa (Wang *et al.*, 1997). Spermatozoa are very poorly equipped to withstand damage caused by ROS. Sperm membranes contain a high level of polyunsaturated fatty acids which are extremely susceptible to peroxidation, and spermatozoa have a very limited capacity to repair damage caused by ROS (Sharma and Agarwal, 1996). Although in healthy males the luminal fluid in the epididymis serves to protect the spermatozoa against ROS damage, inflammation of the male accessory sex organs, and infiltration of the seminal fluids with leukocytes will cause extensive damage to the spermatozoa and to their DNA content, by ROS action.

Heat

The scrotal position of the testis, as opposed to an intra-abdominal position, results in a testicular (and epididymal) temperature that is about two centigrade below body temperature (Mieusset and Bujan, 1995). The testis is extremely temperature sensitive, and a slight elevation of testis temperature will result in massive derailment of spermatogenesis. In addition to the testis, the epididymis also apparently is dependent on slightly lower-than-body temperatures for adequate function (Bedford, 1978; Bedford and Yanagimachi, 1991). Any condition that causes an increase in testis temperature will result in a deterioration of spermatogenesis and male fertility, as is elegantly illustrated by experiments requiring men to wear special retainers which held their testes in an inguinal position for varying amounts of time (Mieusset and Bujan, 1994). In addition, it has been shown that tight fitting underwear can negatively affect sperm counts. Clearly, scrotal temperature is one of the confounding factors in the discussion on declining semen parameters during the last 50 years.

The incidence of varicocele is relatively high in the male subfertile population, and may be an important (co)factor in causing male subfertility (Aafjes and van der Vijver, 1985; Marks *et al.*, 1986). Although other theories exist as to the influence the presence of a varicocele may have on testis function, it has been shown that varicocele results in a higher scrotal temperature, particularly in standing position when the cooling effect of the scrotum is greatest (Lerchl *et al.*, 1993; Hargreave, 1997). Men with a varicocele may have progressively deteriorating testis function, that may only be partially restorable upon ligation of the spermatic vein (Hargreave, 1997). Although it has now been shown that spermatic vein ligation will result in improved semen parameters (Pierik *et al.*, 1998), it remains to be elucidated whether this procedure will actually improve male fertility.

A non-scrotal position of the testis, as in cryptorchidism or retentio-testis, will certainly result in a higher testis temperature and poor spermatogenesis. Men with bilateral cryptorchidism who have undergone bilateral orchidopexy in early childhood have a high incidence of infertility, indicating that high testis temperature in childhood affects later fertility, and/or that an inherent defect in the fetal testis caused both maldescent and a spermatogenic defect, through unrelated mechanisms (Rajfer, 1998).

Fever as a result of any ailment has been shown to have a rapid and devastating effect on spermatogenesis, to the point of azoospermia (Mac-

Leod, 1951; Burch and Havlovec, 1991). Because the effect of fever on sperm counts can be observed only days after its onset, it is highly likely that fever is equally detrimental to the epididymis as it is to the testis.

In the following chapters, two facets of human infertility are studied. First, to ascertain the involvement of deregulated AR expression in male infertility, the immunoexpression of androgen receptor in the testes of subfertile males is explored. Subsequently, the effect of spermatogenic impairment upon sperm nuclear histone composition is studied, using an antibody targeting testis-specific histone 2B in the human testis.

6

Androgen receptor expression in the testes of subfertile men

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Published in: Journal of Andrology 1995; 16: 510-516

Abstract

The localization and intensity of androgen receptor immunostaining was studied in the testes of thirty-seven subfertile men with oligozoospermia and normal serum gonadotropin levels, using a polyclonal antibody raised against a synthetic peptide corresponding to the first 20 N-terminal amino acid residues of the androgen receptor (AR). Furthermore, we investigated whether or not the immunoexpression of the AR in human Sertoli cells, in histologically normal testis tissue, is dependent on the stage of the spermatogenic cycle, as has been found in the rat. In the human testis, AR immunoexpression was observed in Sertoli cells, peritubular myoid cells, Leydig cells, and periarteriolar cells, but not in germinal cells. We found no evidence for a stage dependent immunoexpression of AR in Sertoli cells. The intensity of AR immunoexpression varied substantially between biopsy specimens of different patients. There was, however, no correlation of the intensity of AR immunoexpression in either Sertoli cells or peritubular myoid cells with spermatogenic adequacy as measured by the method of Johnsen. When, in this study, the intensity of peritubular myoid cell staining was used as a standard to evaluate the intensity of Sertoli cell staining, also no correlation was detected. Furthermore, serum gonadotropin levels were not correlated with AR immunoexpression levels in Sertoli cells and peritubular myoid cells. These results indicate that immunodetectability of the AR is not related to the condition of the spermatogenic epithelium in patients with oligozoospermia. Inappropriate expression of the AR is neither a cause, nor a consequence of idiopathic infertility in the present group of patients.

Introduction

Male factor subfertility is a frequent clinical problem (Greenhall and Vessy, 1990). A number of conditions affect spermatogenesis, such as a history of cryptorchidism, an infection, the presence of a varicocele, an abnormal karyotype, pituitary lesions, systemic disorders, and iatrogenic damage to the testes (World Health Organization, 1987). Also, subfertility in the presence of normal spermatogenesis can be found, in case of an obstruction, or an immunological condition, sexual inadequacy, or retrograde ejaculation. There remains, however, a large number of males in whom no cause for their subfertility is apparent (idiopathic infertility). One group of patients with idiopathic male infertility may have defective spermatogenesis caused by inadequate androgen receptor (AR) function; it has been suggested that functional impairment of the AR due to certain mutations might be associated with spermatogenic defects in normally virilized males (Aiman *et al.*, 1979; Aiman and Griffin, 1982; Akin *et al.*, 1991), although this remains a point of discussion (Bouchard *et al.*, 1986). Some forms of insufficient androgen action may become apparent only in the testes.

Recently, well-characterized antisera targeting the AR have become available. Studies in rats have demonstrated that the immunodetectability of the AR in the testis decreases under conditions of low androgenic stimulation (Bremner *et al.*, 1994). We argued that if idiopathic male infertility is associated with an impaired androgenic stimulation of the Sertoli cells, low levels of AR immunoexpression would be observed herein.

Previous studies have specifically located the human testicular AR in the nuclei of Sertoli cells, peritubular myoid cells, Leydig cells, and fibroblasts (Takeda *et al.*, 1990; Ruizeveld de Winter *et al.*, 1991; Iwamura *et al.*, 1994). One report described AR immunoexpression also in germ cells (Kimura *et al.*, 1992). In rat Sertoli cells, AR immunoexpression is dependent on the stage of the spermatogenic cycle (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). However, no report on a possible cyclic pattern of Sertoli cell AR immunoexpression in the human testis is presently available.

This study was designed to retrospectively investigate the intensity of AR immunoexpression in Sertoli cells and peritubular myoid cells in relation to the quality of spermatogenesis, in biopsy specimens of subfertile males. Attention was paid to AR immunoexpression in Sertoli cells in histologically normal testis tissue, to ascertain whether or not AR immunoexpression varies with the stages of the spermatogenic cycle.

Materials and Methods

Biopsy specimens

Thirty-seven recent testicular biopsy specimens were collected in the period 1989-1993, from men who visited our andrology outpatient clinic because of male factor subfertility. All men had an uneventful history, a normal physical exam (including ultrasonography for varicocele), repeated oligozoospermia ($<20x10^6$ sperm/ml), and normal serum FSH, LH, and testosterone levels. Biopsy specimens were scored using the method described by Johnsen (1970) as modified by Aafjes *et al* (1978). Tubular cross sections were given score numbers from 1 to 10 depending on the most advanced stage of spermatogenesis observed. Per tubule, a score of 10 was given for complete and abundant spermatogenesis; 8 for when all stages of spermatogenesis were present, but fewer than 5 condensed spermatids were seen; 7 for no condensed, but many round spermatids present; 6 for no condensed spermatids, and less than five round spermatids present; 5 for no spermatids, but many spermatocytes present; 4 for no spermatids and fewer than five spermatocytes present; 3 for when only spermatogonia were present; 2 for Sertoli cells only; and 1 for no cells in tubular section. The mean score, of 100 tubules, was calculated for each sample. Immunohistochemical analysis of these biopsies was performed in 1994.

Immunohistochemistry

All biopsy specimens were fixed in Bouin's solution, dehydrated in ethanol, cleared in xylene, embedded in paraffin and stored at room temperature. Since, during routine processing, biopsy material is immersed in Bouin's fixative for periods ranging from one to three days, immunohistochemical analyses of AR was performed on a fresh biopsy sample that was divided into three pieces, immersed in Bouin's solution for 24, 48 and 72, hours and subsequently routinely processed to assess if prolonged immersion in Bouin's fluid has a significant effect on AR immunoexpression.

The primary antibody (SP197) is a rabbit polyclonal raised against a synthetic peptide corresponding to the first 20 N-terminal amino acid residues of the human androgen receptor. Its use as a valid immunological probe for human AR has recently been established.

Five µm thick sections were mounted on 3-aminopropyltriethoxisilane (Sigma, St Louis, MO) -treated slides, deparaffinized and rehydrated.

Endogenous peroxidase was blocked by a 20 min incubation in 3% (v/v) H_2O_2 in methanol. An antigen retrieval step was performed by heating the sections, immersed in 0.01M citric acid monohydrate (Merck, Germany) at pH 6.0, for 3 x 5 min in a 700W microwave oven. The material was left to cool down to room temperature, after which the slides were washed in phosphate-buffered saline (PBS) and placed in a Sequenza immunostainer (Shandon, The Netherlands). Non-specific antibody binding was blocked by a 15 min incubation with normal goat serum (Dako, The Netherlands) diluted 1:10 in 5% (w/v) bovine serum albumin (BSA). The primary antibody (SP197), diluted 1:4000 in 5% (w/v) BSA, was applied for overnight incubation at 4C. Sections were rinsed with PBS, and were subsequently incubated with biotinylated goat anti-rabbit antibody (Dako, The Netherlands) diluted 1:400 in PBS, for 30 min, streptavidin-biotin-peroxidase complex (Dako, The Netherlands) for 30 min, and 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Germany) for 7 min, at room temperature. Between incubations, sections were rinsed three times with PBS. Counterstaining was performed with Mayer's hematoxylin (15 sec). All slides were evaluated with a Zeiss Axioskop 20 light microscope. Controls included incubation with no primary antibody (5% (w/v) BSA in PBS) or with pre-immune serum.

Estimation of intensity of immunostaining

AR immunoexpression in Sertoli cells and peritubular myoid cells was evaluated using two methods. First a general score was given for AR immunoexpression in Sertoli cells and peritubular myoid cells in the sample as a whole. A score of 0,1,2, or 3 was assigned for absent, low, medium, or high expression, respectively. Subsequently, maximal intensity of AR immunoexpression in Sertoli cell and peritubular myoid cell nuclei was evaluated semiquantitatively in ten tubules per biopsy. A false impression of AR immunostaining in parts of the testes might be obtained, if its intensity is influenced by the stage of the spermatogenic cyle. As a single cross section of a human tubule reveals 3 or 4 stages of the spermatogenic cycle (Schulze and Rehder, 1984), scoring the maximal intensity of AR expression per tubule might provide a more reliable measure than scoring the mean intensity. Per tubule, expression was scored as 0,1,2, or 3 for: no visible AR expression, faint AR expression, moderate AR expression, and strong AR expression,

respectively. The score per sample was calculated as the sum of the scores of ten tubules for each cell type.
In analyzing our results, we chose two approaches: one was to specifically compare AR immunoexpression from patients with Johnsen scores of 8.0 and higher with those from patients with lower scores. Biopsy scores of 8.0 or higher correlate with a relatively high pregnancy rate, whereas scores below 8.0 correlate with a very poor rate of pregnancy (Aafjes *et al.*, 1978). In addition, we searched for correlations between AR immunoexpression in Sertoli cells and peritubular myoid cells with the respective Johnsen scores and a number of clinically relevant parameters.

Statistics

All statistical calculations were performed using SPSS software (version 6.0 for Windows). The relationship between the quality of spermatogenesis and intensity of AR immunoexpression was evaluated according to Spearman's rank correlation. The significance of differences between groups was determined by Wilcoxon's signed rank test.

Results

Immersion of the biopsy sample in Bouin's solution for a period of 24 to 72 hours did not affect the intensity of AR immunoexpression in the testis. In testis with a biopsy score of 8.0 or higher, AR immunoexpression was specifically found in the nuclei of Sertoli cells, peritubular myoid cells, Leydig cells, and cells associated with arterioles (Figure 1). We found, however, no AR immunostaining in any type of germinal cell. A similar localization of AR was found in tissues with severely impaired or absent spermatogenesis (Figure 2). Some variation in the intensity of the immunostaining of AR in Sertoli cells was observed. This variaton was, however, not associated with the stage of the spermatogenic cycle. In histologically normal testis tissue, all 6 stages, as identified by Clermont (1963), were found, on average, to express the AR in equal abundance (Figure 1). Some random variation in AR immunoexpression was also found in Leydig cells and, to a lesser extent, in peritubular myoid cells.
In our group of patients, thirteen patients (35%) had a Johnsen score below 8.0, and 24 patients (65%) scored an 8.0 or higher. As shown in Table 1, LH,

FSH, and testosterone levels were not significantly different between the two groups. A comparison between the biopsy scores and the intensity of AR immunostaining in Sertoli cells, as estimated by the general and the semiquantitative analysis, revealed no difference (Table 2). The intensity of immunoexpression of AR in peritubular myoid cells is also similar in the two groups, using both scoring methods. No correlation between the intensity of AR immunoexpression in Sertoli cells or peritubular myoid cells and the Johnsen score, and a number of clinically relevant parameters, was found (Table 3). There is clearly a very heterogeneous picture, with AR immunoexpression in Sertoli cells varying widely in biopsies with both high and low Johnsen scores (Figure 3). It is important to note that we found a significant correlation between the intensities of AR immunoexpression in peritubular myoid cells and Sertoli cells. Eventhough all sections were identically processed during a single experiment, a certain amount of variation in accessability of the tissue to antibodies may have occurred. Because our interest involving AR expression in the idiopathic subfertile male involves especially the AR in Sertoli cells, we used the peritubular myoid cell AR immunoexpression as a standard against which Sertoli cell AR immunoexpression was measured. This was done by subtracting the peritubular myoid

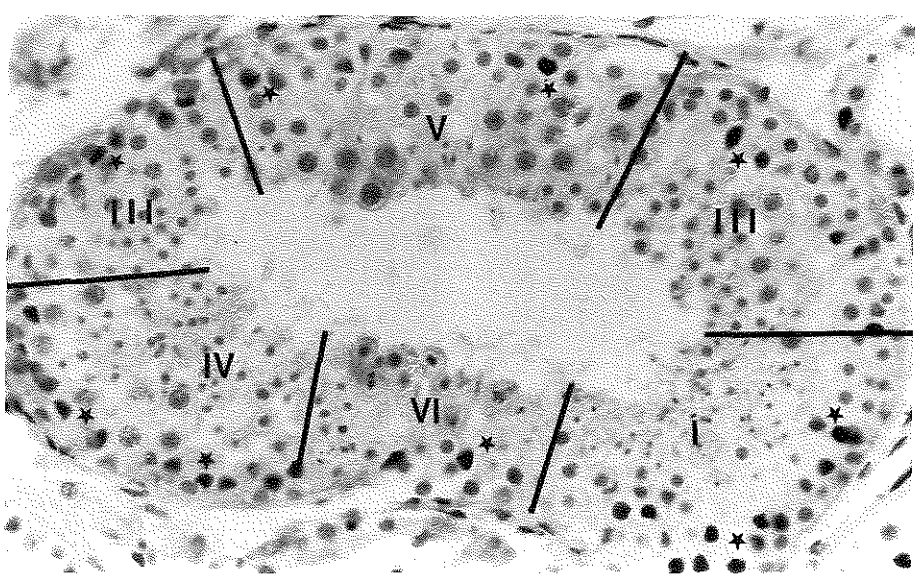

Figure 1. Testis section showing normal spermatogenesis. The stages of the cycle of the seminiferous epithelium are indicated by roman numerals. Sertoli cell nuclei, heavily stained with SP197 (★) were encountered in all epithelial stages. (bar corresponds to 25 μm).

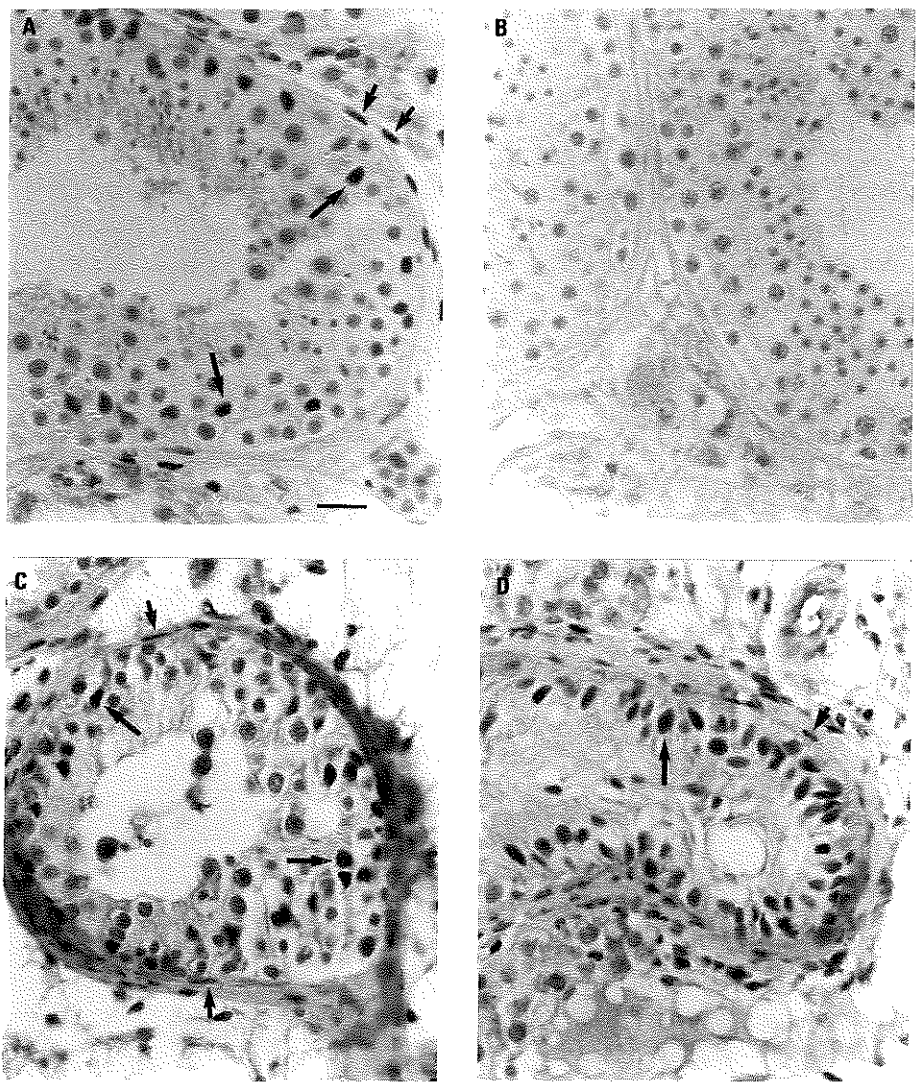

Figure 2. Immunohistochemical staining of the androgen receptor with SP197 of human testicular biopsy material (A,C,E). (B) is a section from the same biopsy sample as (A), but incubated with SP197 pre-adsorbed with an excess of peptide against which the antibody was raised. The figure shows three different biopsies with a Johnsen score of 8.7 (A,B), 3,7 (C), or 2.7 (D). Note similar staining of Sertoli cell nuclei (slender arrows) and peritubular myoid cell nuclei (broad arrows) in all specimens. Bar = 50 μm.

Table 1. Serum FSH, LH and testosterone levels in 37 patients who underwent testicular biopsy (mean±SD).

Johnsen score	<8.0 (n=13)	≥8.0 (n=24)	Significance
FSH	4.8 (±1.4)	4.4 (±1.4)	p=0.50
LH	3.9 (±1.7)	3.0 (±1.2)	p=0.07
Testosterone	18.0 (±6.3)	18.7 (±4.5)	p=.67

Table 2. Comparison of Johnsen score to intensity of androgen receptor immunoexpression in adequete and inadequate spermatogenesis (mean±SD).

Johnsen score	<8.0 (n=13)	≥8.0 (n=24)	Significance
Intensity of AR immunoexpression in:			
General impr:			
Sertoli cells	2.8 (±0.8)	2.8 (±0.8)	p=0.94
Peritubular myoid cells	2.8 (±0.7)	2.9 (±0.8)	p=0.70
Semi Quant:			
Sertoli cells	15.9 (±7.3)	13.3 (±5.6)	p=0.24
Peritubular myuoid cells	15.2 (±4.9)	15.3 (±3.8)	p=0.95

cell score from the Sertoli cell score, and correlating the results with the respective Johnsen scores. We again found no correlation between these values (r=-0.24; p=0.16).

Discussion

Our results indicate that, in a population of patients with oligozoospermia and normal serum gonadotropin levels, inappropriate AR expression in Sertoli cells does not seem to be an important causative factor for, or accompanying phenomenon of, male infertility. The variation in immunoexpression of AR in Sertoli cells is large, in both the histologically normal group (where subfertility is probably caused by an obstructive disorder) and in the hypospermatogenic group. In accordance with a previous report (Janssen *et al.*, 1994), the storage time of the sample did not influence the intensity of AR immunoexpression. Neither was there a change in AR immunoexpression with a number of clinically relevant parameters, including the Johnsen score. The wide variation in the intensity of AR immunoexpression might be the

Table 3. Correlations of intensity of Sertoli cell and peritubular myoid cell AR immunoexpression with a number of clinical parameters (correlation coefficient; significance).

	General impression	
	Sertoli cells	Peritubular myoid cells
Year of biopsy	–0.1029; p=0.54	–0.1901; p=0.25
Patient age	–0.0966; p=0.65	–0.0577; p=0.73
Serum FSH	0.2386; p=0.15	0.2366; p=0.15
Serum LH	–0.0227; p=0.89	–0.0551; p=0.74
Serum testosterone	0.1021; p=0.54	–0.0436; p=0.70
Johsen score	–0.0717; p=0.67	–0.0888; p=0.60
	Semiquantitative method	
n=37	Sertoli cells	peritubular myoid cells
Year of biopsy	–0.1070; p=0.52	–0.1901; p=0.70
Patient age	–0.2702; p=0.10	–0.0577; p=0.68
Serum FSH	0.1936; p=0.24	0.2366; p=0.19
Serum LH	0.2865; p=0.07	0.0551; p=0.11
Serum testosterone	0.2108; p=0.20	–0.0436; p=0.44
Johsen score	–0.2248; p=0.18	–0.0888; p=0.92

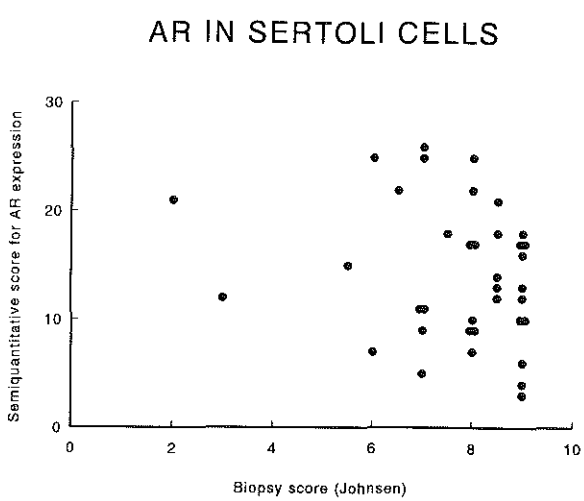

Figure 3. Correlation between biopsy score and Sertoli cell AR immunoexpression in 37 subfertile males. The data were analysed by the semiquantitative method (see Materials and Methods). Correlation coefficient: –0.2248; p=0.18.

result of a difference in accessability of the tissue to antibody. On the other hand, this variation could be real, and might reflect a natural variation in AR immunoexpression in different men, not associated with fertility or hormonal status. In this respect, our results are in agreement with the findings of Wilson *et al.* (1992), who found large variations in androgen receptor expression in cultured foreskin fibroblasts of normal men. Local variations in AR immunoexpression might be caused by damage to the cell nuclei during slicing of the sample on the microtome.

This study was done in a well selected group of patients, with oligozoospermia and serum gonadotropin levels within the normal range. Idiopathic male infertility also encompasses a large number of patients with elevated serum FSH levels (World Health Organization, 1987). Thus, our results should not be applied indiscriminantly upon other groups of patients. As idiopathic male infertility is probably a heterogeneous collection of hypospermatogenic disorders, the possiblility that inappropriate AR expression is indeed a causative factor in some cases of male infertility, cannot be excluded.

Androgen receptor expression in Sertoli cells might be under the influence of a complicated regulatory mechanism in which hormones such as FSH may possibly play an important role (Verhoeven and Cailleau, 1988b; Blok *et al.*, 1989b; Blok *et al.*, 1992b). The role of FSH in human spermatogenesis is, apart from the fact that it is essential (Sharpe, 1994), not well understood. In the premature rat FSH plays a key role, with testosterone, in the initiation of spermatogenesis (Gondos and Berndtson, 1993). *In vitro* and *in vivo*, evidence points to a possible role for FSH as a stimulator of the expression of androgen receptor mRNA and protein in prepubertal Sertoli cells (Verhoeven and Cailleau, 1988b; Blok *et al.*, 1989b; Blok *et al.*, 1992b). Although the importance of FSH seems to diminish in the adult rat (Sharpe, 1994), a role in maintaining spermatogenesis cannot be excluded, in particular in primates including the human. In the adult non-human primate *Macaca fascicularis*, FSH still stimulates spermatogonial proliferation (van Alphen *et al.*, 1988).

We confirm the absence of AR immmunoexpression in human germinal cells at any stage of their development as reported by a number of authors (Takeda *et al.*, 1990; Ruizeveld de Winter *et al.*, 1991; Iwamura *et al.*, 1994), and thus do not support the findings of Kiwamura *et al.* (1992).

In the rat, the question of whether there is AR immunoexpression in germinal cells is also, as yet, unresolved (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). Using the immunohistochemical protocol described above, we have obtained

results similar to those reported by Bremner *et al.* (1994) in the rat testis (results not shown), i.e. no AR immunostaining in germinal cells. Vornberger *et al.* (1994) found AR immunostaining in the cytoplasm of step IX elongating spermatids. The immunohistochemistry protocol used in that study, however, differs considerably from that employed by Bremner *et al.* (1994) and ourselves. In addition, although the antibody used by Vornberger *et al.* (1994) also targets the N-terminus of the AR, there might be a difference in epitope specificity.

In the rat, immunohistochemical data have clearly revealed that AR expression in Sertoli cell nuclei is dependent on the stage of the spermatogenic cycle, while expression of AR in the nuclei of Leydig cells and peritubular myoid cells shows no such pattern (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). There have been several publications reporting AR immunoexpression in the human testis (Takeda *et al.*, 1990; Ruizeveld de Winter *et al.*, 1991; Kimura *et al.*, 1992; Iwamura *et al.*, 1994), but none of these sought to identify specific variations of AR immunoexpression in the 6 stages of the human spermatogenic cycle (Clermont, 1963). Our results clearly show that a stage dependent variation in human Sertoli cell AR immunoexpression was not detectable in the histologically normal testis. The mechanism involved in the stage dependent immunoexpression of the androgen receptor in rat Sertoli cells is not clear. Expression appears to be highest at stages thought to be especially dependent on testosterone (Bremner *et al.*, 1994; Sharpe, 1994). AR function might be modulated through the direct interaction of Sertoli cells with specific germinal cell types. It has been shown, however, that pachytene spermatocytes, and round and elongating spermatids are not instrumental in this phenomenon, demonstrating that the presence of earlier germinal cell types might be sufficient to induce a cyclic pattern of AR immunostaining (Bremner *et al.*, 1994). Why such a phenomenon is not demonstrable in man is not known, though a possible clue may be found in the different architecture of the spermatogenic epithelium. In the rat, the stages of the spermatogenic cycle are arranged sequentially along the length of the tubule, such that a cross section reveals a single stage of development (Leblond and Clermont, 1952). In man, the arrangement may be either in a helical fashion, revealing three or four distinct stages in a single cross section (Schulze and Rehder, 1984) or not exist at all (Johnson, 1994). In this respect, a human Sertoli cell might be exposed to a more heterogeneous environment, obscuring possible stage specific modulatory effects on AR

immunoexpression. Also, the subdivision of the human spermatogenic cycle in only 6 stages (Clermont, 1963) compared to 14 in the rat (Leblond and Clermont, 1952), may be too imprecise to allow detection of certain testosterone-dependent developmental steps.

In conclusion, in men with oligozoospermia and normal gonadotropin levels, the quality of spermatogenesis was not related to the intensity of AR immunoexpression in Sertoli cells or peritubular myoid cells. Moreover, in men with normal spermatogenesis, expression of the AR in Sertoli cells is not dependent on the stages of the spermatogenic cycle.

7

Immunoexpression of testis-specific histone 2B (TH2B) in human spermatozoa and testis tissue

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Published in: Human Reproduction 1998; 13:1559-1566

Abstract

During mammalian spermatogenesis, the chromatin of the spermatogenic cells is profoundly reorganized. Somatic histones are partly replaced by testis-specific histones. These histones are then replaced by transition proteins and finally by protamines. This series of nucleoprotein rearrangements results in a highly condensed sperm cell nucleus. In contrast to spermatozoa from other species, human spermatozoa still contain a significant amount of histones, including testis-specific histone 2B (TH2B). In the present study it is shown that an antibody targeting tyrosine hydroxylase, previously found to cross-react with rat TH2B, also specifically immunoreacts with human TH2B on Western blot, in immunohistochemistry of human testis tissue, and in immunocytochemistry of decondensed human spermatozoa. In human testis tissue, TH2B immunostaining is first apparent in spermatogonia, shows marked variation especially at the pachytene spermatocyte stage, and then reaches an intense signal in round spermatids. Shortly before spermatid elongation, a portion of the spermatid nucleus, corresponding to the acrosomal region, loses its immunoreactivity. During condensation of the spermatid nucleus, the immunodetectability of TH2B disappears gradually, from the anterior region of the nucleus onwards. At the final stages of spermiogenesis, the immunostaining is completely absent. Immunocytochemical staining of spermatozoa revealed no TH2B immunosignal, but immunostaining was observed when spermatozoa obtained from semen were decondensed to make nuclear proteins accessible to the antibody. There was, however, a striking intercellular variability in the intensity of staining of spermatozoa within an ejaculate. In a population of 35 men attending our Andrology Clinic, we observed inter-individual differences in total sperm-TH2B content, which showed a significant, although not very pronounced, negative correlation with normal morphology ($p=0.05$).

Introduction

Spermatogenesis is a complex process, by which diploid stem cells divide, differentiate and mature into haploid, highly specialized spermatozoa. Among the many changes, one of the intriguing developmental characteristics of spermatogenic cells is the replacement of somatic histones, first partly by testis-specific histones, then by transition proteins which are finally replaced by protamines (Meistrich *et al.*, 1978; Balhorn, 1982; Meistrich and Brock, 1987; Balhorn, 1989). This results in a compact nucleoprotamine complex that is highly distinct from the somatic nucleohistone complex, with inter- and intra-protamine disulfide bridges resulting in a highly compact and stable supramolecular structure that fits into a small sperm nucleus and also may render the chromatin relatively resistent to harmful external influences (Marushige and Marushige, 1975; Hunter *et al.*, 1976; Ward, 1993). In rat and mouse spermatogenesis, the histones are completely replaced by protamines, so that mature spermatozoa are devoid of histones (Grimes, 1986). In human spermatozoa, however, about 15% of the basic nuclear proteins are histones (Tanphaichitr *et al.*, 1978).

It is as yet unclear whether the remaining histones in human spermatozoa have a specific role. It has been suggested that the sperm DNA that is complexed as nucleohistone may have a role in the designation of initiation sites for nuclear decondensation, or that the nucleohistone complement may contain genes poised for early expression after fertilization (Gatewood *et al.*, 1987). In contrast to this possibly beneficial effect of sperm nuclear histones, a number of studies have presented evidence that spermatozoa of infertile males contain more histones than that of fertile males, indicating that insufficient removal of histones might be involved in infertility (Chevaillier *et al.*, 1987; Hofmann and Hilscher, 1991; Foresta *et al.*, 1992). Among the histones present in human spermatozoa, TH2B is a major variant (Tanphaichitr *et al.*, 1978).

Recently, an antibody targeting tyrosine hydroxylase (TH) was found to cross-react with rat testis specific histone 2B (TH2B) (Unni *et al.*, 1995). Since the primary structure of TH2B is highly homologous between man and rat (Wattanaseree and Svasti, 1983), we evaluated whether the anti-TH antibody would also specifically bind to human TH2B. After having established that this indeed is the case, we characterized the immunoexpression pattern of TH2B in human testis tissue and in human spermatozoa, and investigated the TH2B content of spermatozoa in a number of males visiting our infertility clinic.

Materials and Methods

Preparation of acid extractable proteins

Testes and epididymides from adult Wistar rats were used. After homogenisation of rat testis tissue, the homogenate was centrifuged at 10,000*g* for 10 min. The pellet was stored at -25C. Rat spermatozoa were obtained from caput and cauda epididymis. Epididymal tissue was minced and shaken in phosphate-buffered saline (PBS) to permit dispersal of luminal contents. After sedimentation of the tissue pieces, the supernatant was layered over a discontinuous Percoll gradient consisting of 10%, 40% and 65% Percoll (v/v in PBS). The gradient was spun at 500*g* for 45 min. Spermatozoa were recovered at the interphase between the 40% and 65% Percoll.

Human semen was layered on top of 40% Percoll and centrifuged at 500*g* for 20 min. Spermatozoa were collected from the bottom of the tube, washed and stored at -25C.

Human peripheral blood mononuclear cells were isolated from heparinized blood of a healthy adult volunteer. A leukocyte-enriched cell suspension was obtained by lysis and removal of red blood cells. Ten ml of whole blood was added to 30 ml lysis buffer (containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM NA_2EDTA, at pH 7.4) at 4C, and was shaken vigorously. The mixture was left on ice for 15 min during which it was periodically shaken. The non-lysed cells were pelletted by centrifugation at 4000 r.p.m. at 4C. The supernatant was removed and the pellet was resuspended in 10 ml lysis buffer at 4C. After vigorous shaking, the remaining cells were pelleted by centrifugation at 4000 r.p.m. and the supernatant was removed. The pellet was frozen in liquid nitrogen for storage until use.

The basic nuclear proteins of spermatozoa and white blood cells were extracted as described previously (Platz *et al.*, 1977). In short, the sample was suspended in 1 ml of cold distilled water. Subsequently 0.33 ml of 1 N HCl was added, followed by 1 ml of 0.25 N HCl. The sample was left to stand on ice for 20 minutes during which the mixture was frequently shaken. The suspension was centrifuged at 12,000*g* for 10 min. The spernatant was removed and proteins were precipitated by adding an equal volume of trichloroacetic acid (TCA). The precipitate was recovered by centrifugation (12,000*g* for 10 min) and washed with acidified acetone (200 ml of acetone + 0.1 ml of 12 N HCl). After subsequent washing in acetone, the sample was dried under vacuum.

Preparation of human sperm proteins

Semen samples were obtained from patients attending the Andrology outpatient clinic of the University Hospital Dijkzigt, Rotterdam. Thirty five consecutive semen samples, with more than $1x10^6$ sperm/ml and a volume of at least 1 ml, were included. All semen samples were first routinely analyzed using the WHO methodology (WHO, 1992). Subsequently, 1 to 2 ml of each sample was layered on top of 2 ml 60% Percoll (v/v in PBS) and centrifuged at 500*g* for 20 min. The pellet was washed twice in 1 ml PBS. The final pellet was resuspended in 1 ml PBS and the sperm concentration was determined in an improved Neubauer counting chamber. A portion containing $0.5x10^6$ spermatozoa was centrifuged in a microfuge (10,000*g*) for 10 min and the pellet was stored at -80C until analysis.
Since the basic nuclear proteins could be efficiently extracted by the Laemmli buffer without previous acid extraction (data not shown), the acid extraction was omitted. The sperm pellets were suspended in 25 ml double strength Laemmli electrophoresis lysis buffer (Laemmli, 1970), containing 0.125 M Tris-HCL pH 6.8, 2% SDS (w/v), 20% glycerol (v/v), and 130 mM dithiothreitol (DTT). From one semen sample with a high sperm concentration ($170x10^6$ sperm/ml), pellets of $0.5x10^6$ Percoll purified spermatozoa were stored, and used as a standard by running one sample per gel alongside the other semen samples.
Each sample was boiled for 3 min in the sample buffer, and was run on SDS-PAGE (Laemmli, 1970) using 15% polyacrylamide gels. The separated proteins were transferred to a nitrocellulose membrane and immunostained as described below. The intensity of the signal was quantified with a Hewlett Packard ScanJet IIcx and analyzed using DeskScan II version 2.0 for Windows software.

Sperm decondensation

Semen samples used for immunocytochemistry were also obtained from the Andrology outpatient clinic. After routine analysis, spermatozoa present in 1 ml of semen were washed twice in 4 ml Ham's medium after which the remaining pellet was resuspended in PBS to obtain a concentration of $50-100x10^6$ spermatozoa per ml. Smears of spermatozoa were fixed in methanol for 5 min and subsequently incubated for 20 min in TRIS-HCl pH 7.2, containing 0.3 mM DTT and 0.1% SDS. The smears were washed in TRIS-HCl and fixed again with methanol for 5 min.

Testis biopsy samples
Samples were obtained from normogonadotropic patients undergoing testis biopsy for evaluation of azoospermia. The tissue was immediately immersed in Bouin's fixative and left for 24 hours, after which the samples were thoroughly washed in 70% ethanol. The sections were dehydrated in ethanol, cleared in xylene and embedded in paraffin, and stored at room temperature.

Electrophoresis and Western blot
The anti-TH antibody (mouse monoclonal anti-tyrosine hydroxylase IgG; Boehringer Mannheim GmbH, Germany) is known to specifically cross-react with rat TH2B, because of sequence homology at the N-termini of TH and rat TH2B (Unni *et al.*, 1995). In order to assess whether anti-TH antibody could recognize human TH2B, we performed two types of gel electrophoreses. First, basic nuclear proteins of human leucocytes, human sperm, rat testis, and rat caput and cauda epididymal sperm were extracted by boiling for 3 min in double strength Laemmli buffer. The samples were run on a 15% polyacrylamide sodium dodecyl sulphate (SDS) gel (Laemmli, 1970). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Non-specific antibody binding was blocked by incubating the membrane in 5% (w/v) BSA in PBS-Tween 0.1% (v/v) for 1 hour. After washing 4x in PBS-Tween, the membrane was incubated with the primary antibody (anti-TH) diluted 1:1600 in 5% BSA (w/v) in PBS-Tween 0.1% (v/v) for 1 hour. The membrane was then again washed 4x in PBS-Tween and subsequently incubated with biotinylated goat anti-mouse antibodies (Dako, Glostrup, Denmark) diluted 1:2000 in 5% BSA (w/v) in PBS-Tween 0.1% (v/v) for 1 hour. The membrane was again washed 4x in PBS-Tween and incubated for 1 min with chemiluminescent reagent (Pierce, Rockford, IL, USA). Excess reagent was removed with blotting paper. The specifically bound antibody was visualized using Hyperfilm^{TM} ß-max paper (Amersham, CEA AB, Sweden). The above samples were also run on a 15% polyacrylamide-acid-urea-Triton (AUT) gel to provide further evidence that the observed immunoreactive band is indeed a histone (Wattanaseree and Svasti, 1983). Samples were dissolved in 0.4% (v/v) Triton X-100, 1% (v/v) mercapto-ethanol and 0.9 M acetic acid. The AUT gels contained 0.4% (v/v) Triton X-100, 1.5 M urea and 0.9 M acetic acid, while the electrode buffer contained 0.4% (v/v) Triton X-100, 0.1% (v/v) mercaptoethanol and 0.9 M acetic acid. Gels were prerun for 4 hours at 130 Volt until a consistent current was

obtained, and run for 30 hours. The separated proteins were transferred on a nitrocellulose membrane in 50 mM acetic acid. Immunodetection of TH2B was performed as described above.

Immunohistochemistry and immunocytochemistry

Immunolocalization of TH2B was performed using the mouse anti-TH antibody. Five micron thick sections were mounted on slides treated with 3-aminopropyltrietoxysilane (Sigma Chemical Company, St Louis, Missouri), deparaffinized and rehydrated. Endogenous peroxidase was blocked by a 20 min incubation in 3% (v/v) H_2O_2 in methanol. Non-specific antibody binding was blocked with normal goat serum (Dako, Glostrup, Denmark), diluted 1:10 in 5% (w/v) BSA in PBS (pH 7.4). The tissues were then placed in a Sequenza immunostainer (Shandon Scientific Ltd., Runcorn, England) and incubated at 4C overnight with the primary antibody (anti-TH), diluted 1:100 in 5% (w/v) BSA in PBS. Immunostaining was performed using biotinylated goat anti-mouse immunoglobulin (Dako) for 30 min, streptavidin-peroxidase (Dako) for 30 min, and metal enhanced diamino-benzidine (Pierce) for 7 min. The sections were counterstained for 15 sec with Mayer's hematoxylin, and viewed with a Zeiss axioscop 20 light microscope at magnifications of 100x and 400x. Control sections were incubated with 5% BSA (w/v) in PBS without the primary antibody and subsequently processed as described above.

Immunocytochemistry was performed as described above, excluding the deparaffinization step.

Statistics

All statistical calculations were performed using SPSS software (version 6.0 for Windows). The relationship between semen parameters and sperm histone content was calculated according to Spearman's rank correlation.

Results

Identification of TH2B in human spermatozoa

In order to elucidate if anti-TH could be used to identify human TH2B, acid extractable proteins from human spermatozoa, human white blood cells, rat testis tissue and rat epididymal sperm were separated by SDS-PAGE, blotted and immunostained with anti-TH. As can be seen in Figure 1a, an immunoreactive protein of 17 kDa was present in human spermatozoa (lane B) but not in human leukocytes (lane A). The other signal in the lane corresponding to the human leukocytes, is non-specific (see figure legend). In Figure 1b, it is apparent that the anti-TH antibody reacts specifically with a 17 kDa protein that is present in rat testis tissue (lane A) but that is not found in rat caput and cauda epididymal spermatozoa (lanes B and C). Lane D

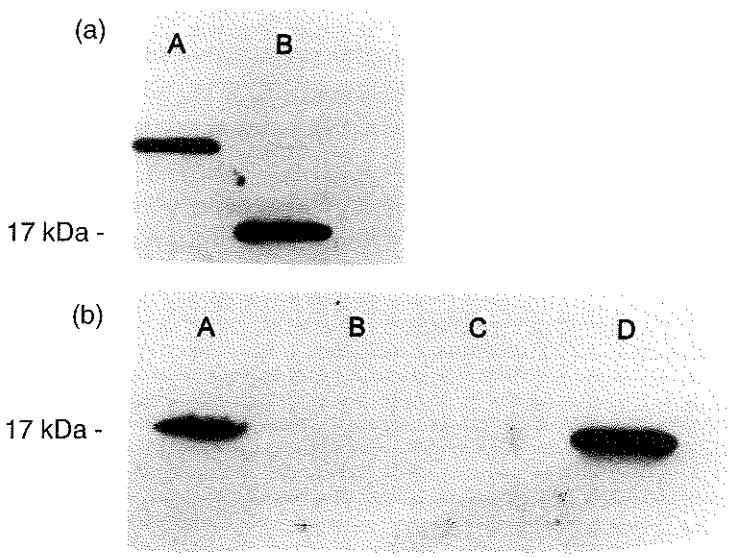

Figure. 1. Immunodetection of proteins, using an antibody targeting tyrosine hydroxylase (TH) on SDS-PAGE and Western blot. (**a**) shows a clear 17-kDa band in lane (B) corresponding to human spermatozoa. This band is not present in lane (A) corresponding to human leukocytes, known to contain somatic type H2B but not TH2B. In lane (A) a non-specific band appears, that is also seen in negative controls where no anti-TH is added, indicating non-specific binding of the second antibody (biotinylated goat anti-mouse Immunoglobulin-G) to a human leukocyte protein (not shown). (**b**) shows a clear 17-kDa band in lanes (A) and (D) corresponding to rat testis tissue and human spermatozoa, respectively. Lanes (B) and (C), corresponding to rat caput and caudal epididymal spermatozoa, respectively, do not show a detectable immunoreactive signal.

corresponds to human spermatozoa, which produce a signal of identical electrophoretic mobility as that of rat testis tissue. Acid extractable proteins of rat testis, human spermatozoa and human leukocytes were further analysed by acid-urea-Triton (AUT) electrophoresis followed by immunoblotting. It appeared that the anti-TH antibody recognized a protein band co-migrating with a H2B marker (standard samples of H2A and H2B were obtained from Boehringer Mannheim) in the extracts from human spermatozoa and rat testis, while no leukocyte protein was recognized (Figure 2). The TH-reactive band in rat testis extract migrated slighly slower than the TH-reactive band in human sperm extract.

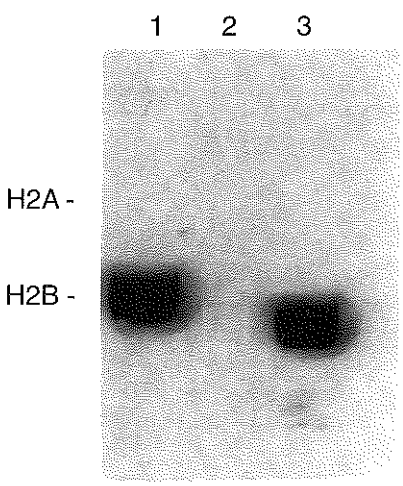

Figure 2. Detection of proteins that immunoreact with antibodies targeting tyrosine hydroxylase (TH), after acid-urea gel electrophoresis and Western blotting. The standard calf thymus H2A and H2B were obtained from Boehringer Mannheim. Aliquots of 3 µg of standard H2A and H2B were loaded on the polyacrylamide acid-urea-Triton gels and run alongside the samples containing human spermatozoa, human leuko-cytes and rat testis tissue. A clear band, represen-ting TH2B is seen in lanes 1 and 3 corresponding to rat testis tissue and human spermatozoa respectively, but is absent in lane 2 corresponding to human leukocytes. Both immunoreactive bands co-migrate with the standard H2B, although the immunoreactive rat testis protein migrates slightly slower that the immunoreactive human sperm protein, indicating that the observed protein is indeed a histone of the 2B type.

Immunohistochemical localization of TH2B in the human testis

Immunohistochemical studies using anti-TH antibodies on human testis tissue displaying normal spermatogenesis, demonstrated strong immunoreactivity in the nuclei of spermatogenic cells (Figure 3). The nuclei of the somatic cells of the testis (Sertoli cells, peritubular myoid cells, interstitial cells, endothelial cells) remained invariably negative. A clear signal was apparent in the spermatogonia lining the basement membrane of the seminiferous tubules. Primary spermatocytes, especially the pachytene spermatocytes, showed a marked variation in nuclear immunodetectability of

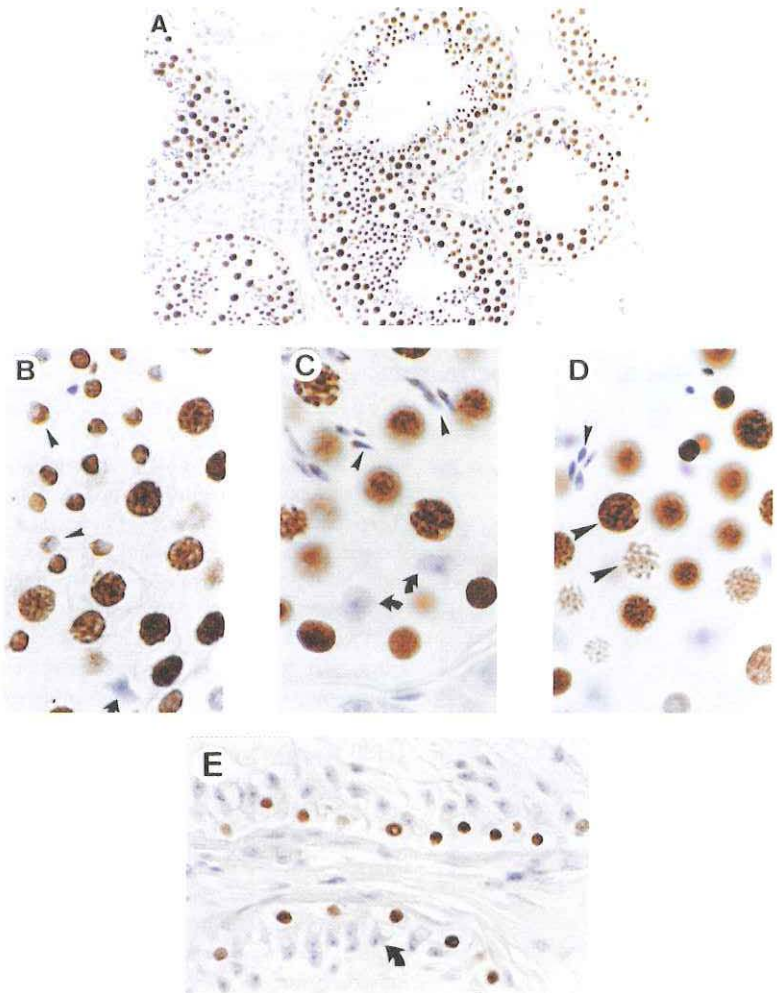

Figure 3. Immunohistochemical localization of TH2B in human testis tissue, stained with anti-TH antibody. The histological sections were counterstained with Mayer's hematoxylin. Immunostaining is localized in the nuclei of spermatogonia, primary and secondary spermatocytes, and round and elongating spermatids. Sertoli cells (curved arrows in panels C and E), peritubular myoid cells, interstitial cells, and cells associated with arterioles were not stained. Panel B, C, and D show human testis tissue at a higher magnification. Note the variation in pachytene spermatocyte immunostaining, especially in panel D (large arrowheads). There is also a change in TH2B immunostaining in elongating spermatids; panel B shows a decrease in TH2B immunostaining at the acrosomal region of the round spermatid nucleus (small arrowheads), and panel C shows a small region of immunodetectable TH2B at the posterior region of the spermatid nucleus (small arrowheads), whereas in panel D no immunodetectable TH2B is seen in the elongated spermatid nuclei (small arrowhead). Panel E shows a detail of a testis biopsy sample from an infertile patient showing only Sertoli cells (curved arrow) and spermatogonia in the tubular lumen; note the presence of TH2B immunoreactivity in all spermatogonia. Panel A, original magnification x200; panels B,C,D, original magnification x1000; panel E, original magnification x400.

TH2B (Figure 3D). The early round spermatids displayed a uniformly intense immunosignal. During early spermiogenesis, a region corresponding to the acrosomal cap of the round spermatid nucleus, displayed reduced TH2B immunostaining as compared to the rest of the nucleus (Figure 3B). During spermatid elongation, this region of reduced immunostaining increased, concomitant with expansion of the acrosome. In the final stages of spermatid nuclear condensation, a small region of immunodetectable TH2B could be discerned in the posterior region of the spermatid nucleus (Figure 3C), after which TH2B became immunologically completely undetectable (Figure 3D).
In human testis tissue from an infertile male, where only Sertoli cells and spermatogonia were present in the seminiferous tubules, again the nuclei of spermatogonia, but not Sertoli cell nuclei, stained positive (Figure 3E). All other somatic cell nuclei did not immunoreact.

Immunocytochemical localization of TH2B in human spermatozoa
Immunostaining of TH2B was absent in all morphologically normal and in most abnormal spermatozoa in human semen samples. However, some of the abnormal spermatozoa and most of the round cells stained intensely (results not shown). When the spermatozoa were subjected to decondensation treatment, a larger number of spermatozoa stained positive (Figure 4).

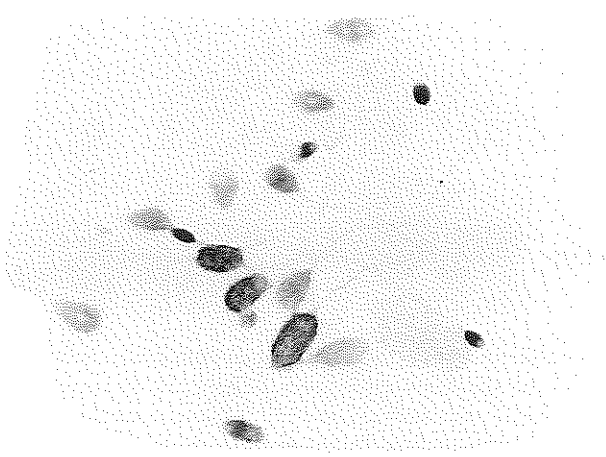

Figure 4. Immunocytochemical localization of TH2B in human spermatozoa. The spermatozoa were treated (see Materials and Methods) to induce decondensation. There is a clear signal in some, but not all, of the decondensed sperm nuclei. All non-decondensed sperm nuclei remain negative. Original magnification x1000.

However, the spermatozoa that remained in the condensed form remained negative. Among the spermatozoa that became decondensed, the intensity and pattern of immunostaining showed variation, which was, however, not correlated with the size of the decondensed sperm nucleus. After decondensation, the original morphology of the spermatozoa was lost, making it impossible to ascertain which spermatozoa might have been morphologically abnormal prior to decondensation. Therefore it was not possible to detect a putative correlation between sperm morphology and sperm TH2B immunostaining at the single cell level.

TH2B level in semen samples

The amount of TH2B present in a fixed ($0.5x10^6$) number of spermatozoa of 35 males visiting our Andrology Outpatient Clinic was determined by measuring the intensity of the TH2B immunosignal on Western blot. Figure 5 shows the TH2B immunoblots for several of these patients. There clearly was a large inter-individual variation in sperm-TH2B content. It should be noted that this data represents the total amount of TH2B per $0.5x10^6$ spermatozoa, and do not give information about the variation of TH2B content among individual spermatozoa. Quantitative analysis of the TH2B level in sperm samples confirmed the qualitative observations (Figure 6). In order to assess if the variation in total sperm-TH2B content might be associated with semen sample characteristics, the TH2B concentration was

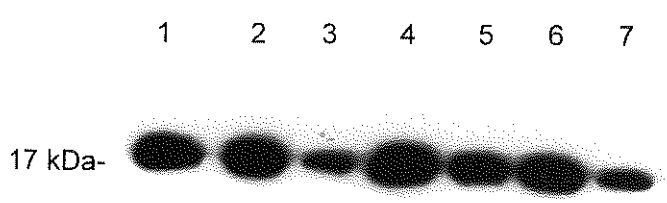

Figure 5. Immunodetection of TH2B in spermatozoa from human infertility patients. Protein was extracted from $0.5x10^6$ spermatozoa from seven different patients attending our Andrology Clinic, separated on SDS-PAGE and transferred on a nitrocellulose membrane. Note the diversity of signal intensity between the different lanes. The gel shown in this figure was exposed slightly longer than normal in order to highlight the patient-to-patient variation in sperm TH2B content. A less exposed version was used for the actual measurements (Figure 6) but was less suitable for photography.

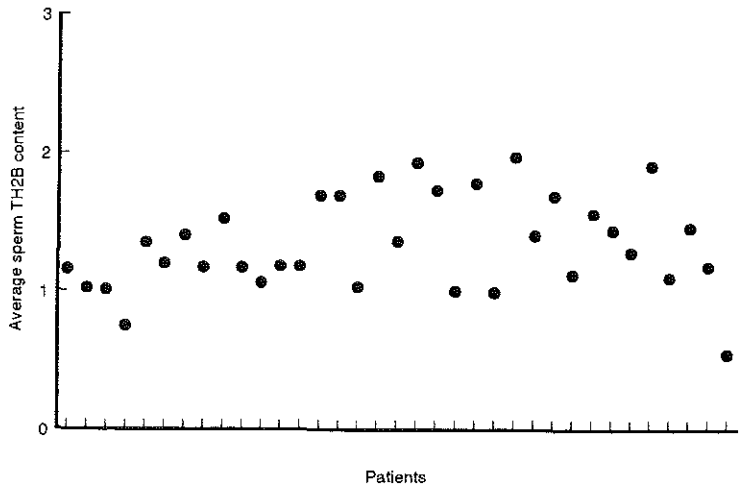

Figure 6. Variation of total sperm TH2B content in 35 infertile males. The total TH2B content of $0.5x10^6$ spermatozoa isolated from a single ejaculate from each of the subfertile males was estimated. All values are relative to the average amount of TH2B in a standard sperm sample obtained from an ejaculate with a high number of spermatozoa. Samples were first washed in Percoll 60%. Proteins were separated on SDS-PAGE, blotted and immunostained. The intensity of the resulting signal was quantified (see Materials and Methods).

compared with the results of the corresponding semen analyses. As is shown in Table 1, there is no correlation between total sperm TH2B content and sperm concentration of the initial sample (r=-0.210, P=0.91) or sperm motility (r=-0.138, P=0.43). In this group of patients, total sperm TH2B content showed a marginally significant correlation with morphology (r=0.328, P=0.05): a higher TH2B concentration was found in ejaculates with more abnormal spermatozoa. In only six samples more than $1x10^6$ round cells per ml were present, thus this factor was not included in the statistical calculations.

Table 1. Results of routine semen analysis (mean±SEM) and correlation with sperm testis-specific histone 2B (TH2B) content in $0.5x10^6$ spermatozoa of 35 subfertile men. The sperm TH2B content of patients were compared with the TH2B content of a standard sperm sample.

		Correlation[a] with histone content	p-value
Total sperm count ($x10^6$)	98.8 (±15.1)	–0.144	0.41
Concentration ($x10^6$)	27.4 (±3.1)	–0.021	0.91
Motility[b] (%)	38.1 (±3.2)	–0.138	0.43
Morphology[c] (%)	19.9 (±2.1)	–0.328	0.05
Histone content[d]	1.3 (±0.06)		

[a] Spearman; [b] Grades a+b (WHO); [c] WHO criteria; [d] Arbitrary units

Discussion

In this study we have shown that the anti-TH antibody, known to cross-react with rat TH2B, can also be used to detect human TH2B in testis tissue and in spermatozoa. A clear and prominent 17 kDa band was detected on immunoblots of acid-soluble proteins extracted from rat testis tissue and human spermatozoa, but not from human leukocytes and rat epididymal spermatozoa. Tyrosine hydroxylase, a 57 kDa protein (Mayerhofer and Russell, 1990), was not encountered in any of the extracts. Additional evidence that the protein recognized by the anti-TH antibody is indeed a histone of the 2B type and not H2A or a totally unrelated protein of 17 kDa was obtained by electrophoresis on AUT-gels. The mobility of the immunoreactive protein was almost similar to rat TH2B and the bovine H2B standard.

In histological sections of human testis, only the nuclei of spermatogenic cells showed immunostaining: spermatogonia, spermatocytes, and round spermatids reacted positively with varying intensity. The immuno-detectability of TH2B in elongating spermatids was confined to the post-acrosomal region of the nucleus, whereas the nuclei of fully condensed spermatids did not stain. All somatic cell nuclei (Sertoli cells, Leydig cells, peritubular myoid cells, and endothelial cells of arterioles) were clearly negative. The absence of immunodetectability of TH2B in the anterior part of the nuclei of elongating spermatids and in spermatozoa is due to the highly condensed condition of the nuclei and not to the absence of TH2B, as Western blotting and immunostaining of acid-extractable proteins from human spermatozoa revealed a positive signal corresponding to TH2B.

Previous studies have shown that about 15% of human sperm nuclear proteins are histones (Tanphaichitr *et al.*, 1978), including TH2B (Wattanaseree and Svasti, 1983; Gusse *et al.*, 1986; Gatewood *et al.*, 1990; Prigent *et al.*, 1996). Using immunocytochemistry, we could not detect TH2B in untreated human and rat spermatozoa. Protein separation on SDS-PAGE followed by immunoblotting, however, confirmed the presence of TH2B in human spermatozoa, and also showed that TH2B is absent in rat spermatozoa. It appears that immunocytochemical detection of TH2B in morphologically normal mature human sperm cells is hampered by inaccessibility of the epitope to the antibody. When the human spermatozoa were decondensed, immunocytochemistry showed the presence of TH2B, albeit with a remarkably diverse pattern of staining. Whether the observed intercellular variation in TH2B immunostaining indeed reflects a difference in sperm TH2B content is a point of concern. During the course of this study, it became clear that a too vigorous decondensation of spermatozoa resulted in loss of immunostaining from all spermatozoa (results not shown). Presumably all TH2B is then removed from the nucleus. The heterogeneous staining pattern of the decondensed spermatozoa can probably not be attributed to loss of TH2B from some cells because many well decondensed and large nuclei stain intensely while other nuclei, also decondensed but smaller, stain lightly or not at all. Conversely, one might argue that the smaller nuclei contain TH2B that is still masked; however, there was no correlation between the size of the decondensed spermatozoa and the signal intensity. Therefore the observed heterogeneity in immunostaining probably is a true reflection of an intercellular variation in sperm TH2B content.

Very little is known about regulation of expression of the testis-specific histones in the human testis. A recent report indicates that during spermiogenesis, the immunodetectability of histones H2B and H3 is constant in the nuclei of early round spermatids, and then increases in intermediate spermatids (step 3-4). The histone H3 immunosignal decreases at the end of the elongation phase (step 5), while the H2B labelling decreases in mature spermatids (step 6) (Prigent *et al.*, 1996). Previous studies in the rat have shown that a number of histones (H1a, H2AX, TH3) are expressed early during the mitotic stages of spermatogenesis , while others (TH2A, TH2B, H1t) are first expressed at a later time when the spermatogenic cells have entered the meiotic phase (Meistrich *et al.*, 1985; Meistrich and Brock,

1987). In the rat, TH2B is first synthesized in the preleptotene or leptotene spermatocytes, and its synthesis continues until the mid or late pachytene stage. TH2B is the major form of H2B in rat round and elongating spermatids, although somatic H2B is still present (Brock *et al.*, 1980; Meistrich *et al.*, 1985; Unni *et al.*, 1995). Our studies show a strong TH2B immunosignal in spermatogonia in human testis tissue, indicating that, contrary to the situation in the rat, first expression of TH2B in the human testis occurs at the early mitotic stages of spermatogenesis.

A number of investigators have shown that ejaculates of subfertile men and/or ejaculates containing a large number of morphologically abnormal cells, contain a relatively large amount of histones (Terquem and Dadoune, 1983; Chevaillier *et al.*, 1987; Foresta *et al.*, 1992). Most studies have used indirect methods to detect the persistence of histones in spermatozoa, such as staining with toluidene blue to test the availability of DNA phosphates (Andreetta *et al.*, 1995), aniline blue to stain all remaining histones (Terquem and Dadoune, 1983), differential nuclear chromatin decondensation using detergents (Calvin and Bedford, 1971a; Bedford *et al.*, 1973a), acridine orange to test for single stranded DNA (Evenson *et al.*, 1980; Tejada *et al.*, 1984), and chromomycin A_3 to test for guanosine rich sequences of DNA (Bianchi *et al.*, 1996). We are now in a position to determine the presence of a single histone variant in human sperm, and to correlate its persistence to routine semen analysis parameters. In our patient population, we found no correlation between total sperm TH2B content and total number of sperm per ejaculate, sperm concentration, and sperm motility. There was a marginally significant negative correlation between total sperm histone content and sperm morphology ($p=0.05$). Although fertile men produce a substantial amount of morphologically abnormal spermatozoa, abnormalities of sperm morphology are encountered more often in subfertile men (Bosofte *et al.*, 1985; Kruger *et al.*, 1988). Sperm morphogenesis is a complex process, which includes the successful compaction of the DNA. An association between defects in DNA packaging and sperm morphology abnormalities have previously been reported (Gledhill, 1970; Bedford *et al.*, 1973; Dadoune and Alfonsi, 1986). Recently it was observed that knock out of the gene encoding ubiquitin-conjugating enzyme HR6B in the mouse is associated with morphological abnormalities of spermatozoa (Roest *et al.*, 1996). These morphological abnormalities may result from impaired

chromatic reorganization during spermatogenesis, which may require ubiquititation of histones. Disruption of the normal dense chromatin structure of spermatozoa in transgenic mice expressing a gene construct that encodes galline, the avian homologue of protamine, under the control of a protamine-1 promoter, causes sperm abnormalities and infertility (Rhim *et al.*, 1995). This is probably explained by impaired DNA compaction in chromatin containing both protamine and galline. Both mouse models demonstrate that a disturbance in chromatin remodelling can lead to marked morphological abnormalities of spermatozoa and to infertility.

The presence of histones in human sperm is also regarded as a consequence of a derailment in the process of chromatin remodeling during spermatid condensation, and is considered an ominous sign with respect to fertility (Terquem and Dadoune, 1983; Chevaillier *et al.*, 1987; Foresta *et al.*, 1992). Our results and those of others (Gatewood *et al.*, 1990), however, show that in virtually all human ejaculates, spermatozoa can be shown to contain histones. It should be kept in mind that, in somatic cells, histones not only play a structural role in organizing nuclear DNA; evidence is accumulating that histones may play a crucial role in regulation of transcription (Ura *et al.*, 1997). Histones in spermatozoa are possibly interacting with a specific part of the genome (Gatewood *et al.*, 1987), and there are indications for early post conception paternal-specific expression of several genes (Sawicki *et al.*, 1981). From this, it is tempting to suggest that sperm histones might be involved in marking the genes that are poised for early expression after fertilization. In addition to the role of nuclear proteins, mRNA's found in human ejaculated spermatozoa have been the focus of a considerable amount of speculation. A number of recent studies have demonstrated the presence of mRNA coding for c-*myc*, b-actin, b_1-integrin, protamine 1, protamine 2 and transition protein 2 in mature human spermatozoa (Kramer and Krawetz, 1997). Whether the highly condensed chromatin within the head of the spermatozoa is actually (partially) transcriptionally active, or whether the observed mRNA's are stable and dormant remnants of past haploid synthetic and equilibrative activity in earlier stages of germ cell differentiation (Miller, 1997), persisting because of lack of degradative mechanisms within the spermatozoa, remains to be elucidated.

In conclusion, we have shown that an antibody targeting tyrosine hydroxylase can be used to specifically identify TH2B in human spermatozoa and testis tissue. Our results indicate that TH2B is first expressed at an early mitotic

stage of human spermatogenesis, and is present throughout spermatogenesis and in mature spermatozoa. There appears to be a marked variation of total sperm TH2B content among subfertile males, that may correlate, to some extent, with abnormalities of sperm morphology. Finally, the present results indicate sperm-to-sperm variation in TH2B immunoexpression within a single ejaculate. The significance of this observation, and the fertilizing potential of human spermatozoa containing varying amounts of TH2B, are subject of present research.

8

Discussion

Introduction

Over the years, a great wealth of knowledge has accumulated in the field of male reproduction, for human and other mammalian species. Experiments on animal models have played a crucial role in the attempt to understand the more basic and intricate mechanisms of regulation of spermatogenesis and sperm maturation. Human testes and epididymides are both organs that are not eagerly offered for experimentation. It has primarily been the study of the male rodent reproductive system, that has led to our present understanding of the workings of the hypothalamus-pituitary-testis axis, and, more recently, of many of the molecular and genetic regulators of male reproductive function. In the quest for an even more intricate insight in this process, however, investigators have removed cells and tissues from the animal or human body for study in a 'test tube environment'. Although these studies have provided us with a great deal of information, it is questionable whether the obtained data indeed reflects cell function under physiological conditions. It is well known that cells taken out of their physical and physiological milieu are different, with respect to morphology and reaction to hormonal or other stimuli (Sharpe, 1993; Sharpe, 1994). When studying testicular cells, the drawbacks of this methodology may be even more compelling. The function of the testicular cells, and indeed the testis as a whole, is dependent on feedback systems that involve essentially an intact and healthy organism (Sharpe, 1994; Grootegoed, 1996). Many of the Sertoli cell functions are highly dependent on their association with germinal cells, and require not only the pituitary endocrine regulators, but are also highly sensitive to factors secreted by other testicular cells [peritubular myoid cells, Leydig cells and germinal cells (Griswold, 1995)]. Similarly, the principal cells of the epididymis probably require a number of testicular factors, in addition to a well functioning mesenchymal layer at their basal side, for normal function (Garrett *et al.*, 1990; Sujarit *et al.*, 1990; Holland *et al.*, 1992; Turner *et al.*, 1995). It is desirable, therefore, to study the processes involved in spermatogenesis and sperm maturation, where possible, in a whole organism. This is especially true for the study of hormonal control mechanisms and hormone metabolism.

Animal model; physiological aspects

The animal studies described in this thesis have explored mechanisms of androgen action in spermatogenesis and regulation of expression of the androgen receptor in the rat testis and epididymis (Chapters 3 and 4). As our experiments were mainly concerned with the function and expression of the androgen receptor, and androgen metabolism, in whole organs, we were able to conduct our research in intact animals. In manipulating any one factor within the highly tuned hypothalamus-pituitary-testis feedback system, changes may result in that system which are not only caused by the primary influence, but also by secondary adaptations of that system. Specifically, injecting varying doses of testosterone in a rat, to study its effect on spermatogenesis, will not only result in a varying concentration of serum testosterone, but will also cause a fall in serum FSH level (Sun *et al.*, 1989). Because FSH is known to influence spermatogenesis, this factor must be controlled. Treating rats with a GnRH antagonist will result in suppression of serum LH and FSH to very low levels without affecting other pituitary hormones (Chapter 3; Rea *et al.*, 1986). The advantage is that, even in rats treated with very low testosterone doses, LH will remain fully suppressed so that the testicular testosterone level can be better controlled. Unfortunately, in the GnRH antagonist-treated rat, a rise in serum testosterone will result in an increase in serum FSH due to a direct effect of testosterone on the pituitary (Rea *et al.*, 1986; Bhasin *et al.*, 1987). Thus, in order to control for a varying FSH level in each treatment group, recombinant human FSH must be supplemented in all rats. Although the serum LH level falls below the detection limit in rats treated with a GnRH antagonist (Chapter 3), mature Leydig cells will continue to secrete a small amount of testosterone in the absence of LH (Sharpe, 1994). This amount is negligible in rats treated with a high level of androgen. However, it has been shown that residual testosterone secretion by non-stimulated Leydig cells does have some stimulatory effect on spermatogenesis (El Shennawy *et al.*, 1998; Russell *et al.*, 1998). To eliminate all mature Leydig cells, rats can be treated with ethane dimethane sulphonate (EDS) (Kerr *et al.*, 1985; Morris *et al.*, 1986). The advantage of LH suppression by a GnRH antagonist in this situation is that the maturation of immature Leydig cells is prevented, resulting, theoretically, in prolonged maximal androgen blockade in the rat testis. Combining the above treatments will result in an animal model, albeit rather complicated, allowing one to study specifically the effect of exogenous androgens on spermato-

genesis in a situation where all mature Leydig cells are destroyed and the influence of LH on development of a new population of mature Leydig cells is negligible, while serum FSH is controlled.
Although the notion that all factors except the factor under scrutiny must remain constant within an experiment to enable one to draw reliable conclusions seems self evident, virtually all of the previous studies regarding the effects of androgens on spermatogenesis fail on this point (see Chapter 2). This renders the data difficult to interpret, and precludes reliable answers to some of the nagging questions relating to androgens and spermatogenesis.

Androgen action in spermatogenesis

How much testosterone is needed for normal spermatogenesis?

The importance of androgens for spermatogenesis has been well recognised for many years. Androgens are, however, important for the adequate function of a number of other tissues in the male body as well. Due to local production, the levels of testosterone found in the testis are far higher than the levels found in the peripheral blood of all studied mammals (Chapters 2 and 3; Sharpe, 1994). Although there is ample discussion about the exact levels of testosterone in the rat testis, and about in which testicular compartment testosterone should be measured, it is accepted that levels are between 25-100 ng/ml (see Chapter 2; Sharpe, 1994). Our own studies have revealed a value of about 46.5 ng/ml of testosterone in whole, flash frozen, adult rat testis, confirming earlier reports.

Testicular testosterone level required for qualitatively normal spermatogenesis

It has become quite clear that the very high levels of testosterone found in the testis are far higher than what is needed for qualitatively normal spermatogenesis, which is attained at relatively low testicular testosterone levels. In a study by Sun *et al.* (1989), qualitatively normal spermatogenesis in rats was maintained at a testicular interstitial fluid testosterone level of about 7 ng/ml over a period of 13 weeks. A more recent study in intact rats treated with low dose testosterone and estradiol indicated that the testicular testosterone level needed for qualitatively normal spermatogenesis may be at about 7-12% of control (O'Donnell *et al.*, 1994). In hypophysectomised rats, however, the interstitial fluid testosterone level needed for qualitatively

normal spermatogenesis was clearly more than 10 ng/ml (Sinha Hikim and Swerdloff, 1993). Many other studies have shown that the ability of testosterone to maintain spermatogenesis is impaired in hypophysectomized rats compared to intact animals (see Sharpe, 1994 for review). As is discussed in Chapter 2, the absence of FSH in hypophysectomized rats may contribute to the need for a higher level of testosterone, although it is likely that other factors are also involved.

Although qualitative maintenance of spermatogenesis can be attained at a relatively low testicular testosterone level, the level needed is still considerably higher than that normally found in serum (1.2 ng/ml in our study; see Chapter 3). At this point, it should be stressed once again that testosterone suppletion at a very low dose, be it in an intact animal, an animal co-treated with estradiol, or in particular in a hypophysectomised animal, results in a significant fall in the serum FSH level (there is no FSH at all in hypophysectomised rats). It is conceivable that in the presence of a normal FSH level, the testicular testosterone level needed for qualitatively normal spermatogenesis would have been even lower. A strong synergistic effect of testosterone and FSH has been described on a number of occasions, especially at low testosterone levels (Kerr *et al.*, 1992). At what dose of testosterone qualitatively normal spermatogenesis can be maintained at a physiologic serum FSH level is currently unknown. In our study, EDS/GnRHa/recFSH treatment without androgen supplementation resulted in a testicular testosterone level of about 1.6 ng/ml, which is comparable to the control serum testosterone level. In the animals treated with this regimen, no condensing spermatids could be detected, indicating that the normal circulating level of testosterone cannot maintain qualitatively normal spermatogenesis even in the presence of FSH (Fig 1, Chapter 3). A testicular testosterone level of 3.4 ng/ml (7% of control) was sufficient to maintain qualitatively normal spermatogenesis in our model, albeit quantitatively markedly reduced, as measured by identification of homogenisation resistant spermatids (unpublished results).

Testicular testosterone level required for quantitatively normal spermatogenesis

Maintenance of quantitatively normal spermatogenesis (i.e. production of a normal amount of mature spermatozoa) requires a relatively high testicular testosterone level. The exact testosterone level needed to maintain normal

spermatogenesis, is not exactly known, due to differences in study design and varying methods of estimating spermatogenic activity (see Sharpe, 1994). On the basis of the available evidence, most investigators accept a value of 20 ng/ml for the rat. An important factor that may be overlooked in some instances, is that spermatogenic efficacy seems to diminish over time, even in animals treated with extremely high doses of testosterone (i.e. Sun *et al.* 1989). In view of this, Sharpe (1994), in his extensive treatise on the subject, indicated that the testicular level of testosterone needed for long term quantitative spermatogenesis might be as high as 40 ng/ml. Our results indicate that quantitative spermatogenesis, as measured by testis weight, never quite reaches 100% in both the testosterone propionate-(TP) and R1881-treated groups (Chapter 3). It must be remembered that these testes are devoid of mature Leydig cells, which may account for the fact that testis weight in the supplemented rats can never quite reach the control level.

However, again it has become clear that the testicular testosterone level that is needed for normal spermatogenesis is far higher than the normal serum testosterone level which is adequate for maintenance of the function of other androgen dependent organs such as the prostate and seminal vesicles.

Why does the testis need a high level of testosterone for adequate spermatogenesis?

Testosterone acts on androgen dependent tissues through the androgen receptor: a ligand dependent transcription factor that, upon activation, can bind to consensus DNA sequences and regulate gene transcription (Brinkmann *et al.*, 1989; Chapter 2). The levels of testosterone normally found in the testis are about five-fold of that needed to occupy 100% of all testicular androgen receptors (Blok *et al.*, 1992). Although it is highly likely that this high level of testosterone may not be necessary for normal spermatogenesis, the lowest level that is required is still more than a magnitude higher than that found in serum. The mechanisms underlying this phenomenon have remained enigmatic, although a number of explanations have been forwarded (see Sharpe, 1994 for review).
The difference in testosterone dependency of the testis compared to other androgen dependent organs may have a metabolic basis. In a number of highly androgen dependent organs in the male, such as the prostate and

seminal vesicles, the primary ligand for the androgen receptor is not testosterone, but its 5α-reduced metabolite dihydrotestosterone (DHT) (Wilson, 1972). In activating the androgen receptor, DHT is about an order of magnitude more potent than testosterone (Grino *et al.*, 1990). The activity of 5α-reductase in these organs thus, in effect, results in a ten-fold amplification of the androgen signal. As testosterone levels within the testis are in excess of ten-fold of that found in serum, it has been proposed that no role exists for 5α-reductase in this organ. Indeed expression of 5α-reductase is very low in adult testis.

Furthermore, it is concievable that a substantial portion of the testicular testosterone is made unavailable to the testicular testosterone-reponsive cells, through binding to extracellular testicular proteins. A candidate protein in this respect, is androgen binding protein (ABP), which is present at a high level within the testis. It is known that the majority of ABP is secreted, by Sertoli cells, into the seminiferous tubular lumen, carrying a substantial amount of biologically unavailable testosterone out of the testis into the epididymis (Gunsalus *et al.*, 1980).

Finally, it cannot be excluded that androgens act on Sertoli cells and perhaps other testicular cells, through an as yet unknown plasma membrane receptor mediated pathway. In recent years, many steroid hormones have been shown to exert rapid actions at the level of the cell plasma membrane, through an unknown mechanism which does not involve the respective nuclear receptor. Androgens have been shown to induce a rapid increase in intracellular calcium in cardiac myocytes (Koenig *et al.*, 1989), mouse kidney cells (Koenig *et al.*, 1982), and human prostate cancer cells (Steinsapir *et al.*, 1991). Gorczynska and Handelsman (1995) published a report describing a rapid increase in Sertoli cell cytosolic calcium as a result of androgen stimulation. This rise in cytosolic calcium results from the induction of a transmembrane influx of extracellular calcium. Both testosterone and its reduced metabolite dihydrotestosterone resulted in this effect, while precursors of testosterone and estradiol had no effect. In addition, conjugation of testosterone to bovine serum albumin (BSA), which limits uptake of testosterone in the cell, did not prevent the calcium influx, indicating that the process is mediated via a membrane bound mechanism (Gorczynska and Handelsman, 1995). The above effects could be abolished using a selective anti-androgen, indicating that the mechanism might involve a putative membrane receptor with a binding domain that is somewhat similar to that

of the nuclear androgen receptor. Although the receptor itself has not been identified, the specificity of the androgen mediated activity as well as the ability of anti-androgens to reverse these effects, point to an androgen binding moiety that closely resembles that of the classic intracellular receptor. Whether other testicular cell types, specifically the germinal cells, display membrane associated androgen regulated activity, or whether this phenomenon is involved in the necessity for a high intratesticular androgen level, is presently not clear. It is important to note, however, that all known androgen effects require the presence of an intact classical androgen receptor, as can be observed in 46,XY individuals with complete androgen resistance, caused by AR mutations.

Is there a role for 5α-reductase in normal adult testis function?

Although it has been shown on a number of occasions that indeed 5α-reductase expression and activity in the adult testis are extremely low (Van der Molen *et al.*, 1975; Turner *et al.*, 1984), a number of observations have raised doubts as to whether testosterone metabolism can fully explain the discrepancy in testosterone dependence between testis and other androgen dependent organs. First, if the ten-fold amplification of the androgen signal, by T to DHT conversion, as occurs in other androgen dependent organs, is absent in the testis, one would expect that a testicular testosterone concentration ten-fold higher than that found in peripheral blood should be sufficient for normal spermatogenesis. Many studies have attested to the fact that this may not be the case (see Sharpe, 1994). Second, a number of studies have shown some, albeit low, 5α-reductase expression and activity in adult human and rat testis, indicating that a low rate of conversion of testosterone to DHT in the testis cannot be excluded (Van der Molen *et al.*, 1975; Turner *et al.*, 1984). Third, men with a genetic defect resulting in a 5α-reductase type 2 deficiency have impaired spermatogenesis. It has been implied, therefore, that the testis expresses a low activity of 5α-reductase, and that its activity seems to be essential for spermatogenesis even at the physiologic high levels of testosterone in the testis.

The study described in Chapter 3, however, provides compelling evidence that indeed a relative lack of 5α-reductase activity in the testis is responsible, at least in part, for the discrepancy in testosterone dependence of the testis compared to the prostate and seminal vesicles. Supplementation with various

doses of R1881 (methyltrienolone), a synthetic androgen that is not metabolised and does not bind to ABP, in rats pre-treated with EDS/ GnRHa/recFSH, resulted in an equal response of the testis, the prostate and seminal vesicles. This indicates that the testis is not less sensitive to androgen compared to the other organs, in a situation where metabolism and binding of the androgen are eliminated. A second experiment, where testosterone was supplemented in EDS-treated rats co-treated with fina-steride (a potent 5α-reductase inhibitor), resulted in a practically identical response of the testis, the prostate and the seminal vesicles, judged by changes in organ weight.
The testis apparently contains a low activity of 5α-reductase, but its functional importance may be very limited. Inhibition of 5α-reductase activity in beagle dogs has no discernable effect on spermatogenesis (Juniewicz *et al.*, 1993). At a low testicular testosterone level, however, inhibition of 5α-reductase does appear to have a negative effect on post-meiotic development of spermatids in the rat (O'Donnell *et al.*, 1996). When testosterone supplementation is increased to a higher dose, however, 5α-reductase inhibition no longer has any discernable effect on any spermatogenic process. This data indicates a very limited role for 5α-reductase in the testis, which becomes apparent only when the testicular testosterone level is very low.
Men with a 5α-reductase deficiency have impaired spermatogenesis, mainly as a result of maldescent of the testis into the scrotum. There is no indication that the enzyme deficiency itself is directly related to impairment of spermatogenesis.

Androgens in the search for a male contraceptive

The observed necessity for a relatively high testicular testosterone level for normal spermatogenesis, has been put to good use in the search for a male contraceptive pill. Since the early 70's, it was realised that administration of exogenous testosterone, inhibiting pituitary gonadotropin secretion, induced a strong decrease in serum FSH level, and inhibition of testicular testosterone production as a result of a low serum LH level (Wu, 1996). Early studies using either androgen alone or in combination with progestogens resulted in azoospermia in 40-80% of Caucasian men (Wu, 1997). Between 1987 and 1994, two important WHO contraceptive efficacy studies, using a weekly regimen of 200 mg testosterone enanthate i.m. inducing a two-fold increase

in the serum testosterone level, resulted in a pregnancy rate of 0.8 per 100 person years, for couples in which the men were suppressed to azoospermia (Pearl rate of 0.8%) (WHO Task Force on Methods for the Regulation of Male Fertility, 1990; WHO Task Force on Methods for the Regulation of Male Fertility, 1996). In the second study, 98% of all treated men were suppressed to azoospermia or deep oligozoospermia (<3 million sperm per ml) (WHO Task Force on Methods for the Regulation of Male Fertility, 1996). Previous *in vitro* studies had indicated that the fertilizing capacity of residual sperm of severely oligozoospermic men due to androgen treatment was impaired (Wu, 1997). The Pearl rate for individuals reaching azoospermia or oligozoospermia of <3 million sperm per ml was 1.4. (this is comparable to the first year failure rate of female oral contraceptives) (Wu, 1997). The above Pearl rates suggest an efficacious contraceptive effect in men in whom semen parameters have been reduced to less than 3 million sperm per ml. However, all pregnancies occurred in men in whom azoospermia was not attained, but who had <3 million sperm per ml in their semen. It is possible that the failure rate in the latter group is deemed higher than acceptable.

Unfortunately these regimens had a number of problems, including a twice normal serum testosterone level resulting in acne and a decrease in HDL-cholesterol. The increased circulating testosterone level also raises concerns about prostatic disease, cardiovascular disease, and behavioural disturbances. In addition, the need for a weekly injection is unpleasant, and resulted in treatment discontinuation.

As a result of the fact that only 50 to 70% of Caucasian men (as opposed to >90% of Asian men; see below) will attain azoospermia with androgen alone, with the above-mentioned side effects, a number of other regimens have been forwarded. Progestogens have been utilised, as they are potent inhibitors of gonadotropin secretion (Goldzieher and Castracane, 1984). In addition, progestogens appear to possess 5α-reductase inhibitory activity, which, in light of data discussed below, would make this substance ideal for use in combination with an androgen (Mauvais-Jarvis *et al.*, 1974). A number of trials using various combinations of androgens and progestogens are ongoing (Wu, 1997). Anti-androgens and androgen combinations and the use of androgen esters with improved pharmacokinetic properties are also currently being studied for use in male contraception (Wu, 1997).

Interestingly, the influence of testicular 5α-reductase on androgen action in

human spermatogenesis may be of some importance in the observed failure of testosterone enanthate to induce azoospermia. In one of the studies on this topic, of 33 men who were treated with testosterone enanthate (200 mg i.m. weekly), only 18 men achieved azoospermia (Anderson and Wu, 1996). The rest maintained a low level of spermatogenesis with 2.0 +/- 0.6 million sperm per ml of ejaculated semen. The serum level of DHT in the oligozoospermic men had increased while the DHT level in azoospermic men had not changed (Anderson and Wu, 1996). Similarly, in the same cohort of men, incomplete suppression of spermatogenesis was associated with a rise in seminal DHT, while for men who responded with azoospermia, no rise in seminal DHT could be detected (Anderson *et al.*, 1997). Although the seminal DHT level is probably the result of an increased production of DHT in the accessory sex organs (epididymis, seminal vesicles, and prostate), and provides no information about inter-individual differences in testicular 5α-reductase activity, the authors conclude that this difference in androgenic milieu might underlie the difference in response to testosterone enanthate treatment. Studies involving treatment of Asian men with androgens alone or progestin/androgen combinations, have indicated a consistently higher rate of suppression to azoospermia (about 90% of men) than that found in Caucasian men (about 75%) (for review see Wu, 1997). The mechanisms behind this observation are presently not clear. One could speculate that Asian men have lower 5α-reductase activity, as is possibly indicated by the less beard growth and a lower incidence of male pattern baldness in Asian men, compared to Caucasian men. This would concur with the observation that 5α-reductase activity in the testis has an influence on spermatogenis in situations of low testicular androgen concentration (O'Donnell *et al.*, 1996). Recent investigations using GnRH agonists and antagonists to suppress gonadotropin release have generated hopeful results (Behre and Nieschlag, 1997). GnRH antagonist treatment in monkeys fails to reduce the testicular testosterone level below 30% of normal after 15 weeks of treatment, even though the serum LH level is suppressed (Weinbauer *et al.*, 1988). In a later study, only a small proportion of men treated with a GnRH antagonist were rendered azoospermic (Behre and Nieschlag, 1997). Combination of GnRH antagonist and testosterone treatment induced azoospermia in 88% of men (Behre and Nieschlag, 1997). Why addition of androgen to GnRH antagonist treatment should further reduce sperm production remains elusive.

It would seem that, for successful hormonal induction of azoospermia in healthy men, a combination of maximal blockade of androgen production (including 5α-reductase inhibition) in the testis with maximal repression of serum FSH, combined with adequate androgen stimulation of peripheral tissues, would be a prerequisite. This is a difficult combination to achieve, and with the necessity for easy and cheap administration modalities, introduction of a hormonal male contraceptive will require much innovative research.

Heterogeneity of androgen receptor expression

Inter-individual variation in androgen receptor expression

Early reports have illustrated the presence or absence of the nuclear androgen receptor within a number of human and animal tissues. With the advent of the realization that some diseases such as Reifenstein's syndrome (see Chapter 5) can be the result of an abnormal expression of the androgen receptor, quantitative analysis of androgen receptor expression in cohorts of men were initiated. Subsequently a number of authors have reported a large variation in androgen receptor expression in cultured foreskin fibroblasts of normal men (Eil *et al.*, 1985; Morrow *et al.*, 1987; Wilson *et al.*, 1992). Our own data on the expression of androgen receptor in the testes of subfertile men with morphologically normal spermatogenesis, where infertility is probably the result of an obstruction of the epididymis or vas deferens, confirm these findings (Chapter 6). In some men, androgen immunoexpression was very low in both Sertoli cells and peritubular myoid cells, whereas in other men both cell types displayed strong immunoexpression. Although some studies have suggested a lower androgen expression pattern in non testicular tissue to be associated with androgen resistance and subsequently subfertility (Morrow *et al.*, 1987; Akin *et al.*, 1991), our findings and those of others (Eil *et al.*, 1985; Bouchard *et al.*, 1986) do not implicate that the level of androgen receptor expression is the cause of spermatogenic failure in a significant percentage of infertile men. The foundation for, and implications of, the apparently large variation in androgen receptor expression in apparently normal men remains to be elucidated.

Cellular variation in androgen receptor expression

Cells within a tissue that display identical morphology are generally considered to perform identical functions. Although there may be some periodic cell-to-cell variation in function because of mitotic activity, generally the basic assumption would be that identical cells show comparable activities. Observations concerning the pattern of expression of androgen receptor and the expression of androgen dependent proteins in both testis and epididymis tissue, now point to a functional variation between individual Sertoli cells and epididymal epithelial cells, which is quite unexpected, at least for the epididymal cells .

The testis

In 1994, two reports were published indicating that androgen receptor immunoexpression is variable in Sertoli cells within one and the same rat testis, and that this variation is tightly correlated with the stages of the spermatogenic cycle (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). It has previously been shown that androgen action seems to be mainly required around stage VII of the rat spermatogenic cycle (Sharpe, 1994), and the pattern of androgen receptor immunoexpression indeed would be in agreement with this. However, the mechanism of regulation of androgen receptor expression in rat Sertoli cells is still largely unresolved. Androgen receptor expression is apparently mainly regulated at the level of gene transcription, as the androgen receptor mRNA level varies with the stages of the spermatogenic cycle in synergy with changes in the AR protein level (Shan *et al.*, 1995). The stage dependence of expression of the androgen receptor suggests that germ cells may exert an important regulatory influence. Experiments using methoxyacetic acid (MAA) to induce degeneration of pachytene spermatocytes and round spermatids failed to disrupt the stage-dependent expression of the androgen receptor in Sertoli cell nuclei, indicating that possibly the earlier germ cell types might be of pivotal importance for this regulation (Bremner *et al.*, 1994). However, our own unpublished data, regarding treatment of rats with busulfan resulting in the destruction of spermatogonia and primary spermatocytes, similarly did not result in disruption of the normal androgen receptor expression pattern (unpublished observations). Possibly, any part of the germ cell complement is capable of regulating this aspect of Sertoli cell function, and only full

elimination of all germ cells might induce a uniform androgen receptor expression pattern in Sertoli cells. Adult Sertoli cells taken out of their testicular milieu (*ex vivo*) and cultured in the presence of androgen, display a more or less uniform androgen receptor staining pattern (unpublished observations).
Interestingly, our study described in Chapter 6 indicates that, contrary to the situation in the rat, Sertoli cells in the human testis display a pronounced, but random variation in androgen receptor immunoexpression, not related to the stage of the spermatogenic cycle. The reason for this difference in androgen receptor expression in Sertoli cells of the human testis *versus* the rat is not clear. Possibly the different organization of the seminiferous epithelium in man compared to that in the rat, involves a more dynamic and heterogeneous series of Sertoli cell-germ cell interactions. Furthermore, the subdivision of the human spermatogenic cycle in only six stages may be too imprecise, obscuring stage dependent differences in Sertoli cell androgen receptor immunoexpression (Clermont, 1963). Further study will be required to comprehend the possible biological significance of these observations.

The epididymis

Our studies of the pattern of androgen receptor expression in the epithelial cells of the rat epididymis, disclosed a regional variation in nuclear androgen receptor imunoexpression (Chapter 7). In the efferent ductules, androgen receptor immunoexpression in the nuclei of the epithelial cells was found to be very low. From the initial segment onwards, androgen receptor immunoexpression was prominent. *In situ* hybridization studies of androgen receptor mRNA showed a high mRNA level in the initial segment, and much lower levels further distal. Apparently, the expression of androgen receptor in the epididymis is regulated, at least in part, at the translational level.
Most intriguing in the study of androgen receptor expression in the nuclei of epididymal epithelial cells, was the observation that this expression was highly heterogeneous for different nuclei within a single tubule cross section, especially in the caput (Chapter 7). *In situ* hybridisation studies revealed a similar variation in cell-to-cell androgen receptor mRNA expression, suggesting that this seemingly cell-autonomous, differential expression might also be regulated at the transcriptional level. As the level of androgen receptor expression may be closely related to androgen driven cell activities, we per-

formed double staining experiments with antibodies targeting mouse epididymal protein 10 (MEP 10), a mouse homologue of rat proteins B/C (Rankin *et al.*, 1992a; Rankin *et al.*, 1992a). The expression of proteins B/C in the rat epididymis, is highly regulated by androgen (Brooks and Higgins, 1980). Surprisingly, a considerable number of cells expressing proteins B/C expressed a very low level of androgen receptor.
It is important to note, that in the studies described in Chapter 7, the epididymides were fixed in Bouin's solution. In our experience, androgen receptor immunoexpression is slightly less prominent in tissues fixed in Bouin's compared to formalin. Nevertheless, variation of androgen receptor expression in the epithelial cells of the rat epididymis was also observed in formalin-fixed tissue, albeit with less variation in intensity. Because all our results were highly reproducible, with a clear regional pattern in the heterogeneity of androgen receptor immunoexpression, we are confident that our results are robust and not due to artifact.

Other non-malignant tissue

Not many reports have attested to similar pronounced cell-to-cell variation in AR expression in other tissues. Articles by Janssen *et al.* (1992) and Kimura *et al.* (1994), do not mention any heterogeneity in androgen receptor expression in any of the tissues studied. A report by Ruizeveld de Winter *et al.* (1991) includes a number of cell types in human organs, where immuno-expression of androgen receptor, observed using a mouse mono-clonal antibody (F39.4), was found to be slightly variable, indicated by +/- or ++/-. These cell types are: myoid cells and fibroblasts in the testis, fibroblasts and smooth muscle cells in the accessory sex organs, foreskin fibroblasts, squamous cells and fibroblasts in the ectocervix and vagina, and the ducts and acini of the mammary gland. Contrary to our findings, the later report classified Sertoli cells as invariably positive. A report by Takeda *et al.* (1990) classifies a number of rat cell types and tissues as having heterogeneic immunoexpression of androgen receptor in a qualitative manner. These include the stroma and smooth muscle of the prostate, the stroma of the seminal vesicles, the epithelium of the coagulating gland, the stroma and smooth muscle of the epididymis, the epithelium of the uterus, female (but not male) hepatocytes, and the endocrine cells and pituicytes of the pituitary. However, Takeda *et al.* (1990) have classified Sertoli cell androgen receptor

immunoexpression to be positive, without heterogeneity. Although the reports by Ruizeveld de Winter *et al.* (1991) and Takeda *et al.* (1990) indicate a limited degree of heterogeneity of androgen receptor immunoexpression in several cell types (the above-mentioned cells varied in androgen receptor immunoexpression from no expression at all, to a variable level of expression), their results actually indicate a much wider range of cell types that vary from low expression to high levels of androgen receptor immunoexpression, indicated by +++/+ or ++/+. It is very likely that upon closer scrutiny many more androgen receptor positive tissues will display a marked heterogeneity in androgen receptor expression. A recent article reporting on androgen receptor immunoexpression in human hepatocytes indicates a heterogeneous expression, with 50% to 90% of hepatocytes staining positively (Hinchliffe *et al.*, 1996). It is presently not clear whether all androgen taget tissues display a heterogeneic pattern of androgen receptor expression. In addition, the functional significance of intercellular varialtion in expression of the androgen receptor in various tissues remains to be disclosed.

Malignancies

Heterogeneity of androgen receptor expression has for some time been observed in malignancies, in particular in prostate adenocarcinoma. Androgen ablation therapy is the indicated treatment in advanced prostate cancer, resulting in only a temporary remission of tumor growth (Schröder, 1998). Prostate tumors that progress under androgen ablation and/or blockade, have become androgen independent. As the androgen signal must involve the androgen receptor, a great deal of effort has been put into trying to correlate the degree of androgen receptor expression in prostate tumors and prognosis, with varying results (Sadi *et al.*, 1991; Prins *et al.*, 1998).
An interesting study where androgen receptor immunostaining was combined with immunostaining for prostate-specific antigen (PSA; a highly androgen dependent protein) in prostate carcinoma, revealed no correlation between androgen receptor positive cells and PSA positive cells (Ruizeveld de Winter *et al.*, 1994). These findings are strikingly similar to our observations in the rat epididymis (Chapter 4). Although malignant tissue is in many ways very different from non-malignant tissue, the mechanism behind the observed variation in androgen receptor expression, and the apparent non-association with the expression of androgen dependent proteins may be similar.

Heterogeneity of androgen receptor expression has also been found in other malignancies including ovarian tumors (Chadha *et al.*, 1993).
Malignant tissue is very different fom healthy tissue, and alternative mechanisms possibly are involved in cellular heterogeneity of androgen receptor expression. Tumor cells vary in the degree of cell differentiation, which may explain the lack of androgen receptor in a part of the tumor cell population. This notwithstanding, it remains remarkable that androgen receptor negative prostate tumor cells continue to express PSA (Ruizeveld de Winter *et al.*, 1994). This observation, and the present observation (Chapter 4), that some androgen receptor-negative epididymal epithelial cells in rats express a highly androgen-dependent protein, may point to a cyclic activity of androgen-dependent cells. This cyclic activity would involve, in a temporal sequence, expression of the androgen receptor, followed by expression of an androgen-dependent protein, where finally the latter protein will still be prominent also after the androgen receptor expression has disappeared. There is, however, very little experimental evidence to support this view. Conversely, the androgen-dependent protein in both the epididymis and in prostate cancer cells might be regulated by factors that can override androgen influence (Ruizeveld de Winter *et al.*, 1994). Further research is needed to clarify this point.

The genetic integrity of the paternal gamete

Why explore the genes within the spermatozoon?

Although male factor infertility is often difficult to treat, the development of artificial reproductive techniques [intra-uterine insemination, *in vitro* fertilization (IVF), intra cytoplasmic sperm injection (ICSI), with or without testicular sperm extraction (TESE) or microsurgical epididymal sperm aspiration (MESA)] have opened up the way for men even with very low sperm counts to father a child. With the advent of these protocols, however, have come concerns with regard to the health of the offspring. Particularly ICSI, where a laboratory technician singles out a spermatozoon with which to fertilize an oocyte, has attracted criticism, because natural barriers to fertilization and possible sperm selection mechanisms are circumvented (Vogt, 1995b). The ICSI technique is indeed one of the very few widely implemented medical treatments, for which the safety has not been

demonstrated. Only recently, animal studies have started to investigate the fundamental aspects of ICSI in mice (Yanagimachi, 1998).

Sperm chromosomal and other genetic defects

Cytogenetic studies of spermatozoa have shown large differences in the genetic quality of spermatozoa from fertile and infertile men. Pang *et al.* (1995) have shown a more than 10-fold increase in the frequency (19.6% versus 1.5%) of diploidy, autosomal disomy, autosomal nullisomy, sex chromosome number disorder, and the total number of chromosomal abnormalities for oligo-astheno-teratozoospermic males *versus* fertile donors (Pang *et al.*, 1995). Moosani *et al.* (1995) found an increase in the frequency of disomy for chromosome 1 and a higher frequency of XY disomy in spermatozoa of infertile men. Noteworthy is that all the infertile men studied in the above two reports had a normal somatic karyotype, indicating that the chromosomal abberrations in the spermatozoa are the result of impaired spermatogenesis. This implicates that when ICSI is employed to concieve a child using sperm from a severely oligozoospermic man, chances of creating offspring with chromosomal abnormalities are increased. A large number of autosomal aneuploidies are not viable and will result in abortion. Sex chromosome aneuploidy, however, is compatible with life. Indeed it has been shown that application of ICSI may result in increased incidence of sex chromosome aberrations in the offspring (In't Veld *et al.*, 1995; Liebaers *et al.*, 1995; In't Veld *et al.*, 1997).

Next to chromosomal abnormalities that are created during deficient spermatogenesis, constitutional genetic anomalies are themselves also the cause of male infertility. It is estimated that about 600 to 700 genes are involved in the reproductive cycle (Vogt, 1995a), although this number may be conservative. Some of these genes are involved in the organogenesis of the male reproductive tissues, while others are involved in a variety of functions in adulthood including gametogenesis. Recent estimates indicate that about 30% of spermatogenic defects are genetically determined (Vogt, 1997b). If these figures are correct, genetically determined male infertility would account for the majority of cases of 'idiopathic infertility'. If spermatozoa can be retrieved from these men and used in artificial reproductive techniques, the defective genes may be passed on to the offspring.

A large number of genetic defects resulting in some form of male infertility

have been identified (see Chapter 5). This lengthy list, however, entails only a small number of men that are seen at an andrology clinic. Recent discoveries of the importance of deletions in specific regions of the long arm of the Y chromosome, are important advances due to intensive investigation and search for the genetic determinants of male infertility (Vogt, 1995; Vogt *et al.*, 1996; Dohle *et al.*, 1998; Roberts, 1998). Although earlier reports suggest an important role for androgen receptor mutations in otherwise normal infertile males (Aiman *et al.*, 1979; Aiman and Griffin, 1982; Morrow *et al.*, 1987), our own study (Chapter 6) and those of others do not support this data (Eil *et al.*, 1985; Bouchard *et al.*, 1986). The putative role of the polymorphic polyglutamine stretch in exon 1 of the androgen receptor in the regulation of its transcriptional activity, with long polyglutamine stretches being associated with relatively poor spermatogenesis, is highly interesting (Tut *et al.*, 1997). More research is needed to disclose whether a mild androgen receptor defect may play an important role in the pathogenesis of infertility in otherwise normally androgenized males.

Identifying the genetic defects behind male subfertility is vital for adequate diagnosis, genetic councelling, and ultimately for the development of treatment modalities. In light of the above, it seems prudent that all couples undergoing ICSI because of spermatogenic defects, regardless of the cause, undergo genetic counselling. It is equally compelling that techniques are developed with which certain aspects of the integrity of the sperm DNA can be evaluated prior to referral of the couple for ICSI.

Spermatogenic derailment: can clues be found within the sperm nucleus?

Spermatogenesis involves complex changes in chromatin arrangement (Baarends *et al.*, 1998). Probably before meiosis commenses, genomic imprints are erased after which a paternal mode of genomic imprinting is embedded into the DNA. Subsequently, homology search of the autosomes, synapsis and homologous recombination takes place. Finally, the entire nucleoprotein structure is altered by first partly replacing somatic histones by testis-specific histones, followed by replacement of histones by transition proteins, and subsequently by protamines (Meistrich and Brock, 1987). Derailment of any one of these processes may well result in isolated male infertility as is elegantly illustrated by a number of transgenic and knockout mouse models (see Chapter 5). Targeted disruption of the *HR6B* gene (an

ubiquitin-conjugating DNA repair enzyme) in the mouse, results in a spermatogenic defect at the level of post-meiotic condensation of spermatids (Roest *et al.*, 1996). Ectopic expression of avian protamine (galline) in spermatids of transgenic mice, results in disruption of sperm nuclear condensation, and infertility (Rhim *et al.*, 1995).

It is well known that, contrary to other mammals studied, 15% of human sperm protein content is comprised of histones rather than protamines (Tanphaichitr *et al.*, 1978). Whether these histones are associated with specific DNA sequences and act as markers for genes that are poised for early expression after fertilization (Sawicki *et al.*, 1981; Gardiner-Garden *et al.*, 1997), or whether the sperm histones are a sign of imperfection of human spermatogenesis, is unknown. The study of the nucleoprotein composition of human spermatozoa and its relation to infertility is still in its early stages. Numerous studies using a number of different techniques have indicated the presence of a larger histone fraction in the spermatozoa of infertile males (Chevaillier *et al.*, 1987; Hofmann and Hilscher, 1991; Foresta *et al.*, 1992). Suboptimal rearrangement of nucleoproteins during spermatogenesis or sperm maturation, resulting in a relatively higher sperm histone content undoubtedly has a multitude of possible causes. It is highly likely that a number of genetic mutations can, directly or indirectly, result in a defective step in the histone-to-protamine transition process. Further evidence for defective processes in nucleoprotein rearrangements during human spermatogenesis is given by a number of studies that have shown differences in the protamine 1/protamine 2 ratio in spermatozoa from fertile and subfertile males (Khara *et al.*, 1997; Yebra *et al.*, 1998). Mutations in transition proteins or protamines, however, have not yet been found in infertile males (Schlicker *et al.*, 1994).

It has been shown that a number of endogenous and exogenous factors can result in the production of morphologically abnormal spermatozoa or in a higher fraction of spermatozoa with DNA damage, none of these studies have attempted to elucidate specific changes in sperm nuclear protein content (el-Gothamy and el-Samahy, 1992; Foresta *et al.*, 1992; Hou *et al.*, 1995; Shen *et al.*, 1997). Differences in the sperm nuclear protein content of subfertile males may provide an insight in human spermatogenic dysfunction. We have characterized an antibody that specifically targets testis-specific histone 2B (TH2B) in human testis tissue and human spermatozoa (Chapter 7). Our studies indicate a highly variable inter-individual sperm TH2B content, that marginally correlates with sperm morphology. Furthermore, a highly hetero-

geneous expression of TH2B was observed in spermatozoa of a single ejaculate. It is tempting to speculate that certain causes of spermatogenic dysfunction (such as genetic or hormonal dysregulation, or exposure to heat or toxins) may result in deviation from normal sperm nuclear protein composition. If this indeed proves to be the case for even a small number of causes of male infertility, determination of the sperm nuclear protein composition may become a useful diagnostic tool.

Summary

Spermatogenesis and sperm maturation are both processes that require fine-tuned interplay of a large number of processes. The mechanisms involved in control of male reproductive physiology have been the subject of intensive research and many important factors have now been identified and studied in laboratory animals and in man. A brief introduction to the current understanding of male sexual development and reproductive physiology is given in **Chapter 1**.

In **Chapter 2** a review is presented, regarding the regulation of spermatogenesis and sperm maturation by androgens. Many years ago, it was realized that testosterone is one of the prime regulators of male reproductive function. Testosterone is synthesized by the Leydig cells of the testis under the influence of pituitary LH, and is of vital importance for the morphogenesis of the male phenotype, including the initiation and maintenance of spermatogenesis and sperm maturation. The prime targets for testosterone in the testis are the Sertoli cells, the peritubular myoid cells, and the Leydig cells. Germ cells do not express the androgen receptor and are thus unlikely to be directly influenced by androgens. The precise role of androgens in spermato-genesis, however, remains unclear. The testis needs a much higher level of testosterone for normal spermatogenesis, compared to what is needed for normal function of other androgen-dependent organs, such as the prostate and seminal vesicles. In many androgen dependent tissues, testosterone is converted into the more potent 5α-dihydrotestosterone (DHT) through the activity of 5α-reductase. The activity of 5α-reductase is very low in the testis. The epididymis, which is highly dependent on 5α-reductase activity for normal function, is a unique organ, in that it obtains testosterone directly from the testicular fluids via the ductuli efferentes, and indirectly via the bloodstream. Stimulation by androgens is essential for the synthesis and secretion of a number of proteins by the epithelial cells of the epididymis, that are vital for sperm maturation and sperm storage.

In **Chapter 3** a study is presented, aimed at disclosing the role of testosterone metabolism and androgen binding protein (ABP) in the rat testis. In order to create an animal model, to specifically study the effect of varying doses of androgen on spermatogenesis, mature Leydig cells were destroyed using ethane dimethane sulfonate (EDS). A GnRH antagonist (GnRHa; Org 30267) was given to reduce LH stimulation, and thereby the differentiation,

of remaining immature Leydig cells. To ensure adequate FSH levels in all treatment groups, all rats were supplemented with recombinant human FSH (rec-hFSH; Org 32489). Rats were treated with different doses of either testosterone propionate (Tp) or methyltrienolone (R1881). R1881 is a synthetic androgen that is not metabolized and does not bind to ABP. The dose-response curve (maintenance of testis weight) of the accessory sex organ weight of rats treated with Tp showed a far more pronounced rise to increasing Tp doses, compared to that of testis weight. At a serum testosterone (T) level of 1.6 ng/ml, the accessory sex organ weight was not different from that of control animals. Testis weight, however, was less effectively maintained, and was still significantly lower than control at a serum T level of 6.1 ng/ml, indicating that the rat testis needs much a higher level of testosterone for maintenance of organ weight, compared to the accessory sex organs. Similar dose-response curves were observed, however, for the accessory sex organs and the testis of rats treated with R1881. Additional experiments, in which Leydig cell depleted rats were sup-plemented with Tp and treated with finasteride (a potent 5α-reductase inhibitor), showed a similar response of the testis and accessory sex organs. These obsevations indicate that the difference in 5α-reductase activity in the accessory sex organs, compared to the testis, results in the need for a relatively high level of testosterone in the testis for normal function.

Epididymal cell function is highly dependent on stimulation by androgens. **Chapter** 4 describes results of experiments on the expression of androgen receptor mRNA and protein, as studied in the rat epididymis. Androgen receptor immunoexpression in the epididymal epithelial cell nuclei is weak in the efferent ducts. The nuclei of the epithelial cells of the initial segment, caput , corpus, and cauda show a more intense immunosignal. Ligation of the efferent ducts did not result in a lower intensity of expression of androgen receptor in the caput or cauda as studied by immunoblotting. Withdrawal of testosterone by castration, resulted in a marked decrease of androgen receptor immunoexpression in all epididymal cells. Interestingly, adjacent and morphologically identical, epithelial cells, display a heterogeneous expression of androgen receptors. Androgen receptor mRNA was visualised by in situ hybridisation in control rat epididymis. AR mRNA level was hight in the intial segment, and is relatively lower further distal. In consecutive 8αm sections, alternately stained for AR protein and AR mRNA, regions with high AR immunoexpression appeared to correlate with regions of high AR mRNA

content, indicating that the cell-to-cell heterogeneity in AR expression may be regulated at the transcriptional level. In addition, double-staining studies were performed using antibodies targeting the AR and a highly androgen dependent protein; proteins B/C. Surprisingly, no correlation was found between the cellular expression of AR and proteins B/C. High levels of nuclear AR staining were observed in epithelial cells devoid of proteins B/C, and vice versa. The implications of these observations are discussed in Chapters 4 and 8.

When any part of the intricately regulated series of molecular events within the male reproductive system dysfunctions, male infertility may ensue. It is estimated that, world-wide, about 60 to 80 million couples are unable to conceive a child. In 50% of these couples, the male partner has abnormal semen parameters according to the WHO criteria. While a number of primary and secondary causes of male infertility are known, a large portion of infertile men who visit the andrology clinic have subnormal semen parameters, for which no cause can be found (idiopathic infertility). It is increasingly clear that many men with idiopathic infertility may have an underlying genetic defect, resulting in spermatogenic impairment. Modern assisted reproductive techniques, especially intracytoplasmic sperm injection (ICSI), may result in transfer of genetic defects to the offspring, possibly resulting in infertility in the male progeny. A better understanding of the genetic determinants of male infertility and possible secondary effects of testis dysfunction on the integrity of sperm DNA, is highly desirable. This knowledge may help the medical community to provide couples with adequate counselling, and assisted reproductive techniques with the lowest possible risk to the offspring.
Androgen insensitivity has been implicated as a causative factor in otherwise well androgenized men. In previous studies on the role of androgen insensitivity in male infertility, the androgen receptor was studied in non-testicular tissue. As shown in Chapter 3, the testis responds differently from other androgen dependent organs when it comes to the response to testosterone. In **Chapter 6**, a study is presented where androgen receptor immunoexpression was studied in testes of 37 subfertile men, using a polyclonal antibody. Androgen receptor immunoexpression is observed in the nuclei of Sertoli cells, Leydig cells, peritubular myoid cells and in cells associated with arterioles. Germinal cell nuclei are negative in every stage of development. Interestingly, while in the rat testis the intensity of androgen receptor

immunoexpression in Sertoli cell nuclei is dependent on the stage of the spermatogenic cycle, no such phenomenon could be discerned in the human testis. In all stages of the human spermatogenic cycle, Sertoli cell nuclei could be found that showed either intense or weak staining. The intensity of androgen receptor immunostaining in Sertoli cell nuclei was not related to the quality of spermatogenesis, as assessed by the Johnsen criteria. There was a wide variation of androgen receptor immunoexpression in Sertoli cells in testes of men with all gradations of spermatogenic defects. A clear relation of androgen receptor expression and male infertility could not be established in this population.

Impairment of testicular function may cause a decrease in sperm production, together with a decrease in sperm motility and morphology; a condition known as oligo-astheno-teratozoospermia. Many infertile men presenting with such semen parameters have no apparent other abnormalities, nor can a cause for this condition be found. Derailment of spermatogenesis, independent of its cause, often results in imperfections in the replacement of histones by protamines in the sperm nucleus, possibly resulting in a higher risk of sperm DNA damage. In **Chapter 7**, a study is presented where an antibody targeting tyrosine hydroxylase, which cross-reacts with rat testis-specific histone 2B (TH2B), was used to study the expression of TH2B in the human testis. First, it was established that the antibody indeed specifically stains TH2B in the human testis, using Western blot, acid-urea-Triton gels and immunohistochemistry. TH2B immunoexpression was found in all human germinal cells, including spermatogonia. In condensed spermatids and spermatozoa, TH2B immunoexpression could only be discerned after decondensation of the nuclei. Spermatozoa of 35 infertile men were analysed for TH2B content. Using Western blot, a marked inter-individual variation of TH2B content in spermatozoa was found, that marginally correlated with sperm morphology. The lower the fraction of morphologically normal sperm within an ejaculate, the more prominent the TH2B signal. Furthermore, using immunocytochemistry, a clear intercellular heterogeneity in sperm TH2B signal was observed within ejaculates. The significance of this observation, and the fertilising ability of spermatozoa with high TH2B content remains to be established.

In **Chapter 8**, the findings presented in this thesis, and their clinical implications are discussed in relation to current literature and knowledge, in the area of molecular and clinical andrology.

Samenvatting

Spermatogenese en zaadcelrijping zijn afhankelijk van een nauwkeurig gestuurde wisselwerking tussen een groot aantal moleculaire processen. De moleculair biologische grondslag van de mannelijke voortplantingsfysiologie is al jarenlang onderwerp van intensief wetenschappelijk onderzoek. Veel van de factoren die hierbij zijn betrokken zijn inmiddels bekend en bestudeerd, in mensen en proefdieren. **Hoofdstuk 1** betreft een korte introductie van de huidige kennis met betrekking tot de mannelijke geslachtsontwikkeling en voortplantingsfysiologie.

Hoofdstuk 2 geeft een overzicht van de regulatie van de spermatogenese en zaadcelrijping door androgenen. Het is inmiddels vele jaren bekend dat het steroidhormoon testosteron, een androgeen, een van de belangrijkste regulatoren van mannelijke voortplantingsfuncties is. Testosteron wordt door de Leydig cellen in the testis aangemaakt en uitgescheiden, na stimulatie van de Leydig cellen door het hypofysaire LH (luteïniserend hormoon). Het hormoon testosteron is van essentieel belang voor de ontwikkeling van het mannelijk fenotype, inclusief de totstandkoming en regulatie van de spermatogenese en zaadcelrijping. In de testis zijn het met name de Sertoli cellen, de peritubulaire myoid cellen en de Leydig cellen, die in hun functioneren door testosteron worden beïnvloed. De germinale cellen brengen geen androgeenreceptor tot expressie, zodat het niet waarschijnlijk is dat hun functie direct door androgenen wordt gereguleerd. De preciese rol van androgenen in de regulatie van de spermatogenese is echter nog steeds een raadsel. De testikels bevatten een zeer hoge concentratie testosteron, en het blijkt dat een hoge concentratie nodig is om spermatogenese te laten plaatsvinden. Andere androgeenafhankelijke organen, zoals de prostaat of de zaadblazen, kunnen normaal functioneren bij een relatief lage testosteron concentratie, zoals die in de circulatie wordt aangetroffen. In de meeste androgeengevoelige organen wordt testosteron door het enzym 5α-reductase omgezet in een meer actieve metaboliet, 5α-dihydrotestosteron (DHT), maar in de testis is de 5α-reductase activiteit laag. De epididymis (meervoud: epididymides), die voor adequate functie wel afhankelijk is van 5α-reductase activiteit, is een uniek orgaan, omdat het via twee routes testosteron krijgt aangeboden. Hoge concentraties testosteron komen vanuit de testis de epididymis binnen, via de ductuli efferentes. Tevens wordt de epididymis gestimuleerd door testosteron afkomstig uit de bloedvoorziening. Stimulatie

door androgenen is een absolute noodzaak voor de normale zaadcel-rijpings en -opslag functies van de epididymis.

In **Hoofdstuk 3** wordt een studie gepresenteerd, die beoogt een beter inzicht te geven in de rol van het metabolisme van testosteron, en de rol van androgeen bindend eiwit (ABP), in de spermatogenese. Om een proefdier-model te verkrijgen, waarmee specifiek het effect van verschillende hoeveelheden androgeen op spermatogenese kon worden bestudeerd, werden de mature Leydig cellen verwijderd door behandeling van volwassen ratten met EDS. Vervolgens werd een GnRH antagonist (GnRHa; Org30267) toegediend om de LH secretie, en daarmee de differentiatie van immature Leydig cellen, te remmen. Om adequate FSH spiegels te behouden, werden alle ratten met een humaan recombinant FSH (rec-hFSH; Org32489) behandeld. De ratten werden gesupplementeerd met verschillende doses testosteron propionaat (Tp) of een synthetisch androgeen, methyltrienolon (R1881). Van belang bij dit experiment, is dat R1881 niet wordt gemetaboliseerd en niet bindt aan ABP. Voor het effect van Tp op behoud van gewicht, was de dosis-response curve van de accessoire geslachtsklieren was beduidend steiler dan die van de testis. Dit impliceerd dat de testis minder gevoelig is voor testosteron dan de andere androgeen-afhankelijke organen. Echter de dosis-respons curves toonden in met R1881 behandelde ratten een vrijwel identieke helling, waarmee werd aangetoond dat de testis en de accessoire geslachtsklieren in gelijke mate gevoelig zijn voor dit synthetisch androgeen. Aanvullende experimenten, waarbij ratten werden behandeld met EDS en gesupplementeerd met Tp, met of zonder finasteride (een krachtige 5α-reductase remmer), maakten aannemelijk dat het verschil in testosteron-gevoeligheid tussen testikels en andere geslachtsklieren groten-deels wordt veroorzaakt door een verschil in 5α-reductase activiteit.
De epididymale epitheliale cellen zijn voor hun functie sterk afhankelijk van androgeen. In de experimenten beschreven in **Hoofdstuk 4** word de expressie van de androgeenreceptor (AR) onderzocht, door bestudering van de aanwezigheid van het AR mRNA en eiwit in de epididymis. De immuno-expressie van AR eiwit in de celkernen van de epitheelellen van de ductuli efferentes was zwak. De nuclei van de epitheliale cellen van de rest van de epididymis was duidelijk sterker gekleurd. Ligatie van de ductuli efferentes had geen duidelijk effect op de intensiteit van immunoexpressie van AR in de epididymis. Bij sterke verlaging van de serum testosteron waarden door middel van castratie, werd een duidelijke afname van de intensiteit van AR

immunoexpressie in alle epididymale cellen waargenomen. De epitheliale cellen van epididymides van onbehandelde ratten toonden een opvallende heterogeniteit in de mate van AR immunoexpressie. De intensiteit van de immunokleuring verschilde sterk, voor kernen van naast elkaar gelegen morfologisch identieke cellen. Expressie van androgeenrecepor mRNA in onbehandelde ratten, werd geanalyseerd door middel van *in situ* hybridisatie. AR mRNA expressie was hoog in de ductuli efferentes, en lager in de rest van de epididymis, waarbij eveneens een relatief intercellulaire heterogeniteit van de hoeveelheid AR mRNA werd gezien tussen aanliggende epitheliale cellen. Met opeenvolgende 8 µm dikke coupes, die alternerend werden gekleurd op AR immunoexpressie en AR mRNA expressie, werd aannemelijk gemaakt dat cellen met een lage intensiteit van AR immunoexpressie ook een lage AR mRNA expressie hebben. Dit zou een indicatie zijn dat de variatie in nucleaire AR eiwit expressie, in epididymale epitheliale cellen, op het niveau van de transcriptie wordt gereguleerd. Tevens werden er dubbelkleuringen uitgevoerd waarbij op dezefde coupes AR werd gekleurd alsmede het androgeenafhankelijke eiwit Proteins B/C. Opvallend genoeg was er geen enkele correlatie tussen de expressie van de AR en de expressie van Proteins B/C. Over implicaties van deze bevindingen worden in Hoofdstuk 4 en 8 gediscussieerd.

Het nauwkeurig gestuurde moleculaire samenspel dat ten grondslag ligt aan de mannelijke voortplantingsfuncies, kan ontregeld raken, resulterend in mannelijke subfertiliteit. Wereld-wijd, zijn naar schatting ongeveer 60 tot 80 miljoen paren onvruchtbaar. Bij de helft van deze paren heeft de mannelijke partner, volgens criteria van de werldgezondheidsorganisatie (WHO), semenafwijkingen. Ook al is tegenwoordig een groot aantal oorzaken van mannelijke infertiliteit bekend, een groot deel van de mannen die naar een andrologie polikliniek komen hebben semenafwijkingen met onbekende oorzaak (idiopathische infertiliteit). Het wordt in toenemende mate duidelijk dat een genetische afwijking ten grondslag ligt aan een groot percentage infertiliteitsproblemen bij mannen. Moderne geassisteerde voortplantings-technieken, met name intracytoplasmatische sperma injectie (ICSI), zullen er zonder twijfel aan bijdragen dat enkele defecten worden doorgegeven aan het nageslacht, met als mogelijk resultaat infertiliteit bij mannelijke nakomelingen. Een beter begrip van de genetische determinanten van mannelijke infertiliteit, en van mogelijke effecten van een afwijkende spermatogenese op het DNA van zaadcellen, is van groot belang om paren met een nog onvervulde kinderwens goed te kunnen informeren, alsmede om

de meest geschikte en veilige geassisteerde voortplantingstechnieken aan te kunnen bevelen.
Androgeenongevoeligheid door mutatie van de AR is een van de genetische afwijkingen die mogelijk aan mannelijke infertiliteit ten grondslag ligt. Voorgaande studies die soms wel en soms niet een verband aantoonden tussen een verminderde expressie van de androgeenreceptor en mannelijke infertiliteit, maakten gebruik van (genitale) huidfibroblasten. Zoals in Hoofdstuk 3 werd aangetoond, is de respons op testosteron van de testis anders dan die van andere androgeen-afhankelijke organen. Mogelijk komt een mild androgeenreceptor defect, dat kan leiden tot infertiliteit zonder aantasting van andere aspecten van het mannelijke fenotype, duidelijker tot uiting in de testis. In **Hoofstuk 6** wordt een studie beschreven waarbij, in testisbiopten van infertiele mannen, middels immunohistochemie androgeen receptor eiwit werd gekleurd en geanalyseerd. AR immunoexpressie werd waargenomen in de kernen van Sertoli cellen, Leydig cellen, peritubulaire myoid cellen en cellen geassocieerd met arteriolen. Germinale cellen waren negatief tijdens alle ontwikkelingsstadia van de spermatogenese. Tijdens de z.g. spermatogenetische cyclus werd geen stadium-afhankelijke variatie in intensiteit van AR immunoexpressie in de kernen van de Sertoli cellen aangetroffen, zoals dat wel bij knaagdieren wordt gevonden. Vanuit klinisch perspectief, was het interessant dat de intensiteit van AR immunoexpressie in de humane Sertoli cellen niet was gecorreleerd met de kwaliteit van de spermatogenese, zoals beoordeeld middels de Johnsen criteria. Er was een groot interindividueel verschil in de mate van AR immunoexpressie in alle gradaties van hypospermatogenese en microscopisch normale spermatogenese. Een duidelijke relatie tussen de mate van AR immunoexpressie en mannelijke infertiliteit kon in deze groep niet worden aangetoond.
Een aangetaste spermatogenese, door welke oorzaak dan ook, kan een verminderd aantal, verminderde beweeglijkheid, en een verslechterde morfologie van zaadcellen teweeg brengen; een beeld dat bekend staat als oligo-astheno-teratozoospermie. Mannen met een dergelijk semenbeeld hebben meestal geen andere afwijkingen, en vaak kan geen oorzaak voor deze situatie worden gevonden. Een aangetaste spermatogenese, ook al is de oorzaak niet primair genetisch, kan imperfecties in de compactie van het DNA in de zaadcellen veroorzaken, met een minder volledige vervanging van histonen door protamines. Dit zou een vergroting van de kans op zaadcel-DNA schade kunnen veroorzaken. In **Hoofdstuk** 7 wordt een studie gepresenteerd waarin immunoexpressie van testis-specifiek histon 2B (TH2B) in menselijke testis

en zaadcellen is bestudeerd, middels een antilichaam tegen tyrosine hydroxylase, dat een kruisreactie vertoond met TH2B van de rat. Allereerst werd aangetoond dat anti-tyrosine hydroxylase inderdaad ook kruisreageert met menselijke TH2B met behulp van 'SDS-PAGE' en Western blot, 'acid-urea-Triton gels', en immunohistochemie. TH2B werd aangetoond in alle germinale celstadia, inclusief de spermatogonia. In gecondenseerde zaadcellen was de TH2B immuno-expressie zichtbaar decondensatie van de zaadcelkern. Zaadcellen van 35 mannen werden geanalyseerd op TH2B inhoud. Door middel van Western blot werd een duidelijke variatie in zaadcel TH2B inhoud tussen de ejaculaten van de verschillende mannen gevonden, met een geringe correlatie met zaadcelmorfologie. Hoe beter de zaadcelmorfologie, hoe minder de TH2B inhoud. Tevens was er een duidelijke heterogeniteit in TH2B immunoexpressie in gedecondenseerde zaadcellen uit één ejaculaat. Het belang van deze waarnemingen, en of er een verschil in bevruchtend vermogen is tussen zaadcellen met een hoge danwel een lage TH2B inhoud, moet nog worden onderezocht.

In **Hoofstuk 8** worden de in dit proefschrift beschreven bevindingen besproken, in relatie tot de huidige literatuur en kennis, en in relatie tot klinische implicaties van het uitgevoerde onderzoek.

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Curriculum Vitae

The author of this thesis was born on December 27th 1964 in The Hague. In 1982 he successfully completed the International Baccalaureate diploma requirements at Ashbury College (Ottawa, Canada). After spending a year at the Vrije Hoge School in Driebergen, he started his medical training at the University of Utrecht in 1983. His medical degree was obtained in August 1992. In this time period, he participated in various research electives: a phase II study to evaluate a chemotherapy protocol in children with osteosarcoma (Prof. Dr P.A. Voûte, Emma Children's Hospital/AMC, Amsterdam); the role of Tuftsine in the activation of macrophages and secretion of interleukin-1 (Dr E. Tsehoval, Weizmann Institute of Science, Rehovot , Israel); *in vivo* distribution of liposomes in mice (Dr K.K. Matthay, University of California at San Francisco, U.S.A.); the role of Substance-P in activation of leukocytes (Dr C.J. Heijnen, Wilhelmina Children's Hospital, Utrecht). He was drafted into the military service in September 1992, where he served as a medical officer in the Royal Dutch Army at Oirschot. In September 1993 he started the research project presented in this thesis, at the department of Endocrinology and Reproduction, at the Erasmus University Rotterdam, under the supervision of Dr J.T.M. Vreeburg and Prof. Dr J.A. Grootegoed. The research was combined with clinical work at the department of Andrology, at the University Hospital Rotterdam, Dijkzigt, under the supervision of Dr R.F.A. Weber. In May 1997 he successfully completed the European Academy of Andrology (E.A.A.) registration examination for 'Clinical Andrologist'. Training in urology started in January 1996, with a two-year rotation at the department of General Surgery at the University Hospital Rotterdam, Dijkzigt (Prof. Dr H.A. Bruining). Since January 1998 he is a resident at the department of Urology at the University Hospital Rotterdam, Dijkzigt (Prof. Dr F.H.S. Schröder).

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Dankwoord

Graag wil ik iedereen bedanken die, op welke manier dan ook, mij heeft gesteund in het voltooien van dit proefschrift. Ik wil er graag enkele met name noemen.
Jan Vreeburg, als co-promotor en directe begeleider van dit proefschrift, ben jij het die mij de kunst van het wetenschappelijk onderzoek heeft bijgebracht. Jou kennis, alsmede jou immer geduldige en zeer zorgvuldige aanpak van wetenschappelijke vraagstukken zijn inspirerend en een belangrijk voorbeeld voor mij.
Anton Grootegoed, je encyclopedische kennis, de snelheid waarmee je gecompliceerde problemen overziet en het enthousiasme en de gedrevenheid die je altijd uitstraalt dwingen bewondering af. Ik heb je ondersteuning en kritiek altijd bijzonder op prijs gesteld. Ik ben je erg dankbaar voor het feit dat tegen het einde van de rit, je nimmer aflatende bereidheid om nog eens een manuscript te beoordelen tot een spoedige afronding van dit proefschrift heeft kunnen leiden.
Rob Weber, als hoofd van de Polikliniek/Laboratorium Andrologie en als co-promotor ben jij niet alleen mijn mentor geweest bij mijn opleiding tot klinisch androloog, maar ben je ook van grote steun geweest bij het ontwerpen en uitvoeren van de patiënt-gebonden experimenten. Je kritische kijk op voordrachten en artikelen heeft mij veel bijgebracht. Je gedrevenheid en visie ten aanzien van het ontwikkelen van een gedegen medische benadering en behandeling van mannen met andrologische problemen is zonder parallel in Nederland en dwingt respect af.
Marja Ooms, vele zorgvuldig uitgevoerde proeven zijn van jou hand. De gezelligheid in het lab en je morele ondersteuning in de tijd dat ik twee keer per dag bijtgrage ratten moest injecteren zal ik niet snel vergeten.
Andere collega's van E&V: Willy, Jos en Henk, bedankt voor de gezellige tijd en de kritische discussies tijdens de lab besprekingen.
Op de Polikliniek/Laboratorium Andrologie wil ik Gert Dohle, Frank Pierik en Salem Aid hartelijk danken voor de prettige en leerzame samenwerking.
De efficiëntie, patiënt-vriendelijkheid en gezelligheid op de Polikliniek/ Laboratorium Andrologie zijn voor een groot deel het resultaat van het werk en de aanwezigheid van Marijke, Chris en Mieke. Jullie bereidheid om extra werk te verrichten om de patiënt-gebonden studies gladjes te laten verlopen waren cruciaal voor het welslagen hiervan.

Graag ik ook graag een woord van dank richten aan professor dr H.A. Bruining. De mogelijkheid die u mij heeft geboden om in het Academisch Ziekenhuis Rotterdam, Dijkzigt mijn chirurgische vooropleiding te volgen heeft mij de gelegenheid gegeven om tijdens de drukke werkzaamheden regelmatig een sprint naar de faculteit te trekken om aldaar het nodige te verrichten om uiteindelijk dit proefschrift te kunnen voltooien.
Graag wil ik de leden van de promotiecommissie bestaande uit prof. dr Th.H. van der Kwast, prof. dr F.H. Schröder en prof. dr F.H. Comhaire hartelijk danken voor de snelle en kritische beoordeling van dit proefschrift.
Bij voorbaat is ook veel dank verschuldigd aan Hak van Nispen tot Pannerden en Bernard Noothoven van Goor die het op zich willen nemen als mijn paranymfen op te treden.
Theodora en Anne, mijn twee zusjes met wie ik in mijn schooljaren de wereld door reisde. Hun wil ik danken voor alle goede tijden en bijzondere momenten die er zijn geweest en die nog in het verschiet liggen.
Een bijzondere plaats in dit dankwoord verdienen mijn ouders. Samen hebben zij mij een zorgeloze, maar boeiende en uitdagende jeugd gegeven. Ik wist mij altijd gesteund in alles wat ik ondernam. Daarnaast is het ouderlijk huis nog steeds een warme en een gastvrije 'thuis'.
Sophie, jij hebt zonder twijfel het meest geleden onder dit boekje. Ik ben je dankbaar voor alle tijd die je mij hebt gegund om dit proefschrift in onze schaarse uurtjes samen thuis te schrijven. Je interesse, aanmoedigingen en steun zijn onmisbaar geweest. Ik verheug mij op de toekomst.