Male Genital Tract Development

looking at the protein side of life

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Promotor:
Prof. Dr. J. A. Grootegoed

Overige leden:
Prof. Dr. J. Trapman
Prof. Dr. S. L. S. Drop
Dr. N. Galjart

Co-promotor:
Dr. A. O. Brinkmann

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Often, all of the hard work produces little or nothing new…
Robert Longtin, 2003

Annem ve babam için
en voor Robin

Contents

Abbreviations

ACN	acetonitrile
AD	activation domain
AF	activation function
AFP	α-fetoprotein
AIPC	androgen-independent PC
AIS	androgen insensitivity syndrome
ALK	activin receptor-like kinase
AMH	anti-Müllerian hormone
AMT	accurate mass and time
AR	androgen receptor
ARA	AR activator
ARC	activator-recruited cofactor
ARE	androgen response element
C	degrees Celsius
p/CAF	p300/CBP-associated factor
CAIS	complete AIS
CBF-A	CArG-binding factor
CBP	CREB-binding protein
Cdc	cell division cycle
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovary
COS-1	monkey kidney
CREB	cAMP response element binding protein
2D	two-dimensional
DAX	dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome
DBD	DNA-binding domain
DCC	dextran-coated charcoal
2DE	two-dimensional gel electrophoresis
Dhh	desert hedgehog
DHT	5α-dihydrotestosterone
DMEM /F12	Dulbecco's modified Eagle's medium/F12 nutrient mix
DREAMS	dynamic range enhancement applied to MS
DRIP	VDR-interacting proteins
DTT	dithiothreitol
E	embryonic day, days post coitum
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
EST	expressed sequence tag
FCS	fetal calf serum
FGF	fibroblast growth factor
FHL	four-and-a-half LIM domain protein
FK506	immunosuppressive drug
FKBP	FK506-binding immunophilin
FOG	friend-of-GATA
FRAP	fluorescence redistribution after photobleaching
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
FTICR	Fourrier-transform ion cyclotron resonance
FTZ-F	Fushi tarazu factor
GAF	guanine nucleotide activating factor
GATA	zinc-finger transcription factor
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
G3PD	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor
GSF	genital skin fibroblast
GTF	general transcription factors
GTP	guanine triphosphate
HAT	histone acetyl trandferase
HDAC	histone deacetylase complexes
HGP	human genome project
HMG	high mobility group
HMS-PCI	high-throughput mass spectrometric protein complex identification
HPLC	high performance LC
hnRNP	heterogeneous nuclear r ibonucleoprotein
HSD	hydroxy steroid dehydrogenase
HSP	heat shock protein
HUPO	human proteomics organization
ICAT	isotope-coded affinity tag

IEF isoelectric focussing
Insl insulin-like growth factor
IPG immobilized pH gradient
KGF keratinocyte growth factor
large T large tumor antogen
LBD ligand0binding domain
LBP ligand binding pocket
LC liquid chromatography
LCM laser capture microdissection
LH luteinizing hormone
Lhx1 LIM homeobox domain
LIM cycteine rich consensus sequence
LNCaP lymph node positive prostate cancer
MALDI-TOF matrix-assistedlaser/desorption ionization time-of-flight
MAPK mitogen-activated protein kinase
MD Müllerian duct
MFVD mouse fetal vas deferens
MIS Müllerian inhibiting substance
MMTV-LUC mouse mammary tumor virus-luciferase
MR mineralocorticoid receptor
MS mass spectrometry
MSDB matrix science data base
MudPIT multidimensional protein identification technology
NADPH nicotinamide adenine dinucleotide phosphate
NCBI national center for biotechnology information
N-CoR nuclear receptor co-repressor
NLS nuclear localization signal
NR nuclear receptor
NTD NH2-terminal domain
OHF hydroxyflutamide
ORESTES open reading frame EST
ORF open reading frame
PAGE polyacrylamide gel electrophoresis
PAIS partial AIS
PAK p21 activated kinase
PC prostate cancer
PCR polymerase chain reaction
pI isoelectric point
ppm parts per million
PR progesterone receptor
PRK protein kinase C-related kinase
PSA prostate specific antigen
PSF penicillin/streptomycin/fungizone
RAR all-trans retinoid acid receptor
RhoGDI Rho guanine nucleotide dissociation inhibitor
RNA Pol II RNA polymerase II
RPLC reversed phase LC
RXR 9-cis retinoid acid receptor
R1881 methyltrienolone
SA serum albumin precursor
SAGE serial analysis of gene expression
SDS sodium dodecyl sulphate
SELDI surface enhanced laser desorption/ionization
SF1 Steroidogenic factor 1
SHR steroid hormone receptor
SMRT silencing mediator of RAR and TR
SOX9 SRY related HMG box 9
SRC steroid receptor co-activator
SRE steroid response element
SRY sex determining region on the Y
SSCP single-strand conformation polymorphism
StAR steroidogenic acute regulatory protein
SV simian virus
TFIIF transcription factor II F
TIF transcription intermediary factor
TIS transcription initiation site
TPI triosephosphate isomerase
TR thyroid hormone receptor
TRIP TR-interacting proteins
UGS urogenital sinus
UTR untranslated region
VDR 1,25-dihydroxy-vitamin D receptor
WD Wolffian duct
WT Wilm's tumor-associated protein

Chapter 1

General Introduction

Introduction

Androgens regulate a large number of developmental processes, starting in fetal life and going on all the way through puberty. The action of androgens is essential for development of the male genital tract in the embryo, and development of secondary male characteristics at puberty. In addition, androgens are important for maintenance of normal male functions in adulthood, such as spermatogenesis, sexual libido, and other aspects of the male phenotype. Lack of androgens or androgen action during embryonic development inevitably results in partial or more complete lack of virilization or feminization of the genital tract. Primary sex determination is strictly chromosomal, while gonadal hormones and their downstream effects are responsible for differentiation of genital tract tissues. This chapter describes the ontogeny of sex determination and differentiation, and the factors involved in androgen-regulated genital tract development. Furthermore, androgen receptor function and mechanism of action is described in more detail, as well as the involvement of the androgen receptor in different pathological conditions. Mouse models are being widely used to study processes and factors involved in genital tract development, and therefore throughout this thesis the situation for the mouse will be outlined and, where applicable, observations in humans will be referred to.

1.1 Sex determination

Development of a new individual starts with fertilization of an oocyte by a sperm cell. While the genome resulting from the two meiotic divisions in the oocyte will always contain an X chromosome, the sperm cell contains either an X or a Y chromosome. The subsequent sex determination of the embryo depends on the two inherited sex chromosomes. XX individuals are genetically predetermined to become females, whereas the presence of a Y chromosome determines male sex. The Y chromosome harbors the testis-determining region, to which the *Sry* gene was mapped, which triggers the undifferentiated gonads to develop as testes [1]. Regardless of chromosomal sex, sexual development in the embryo is preceded by an indifferent stage, in which bipotential gonads and anlagen for both the male and female reproductive tract, Wolffian and Müllerian ducts respectively, are present. At this stage, the bipotential gonad can still develop into either an ovary or a testis, depending on the regulatory mechanisms [2-4]. In humans, embryonic and fetal development of a new individual takes 9 months, with the critical period of gonadal development between days 37 and 43, whereas development in mice takes 19-21 days, in which development of the gonadal anlage occurs around embryonic day 9 (E9) to E11. Morphologically, the gonads are formed as part of the urogenital system, which is derived from the intermediate mesoderm and can be divided into the pronephros, mesonephros, and metanephros, and associated duct systems. The indifferent gonads originate from a thickening of the ventrolateral surface of the embryonic mesonephros, the so-called genital ridge, which is visible in mice at E10. The genital ridge is composed of somatic cells and primordial germ cells, which originate from the epiblast, and move to the extraembryonic ectoderm in the yolk sac, from where they migrate to the site of the genital ridge [5,6] (Figure 1.1). Genotype analysis of human sex-reversed individuals, mutation analysis, and genetically engineered mouse-model systems, have revealed that the formation of the gonads and the process of sex determination and differentiation is tightly controlled by genes. What role these genes play in sex determination is not always clear, but it is likely that multiple factors collaborate in a network that activates downstream targets, which in turn feed back on upstream factors. So far, at least four autosomal genes, *Sf1, Wt1, Lhx9,* and *Lim1,* have been identified that are

required for proper development of the indifferent or bipotential gonad, and more are being identified for which a role in gonadogenesis is implicated but not yet completely understood, as reviewed in [3,4,7,8].

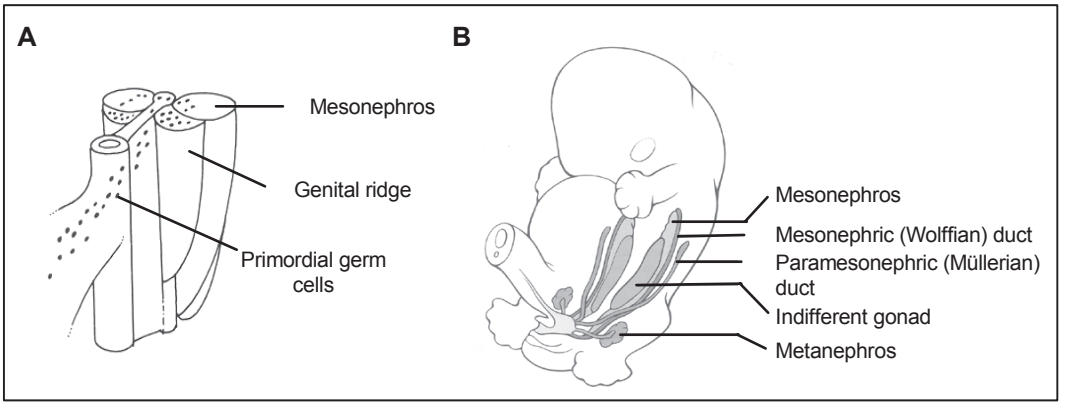

Figure 1.1. Schematic representation of urogenital tract development
A) Migration of the primordial germ cells from the yolk sac to the genital ridges.
B) The genital ridges, which are composed of somatic cells and primordial germ cells, develop into indifferent gonads. Figure adapted from Larsen, 1993 [9].

Genes involved in gonad formation

Expression of steroidogenic factor 1 (SF1) is associated with tissues and organs of endocrine function, such as gonads, adrenals, pituitary, and hypothalamus. SF1 is detectable in genital ridges of both sexes from approximately E9 [10] and is essential for gonadogenesis, since *Sf1* gene knockout mice lack adrenal glands and gonads [11,12]. SF1, encoded by the *Fushi tarazu factor 1 (Ftz-f1)* or *Sf1* gene, belongs to the family of orphan nuclear receptors (see Chapter 1.3) and regulates expression of a variety of steroidogenic enzymes in adrenal glands and gonads [13]. The exact role of SF1 in gonadal development remains unclear, since *Sf1* gene knockout mice develop genital ridges which are colonized by primordial germ cells, but the gonads subsequently fail to develop further after E11.5 and degenerate due to apoptosis [11]. Recent studies in humans with mutated SF1 revealed that SF1-dependent developmental pathways are exquisitely sensitive to gene dosage [14].

The Wilm's tumor-associated gene (*Wt1*) is very complex and encodes a variety of protein products with different functions. The WT1 proteins are zinc-finger containing factors, which are homologous to members of the early growth response (EGR) family, and can act either as repressors or activators of transcription [15]. WT1 mRNA is detected in the genital ridge of both male and female mice as early as E9.5 and remains highly expressed during gonadal development [16]. The involvement of WT1 in gonadal development is demonstrated by *Wt1* knockout mice, which show an arrest in gonadal and kidney development [17]. While heterozygous *Wt1* +/- mice are unaffected, children with a heterozygous mutation in the *Wt1* gene exhibit genital malformations and are predisposed to developing Wilm's tumors, a childhood tumor of the kidney [18]. These studies in humans suggest that WT1, like SF1, functions in a gene-dosage dependent manner.

The LIM homeobox domain gene *Lhx9* is essential for mouse gonadal development, since lack of Lhx9 function results in absence of gonads, and consequently male-to-female sex reversal and infertility [19]. In both sexes, Lhx9 is expressed in the genital ridge from E9.5

onwards, and by E13.5 Lhx9 is highly expressed in the mesothelial layer and outer part of the testis. In the E13.5 ovary, Lhx9 is mainly expressed in the cortical region [19]. Lhx9 expression is down-regulated at the time that epithelial cells in the gonads differentiate into either Sertoli or granulosa cells. Therefore, Lhx9 expression can be inversely correlated with the commitment into a differentiation pathway [20]. Unlike other genes important for gonadogenesis, such as *Wt1* and *Sf1*, knocking out *Lhx9* does not result in any other major developmental defects, implicating a specific role for Lhx9 in gonadal development. Furthermore, Lhx9 seems to function upstream of SF1 in the developmental cascade, since SF1 is not detected in Lhx9-deficient genital ridges [19].

Other homeobox proteins such as LIM1, Pax2 and EMX2 are also involved in early gonadal development [21-23], but their precise role is not yet determined. Like the other genes, *Lim1, Pax2,* and *Emx2* are expressed in the urogenital region during early urogenital development. *Lim1* knockout mice fail to develop kidneys and gonads [24], *Pax2*-deficient mice lack kidneys and urogenital ducts, but the gonads and adrenals are formed [22], and *Emx2* knockout mice also show impaired gonadal and kidney development [23].

Genes involved in gonadal sex determination

Once the gonads have been established from the genital ridge, they are morphologically identical for XX and XY mouse embryos until E12, and then they develop into either ovaries or testes, a process that is tightly controlled by programmed gene expression. The gonads harbor three cell lineages that have a bipotential fate depending on the sex of the organ. The supporting cell precursors give rise to Sertoli cells in the testis and granulosa cells in the ovary, the steroidogenic cell precursors differentiate into sex hormone producing Leydig cells or theca cells, respectively, and the connective cell lineages contribute to the formation of the organ as a whole. While testis formation is morphologically visible in the mouse at E12.5 by the appearance of testis cords, which include the primordial germ cells, differentiation of the ovary takes place later, and is morphologically visible in the mouse by E14.5 [3].

Sry is the only Y-chromosomal gene necessary for male sex determination. This was shown by experiments in which the genomic fragment, containing the *Sry* gene and flanking sequences, induced male sexual development in transgenic XX mice [25]. SRY is expressed in the urogenital ridge between a limited time window of E10.5-E12.5 [26,27]. This expression along the genital ridge occurs in a dynamic wave. As a result, individual cells are exposed to SRY at different time points and for a different length of time [28]. The action of SRY is at least three-fold: 1) triggering Sertoli cell fate for cells that would otherwise become granulosa cells [29], 2) inducing migration of cells from the mesonephros into the gonad [30], 3) inducing proliferation of cells within the genital ridge [31]. These actions are most likely mediated at the level of transcriptional control, since SRY is capable of bending DNA, by binding of its HMG (high-mobility group) domain, a domain which is also found in other transcription factors [32,33], to specific sequences. Although SRY acts as a switch for Sertoli cell identity, it is not involved in maintaining this identity, because its expression is only transient. Therefore, other genes downstream of SRY must be involved. One gene directly regulated by SRY is SOX9.

SOX9 (SRY-related HMG box) resembles SRY as it contains a similar HMG domain, but additionally two transcription activation domains are present within the SOX9 protein [34,35]. During early gonadal development, SOX9 is expressed at a low level in both male and female genital ridges. Around E11.5, following the onset of SRY, SOX9 is up-regulated in

the male gonad and down-regulated in the female, and continues to be expressed in Sertoli cells of the developing testis [36,37]. Because SOX9 is male-specifically up-regulated just before testis development and subsequently in Sertoli cells, it was proposed that SOX9 is a downstream target of SRY in the male-sex determining pathway. However, since females do not have SRY but do express SOX9 initially in the genital ridges, there must be other factors involved in switching on SOX9 [38]. All species tested so far show a conserved male-specific expression pattern of SOX9, which implies that SOX9 is a pivotal sex determining gene in all vertebrates. Ectopic expression of SOX9 in XX mouse gonads showed that SOX9 is sufficient to induce male development and indicates that it can substitute for SRY function [39].

Female sexual development has long been considered a default pathway, since XY embryos show male-to-female sex reversal in the absence of male determining factors. However, the identification of proteins like DAX1 and Wnt4 has changed this view, since they are considered to be necessary for the initiation of the female pathway of sex determination. DAX1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome, gene 1) is expressed in the genital ridge of both sexes at the same time as *Sry*, at E10.5, and becomes ovary-specific after E12 [40]. Duplication of the X-chromosomal region where *Dax1* resides is associated with dosage-sensitive sex reversalin humans, causing XY femaleness [41]. This suggests that DAX1 functions as an ovary-determining factor. However, *Dax1*-/- female mice have normally developed ovaries with only minor defects observed in the adult ovary, but *Dax1*-/- male mice have impaired spermatogenesis [42]. In addition, crossing *Dax1*-/- mice to a mouse strain known to be susceptible to XY sex reversal because of an altered *Sry* allele, resulted in anovulatory XY females [43]. Thus, DAX1 appears to play a role in both ovary-determination, as well as testis development, and these effects are highly dosage-sensitive. DAX1 is an X-linked member of the nuclear hormone receptor family [44], and is shown to inhibit transcriptional activation by SF1 *in vitro* [45]. This inhibition most likely occurs through protein-protein interaction between DAX1 and SF1. The interaction functionally opposes the effect of WT1 on testis development mediated by SF1 transactivation [46]. It is proposed that a double dose of DAX1 inhibits the effects of SF1 and SRY, resulting in ovary development [43,47], whereas a single dose DAX1 stimulates SOX9 to induce testis development, and as a result absence of DAX1 results in defective testis development [43]. In the developing ovary, *Dax1* gene transcription is activated by β-catenin, a key signal transducing protein in the Wnt signaling pathway, acting in synergy with SF1 [48]. *Dax1* expression is down-regulated in *Wnt4*-gene disrupted female mice, which show masculinized internal genitalia, because of activation of steroid biosynthesis in the fetal ovary [49]. *Wnt4*-null ovaries, however, do not express Sertoli cell-specific genes, indicating that Wnt4 is not a general repressor of testis determination but rather a suppressor of steroidogenesis. Thus, Wnt4 signaling mediates increased expression of DAX1 in the differentiating ovary and is crucial for female sexual development [48,49].

More and more factors are being identified that appear to play a role in sex determination and differentiation, acting either upstream and/or downstream of Sry. One such a factor is GATA4, a member of the GATA zinc-finger transcription factors, that is present in the developing gonads around E11.5 in both males and females. GATA4 is highly expressed in developing Sertoli and granulosa cells, but is down-regulated in the XX embryo at E13.5 during histological differentiation of the ovary [50]. Although *Gata4* deficient mice die *in utero* between E8.5 and E10.5, before gonadal development takes place [51], studies in mice transgenic for a mutated *Gata4* show defective gonadogenesis due to an abbrogated

interaction with FOG2 (friend-of GATA 2), a co-factor for GATA4 [52]. Furthermore, in both *Gata4* mutated mice as well as *Fog2* -/- mice, SRY, SOX9, and steroidogenic enzymes could not be detected, while WT1 and SF1, which act upstream of SRY, were still expressed. Wnt4 expression in the developing ovaries was also unaffected [52]. These studies indicate that an interaction between FOG2 and GATA4 is required for gonadal development and that GATA4 has a role in male sex differentiation.

Disruption of the *M33* gene, encoding the M33 protein, in mice results in retardation of genital ridge development and male-to-female sex reversal. Gonadal growth defects are obvious at the time of *Sry* gene expression, suggesting that M33 may act upstream of SRY [53].

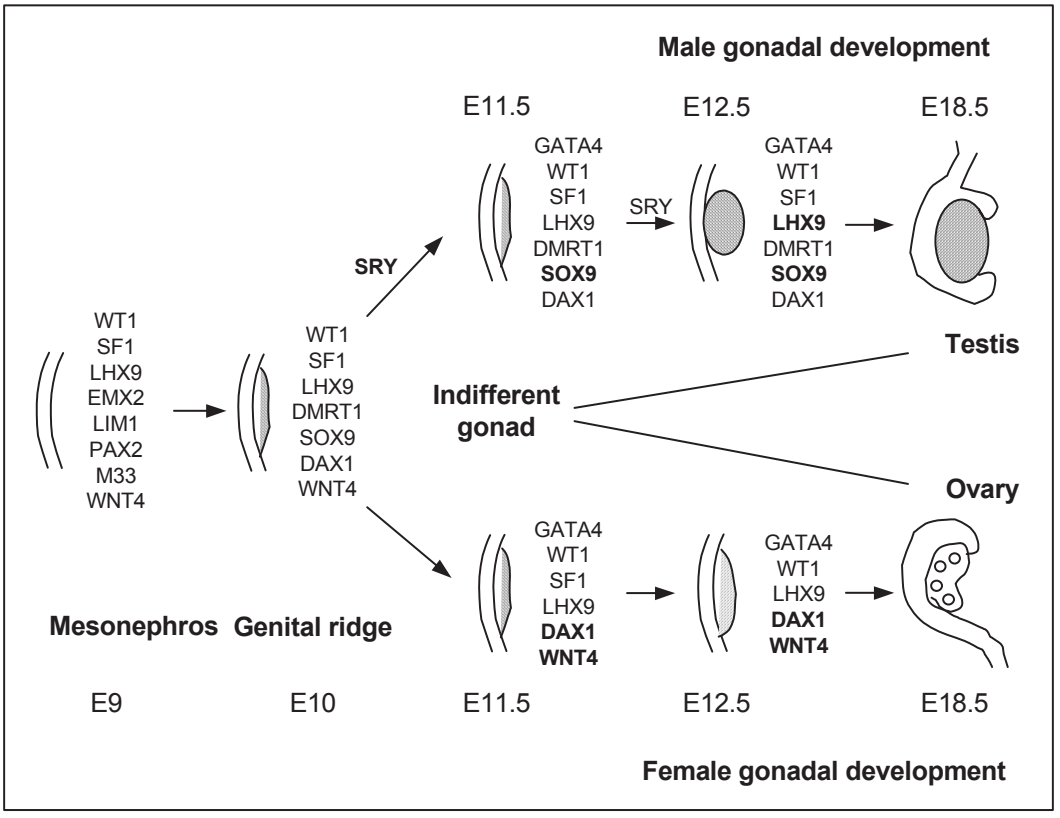

Figure 1.2. Factors involved in mouse gonad formation and differentiation
In both male and female gonadogenesis, formation of the genital ridge and indifferent gonad is under the control of the same set of genes. Around E11.5, male and female specific genes are either turned on or off in the respective tissues resulting in the development of ovaries and testes. Sex-specific up-regulatied genes are marked in **bold**.

Another gene that may function as a sex-determining factor in vertebrates, is *Dmrt1*, which is probably a target for *Sox9* [54]. DMRT1 is a protein with a DNA-binding motif (so-called DM domain), which is related to the sex-determining DM-domain proteins MAB3 in the nematode *Caenorhabditis elegans* and Double-sex in the fruitfly *Drosophila melanogaster*. DMRT1 is expressed at E9.5 exclusively in genital ridges of both XX and XY embryos but becomes male-specific during gonadal differentiation and its expression is restricted to the testis [55]. *Dmrt1* null mutations cause severe defects in testis differentiation, demonstrating

that its role is relatively downstream in mammalian sex-determination [56]. Recently, other DM-domain proteins have been identified, of which DMRT3 is highly expressed in males and DMRT7 is more highly expressed in females [57]. Thus, DM-domain proteins may be involved in both male and female sexual differentiation.

Other factors involved in fetal testis development include fibroblast growth factor 9 (FGF9) and desert hedgehog (Dhh). Fgfs regulate embryogenesis of several organs, including lung, limb, and anterior pituitary. FGF9 plays an important role in testicular embryogenesis, since lack of FGF9 results in male-to-female sex reversal in mice [58]. Dhh is expressed in Sertoli cells and Patched 1 in Leydig cells and peritubular cells [59,60]. Absence of Dhh in mice results in male infertility. Dhh-/- mice fail to up-regulate SF1, which results in defective Leydig cell differentiation and impaired spermatogenesis [61].

Recently, screening of sex-specific libraries has resulted in identification of 13 male and 2 female specific clones, of which 60% were novel sequences [62]. This implies that sex determination and differentiation involves many factors of which only a few have been identified so far (depicted in Figure 1.2).

1.2 Male sex differentiation

While sex determination is strictly controlled by genes, once the gonads have committed themselves to either male or female differentiation, gonadal hormones play an essential role in maintaining that commitment [2]. In the XY embryo that has received proper signals, the developing testis starts producing anti-Müllerian hormone (AMH), testosterone, and later insulin-like factor 3 (Insl3). AMH causes the Müllerian ducts (MD) to regress, while testosterone stabilizes the Wolffian ducts (WD) and triggers their differentiation into epididymides, vasa deferentia, and seminal vesicles, as reviewed by [4,63]. Insl3, together with testosterone, is responsible for the first phase of testicular descent, from the urogenital ridge to the abdominal bottom [64,65], which is followed by translocation of the testis into the scrotum. In the female genital tract, lack of testosterone results in the regression of WD, whereas absence of AMH triggers the MD to develop into Fallopian tubes, uterus and the upper part of the vagina. Furthermore, the urogenital sinus (UGS), genital tubercle, genital fold and swelling develop into the prostate gland, urethra, and external genitalia in males, and in the lower part of the vagina and external genitalia in females [4,63] (Figure 1.3, on page 21). The transition from the bipotential state to initiation of sex differentiation for murine WD and MD occurs at E14-15, while the earliest morphological differences between male and female UGS occurs at E17.5, when the prostate starts to differentiate.

Male development (virilization) of the genital tract starts with testicular production of AMH at E11.5 in the mouse fetus [66], which is responsible for the regression of the MD [67-69]. AMH, or MIS (Müllerian inhibiting substance), expression persists in the Sertoli cells of the male gonads and decreases after birth, whereas in female mice AMH is first expressed in granulosa cells of the ovary 4 days after birth [66,70,71].

The role of anti-Müllerian hormone

AMH is a glycoprotein belonging to the transforming growth factor β (TGFβ) gene family of growth and differentiation factors [73,74]. TGFβ family members mediate their actions via a membrane-bound heteromeric typeI/typeII serine-threonine kinase receptor complex. The AMH type II receptor is expressed at E15 in mesenchymal cells surrounding the MD in both males and females [75,76]. Recently, targeted disruption in mice has identified the bone morphogenetic protein receptor 1a, activin receptor-like kinase 3 (ALK3), as the

predominant AMH type I receptor in Müllerian duct regression [77]. It was suggested that ALK3 acts together with ALK2, which was previously identified as the type I receptor, to mediate AMH signals [77-79]. Both ALK2 and ALK 3 are ubiquitously expressed and also present in the mesenchymal cells surrounding the MD. The specificity of the AMH signal is thus regulated by sex specific, temporal control of the expression of AMH and tissue specific expression of its type II receptor in target tissues. The *in vivo* role of AMH and AMH type II receptor in MD regression was shown using knockout mouse models. These studies show that AMH is required for elimination of the MD, and also plays a role in male fertility by acting as a negative regulator of Leydig cell proliferation [80,81]. Regression of the MD is induced by AMH indirectly via the AMH type II receptor-expressing mesenchymal cells, eventually resulting in apoptosis of the epithelial cells [69]. Apoptosis of the duct occurs in a cranial to caudal fashion and is accompanied by accumulation of cytoplasmic β-catenin, which may mediate the action of AMH [69].

Expression of AMH during sexual differentiation is regulated by several of the factors involved in gonadal development described above (**Chapter 1.1**). These factors bind to the 180bp promoter fragment of the *Amh* gene [82], where SF1 plays a central role in transcription activation by cooperative interaction with SOX9 [83], GATA4 [84], or WT1 [46]. On the other hand, DAX1 interaction with SF1 results in repression of *Amh* gene expression [46,85], consistent with its role in ovary-determination. Recently, another factor, SOX8, has been identified that also synergizes with SF1 to enhance *Amh* expression [86]. SRY, SOX8, SOX9, and AMH show a similar spatial and temporal dynamic expression pattern in the developing gonads, providing a link between these genes in the molecular pathway of testis development.

In the adult testis, AMH is expressed at low levels [66,87], where it may play some role in regulating androgen synthesis in the Leydig cells. It has been found that AMH directly suppresses transcription of key enzymes involved in testosterone synthesis [88]. In the adult ovary, AMH may have at least two functions: inhibiting initial recruitment of primordial follicles into the pool of growing follicles [71], and decreasing the stimulatory effect of follicle-stimulating hormone (FSH) on follicle growth [89].

Role of androgens

Once Leydig cells have developed in the fetal testis, they start synthesizing testosterone as early as E13 in the mouse [90]. Testosterone is a steroid hormone and its biosynthesis takes place in the mitochondria of the Leydig cells through enzymatic conversion of cholesterol in a series of steps. Cholesterol is transported through the inner mitochondial membrane by the steroidogenic acute regulatory protein StAR, where it is cleaved by the cholesterol side-chain cleavage enzyme P450scc [91], and further processed by 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/c17,20-lyase (P450c17), and 17β-hydroxysteroid dehydrogenase (17β-HSD) [90,92]. In certain tissues, testosterone can be further reduced into the more potent 5α dihydrotestosterone (DHT) by 5α-reductase type 1 and type 2 enzymes, or aromatized into estradiol by P450 aromatase. In the fetal testis, all steroidogenic enzymes including P450 aromatase can be detected, showing the potential of fetal testis to convert androgens into estrogens. Although estrogens are not necessary for gonadal development or for the male urogenital structures, male mice deficient in estrogen receptor α are infertile because of abnormal sexual behaviour and reduced sperm counts [93,94]. Furthermore, mice lacking aromatase display arrested spermatogenesis [95]. This suggests a direct effect of estrogens on male germ-cell development and fertility [96]. In contrast, in

fetal ovaries none of the steroidogenic enzymes could be detected except for 3β-HSD [90], which explains why developing ovaries are not steroidogenically active.

Testosterone production and secretion in newborn mice and adult animals is regulated by pituitary gonadotropins and luteinizing hormone (LH), unlike initial fetal testosterone synthesis, which appears to be independent of LH since it already occurs before LH transcripts are detected in the pituitary [97]. Thus, testosterone dependent male fetal sexual differentiation does not require pituitary hormones [98]. Rather, initiation of steroidogenesis is considered to be regulated by sex determining factors, with SF1 as key regulator, because promoters of most of the genes encoding steroidogenic enzymes have binding sites for SF1 [12].

Testosterone delivery to its target tissues may proceed in two ways: 1) passive diffusion from the testis into the lumen of the epithelial duct, which results in a concentration gradient of hormone that is relatively high cranially in the WD and lower in the caudal region of the duct, or 2) secretion into the circulation, thereby reaching the UGS in only low amounts [99]. Testosterone is the principle androgen preventing the WD from regression by apoptosis, promoting differentiation of the WD into epididymis, vas deferens, and seminal vesicle. DHT is the major androgen involved in differentiation of the UGS, and tubercle into prostate gland, urethra, and external genitalia. However, conflicting data from human and rodent studies indicate that this concept of differential roles of testosterone and DHT is rather simplistic, and may not be true for all mammalian species.

Conversion of testosterone into DHT is catalyzed by two distinct isozymes, 5α-reductase type 1 and type 2 [100,101]. The type 1 and 2 enzymes have different biochemical and pharmacological properties, in a tissue-specific and cell type-specific manner [102,103]. The type 2 enzyme is expressed predominantly in tissues of the male urogenital tract and is thought to have a developmental anabolic role, whereas type 1 appears to be expressed mainly in peripheral androgen target tissues and has a catabolic role in androgen metabolism [103-105]. Furthermore, the type 1 enzyme seems to have a greater role in female development, as compared to the type 2 enzyme [106]. In the male urogenital tract, type 2 is present in mesenchymal cells and shows regional differences in expression level during development. The importance of 5α-reductase type 2 and the action of DHT in the developing UGS and external genitalia is observed in human individuals with steroid 5α-reductase type 2 deficiency. In this type of male pseudohermaphroditism, there is defective virilization of the external genitalia, and the prostate gland is underdeveloped, whereas WD structures have developed normally, demonstrating dependence of WD differentiation on testosterone [101,107,108] (**Chapter 1.4**).

In mice at E14.5 and at birth, very little type 2 activity is detected in the testis and in the cranial portion of the WD, the future epididymis. The caudal portion of the WD, which is to become the seminal vesicle, has much higher enzyme activity, and the middle portion, the prospective vas deferens, has intermediate enzyme activity. In the UGS, high type 2 activity can be measured [102]. Thus, along the WD a gradient of type 2 activity can be measured, increasing in cranial to caudal fashion, resulting in comparable high levels of DHT in the caudal WD and UGS. From these experiments in mice it was concluded that DHT may indeed be the major androgen responsible for virilization of the UGS, but also for initiation of seminal vesicle development, whereas both testosterone and DHT could be responsible for cranial WD development, although testosterone must have a dominant role because DHT levels may be too low [102,109].

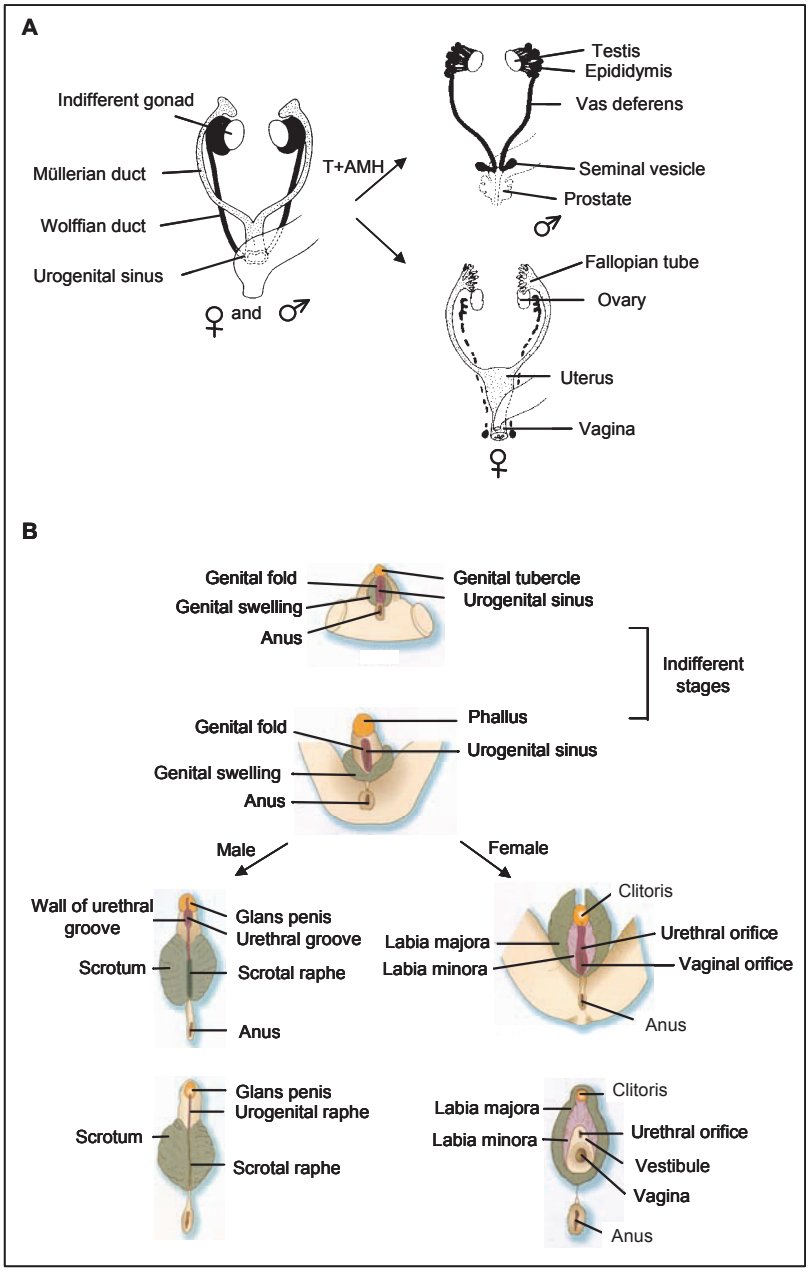

Figure 1.3. Schematic representation of sex differentiation of male and female internal and external genitalia

A) Early genital tract development is characterized by the presence of bipotential gonads, and the anlagen of both the male (Wolffian) and female (Müllerian) duct systems. Once the gonads differentiate into testes, testicular hormones are produced. AMH production causes regression of the Müllerian duct, whereas testosterone production results in the stabilization and development of the Wolffian duct into epididymis, vas deferens, and seminal vesicle. Absence of AMH and testosterone production by the ovaries results in the regression of the Wolffian duct and stabilization and development of the Müllerian into Fallopian tubes, uterus and the upper part of the vagina.

B) Development of the male and female external genitalia from the urogenital sinus, genital tubercle, genital fold, and genital swelling. Figure adapted from Carlson, 1999 [72].

However, gene knockout experiments targeting both type1 and type 2 enzymes in the mouse resulted unexpectedly in quite complete virilization of male mice [110]. Double-knockout males have only mild virilization defects, marked by a reduction in size of the accessory sex organs, while females were not affected. This phenotype demonstrates that, in mice, testosterone is sufficient for virilization of internal and external sex organs, and suggests that the conversion of testosterone to DHT represent a signal amplification mechanism, which is not of crucial importance in mice. For rats, this was already suggested by studies using 5α-reductase inhibitors [110,111]. Thus, the role of testosterone and DHT in male sex differentiation may be somewhat different between humans and rodents.

Although testosterone and DHT may act in different target tissues, they both bind to and exert their action through the same intracellular receptor, the androgen receptor (AR) [112,113], which will be discussed in more detail in **Chapter 1.4**. DHT binds to the AR with 10-fold higher affinity and biological efficacy than testosterone [114,115]. This explains how DHT can function as a signal amplifier of testosterone in tissues where the androgenic signal is low. However, when present at a high concentration, testosterone can interact with the AR in a similar manner as DHT [116].

During differentiation of the WD and UGS, the AR is expressed initially only in the mesenchymal cells surrounding the epithelial duct and sinus. In the mouse, AR expression is first detected at E12.5, prior to testosterone secretion by the testis, and this first expression is thus independent of androgens [117,118]. Continued AR expression, however, is androgen-dependent [119]. The differentiation of the WD occurs in a spatio-temporal and cranial to caudal fashion, starting with the development of the epididymis at E14.5, followed by the vas deferens and finally the formation of the seminal vesicle rudiment at E16.5. Development of the UGS starts at E17. During the initial period of WD and UGS development, no epithelial AR can be detected, although action of androgens at this stage is essential for virilization. Epithelial AR expression starts in the efferent ductules (located between the testis and the epididymis) at E16, becomes detectable in the epididymis at E19, in the seminal vesicle 1 day after birth, and in the prostate gland 4 days after birth. [117,118]. This observation led to the hypothesis that androgenic effects must be mediated through paracrine interactions between the AR-positive mesenchyme and the AR-negative epithelium [120,121]. Tissue recombination experiments using urogenital tract epithelium and mesenchyme from either wild-type mice or mice carrying a non-functional AR (testicular feminization mice, Tfm) have provided valuable information about the inductive nature of the mesenchyme. For example, seminal vesicle mesenchyme induces morphological and functional seminal vesicle development of epithelium from the embryonic and postnatal WD, regardless whether it is the caudal or the cranial part of the WD [109,122]. Even differentiated epithelium from the adult urinary bladder can be induced by urogenital sinus mesenchyme to undergo prostatic development [123]. These studies demonstrate that the mesenchyme is a key androgen target tissue during development, and many androgenic effects in the epithelium are elicited through paracrine influences from the mesenchyme (Figure 1.4). In addition, it has been shown that androgens regulate differentiation of a smooth muscle layer in the developing prostate, which is formed by differentiating mesenchymal cells probably in response to epithelial signals [124]. Differentiation of this smooth muscle layer may regulate signaling between mesenchyme and epithelium.

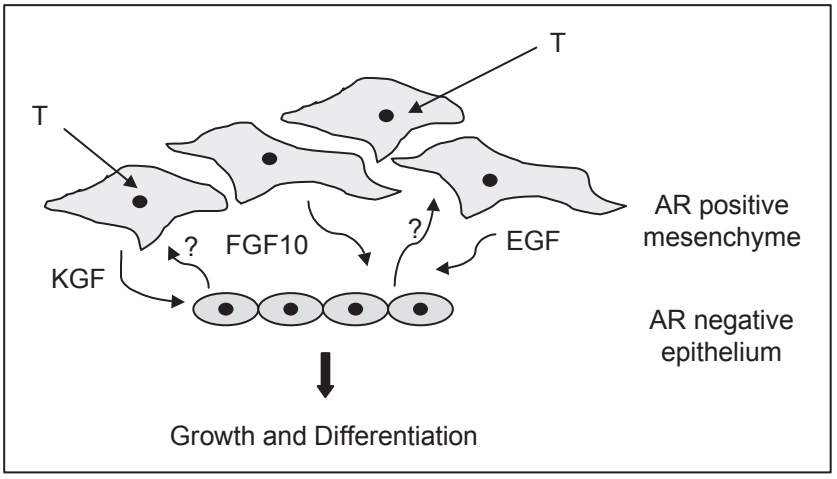

Figure 1.4. Schematic representation of mesenchymal-epithelial interaction
Androgens exert their actions on the developing genital tract tissues through the AR positive mesenchymal cells. Upon an androgenic signal, the mesenchymal cells produce and secrete paracrine factors, such as KGF, FGF10, and EGF, that act on the adjacent epithelial cells. It is believed that the epithelial cells also communicate with the mesenchymal cells.

Which and how paracrine factors are actually regulated by androgens is not yet clear. In the developing prostate several members of different growth factor families, such as insulin-like growth factors, platelet-derived growth factor, nerve growth factor, heparin-binding growth factor, transforming growth factor β, hepatocyte growth factor, epidermal growth factor (EGF), and fibroblast growth factors (FGFs), have been suggested to play a role in mesenchyme-epithelium interactions [122,125]. FGF7, also known as keratinocyte growth factor, was shown to play a key role in seminal vesicle [126] and ventral prostate development [127]. FGF7 is synthesized and secreted by mesenchymal cells and acts as a mitogenic factor upon adjacent epithelial cells of seminal vesicle and prostate, which express its receptor FGFR2iiib [128]. *In vitro* studies have shown that FGF7 can be regulated by androgens [129], but the gene is not a direct target for androgen action *in vivo*, although FGF7 may interact with androgen signalling [128]. Furthermore, *Fgf7* gene knockout mice do not appear to have an impairment of male reproductive tissues [130]. Thus, although FGF7 plays an important role in epithelium-mesenchyme interactions in the prostate, it is not regulated by androgens *in vivo*. FGF7 displays high homology and biochemical similarities with its family member FGF10 [131], which could make them functionally interchangeable and redundant. FGF10 is also expressed in mesenchymal cells and is associated with areas of active epithelial growth. In addition, FGF10 was shown to be regulated by androgen *in vitro* [132], but this observation does not hold for the *in vivo* situation [133], and it is not likely that the Fgf10 gene is a direct target for androgens. However, FGF10 is a key factor in seminal vesicle and prostate development, as deletion of the *Fgf10* gene results in loss of these organs [134]. It can be concluded that FGF10 functions as a paracrine regulator of growth and development of seminal vesicle and prostate [133].

The growth factor EGF was shown to be up-regulated by androgens in androgen responsive tissues, where it plays an important role in sex differentiation [135-139]. From E14 to E16, EGF is expressed in both epithelial and mesenchymal cells of the developing WD, but by E18 predominantly in epithelial cells [140]. EGF induces stabilization and differentiation of

the WD by binding to its receptor, EGFR, which is expressed in epithelial cells [141]. The involvement of EGF/EGFR in WD development was demonstrated in an *in vitro* organ culture system, where anti-EGFR antibodies blocked WD differentiation [142]. EGF may mediate the effect of androgens by acting as a paracrine factor on epithelial cells, but it could play a role in enhancing androgen-induced transcriptional activity in the mesenchymal cells, to potentiate the testosterone-effect [143].

Although many questions remain to be solved regarding the factors that are regulated by androgens in mesenchymal cells and the mediators that act on the epithelial cells, it is likely that many factors are involved in androgen-regulated development and differentiation of the male genital tract.

1.3 The androgen receptor

The androgen receptor (AR) belongs to the super-family of nuclear receptors (NRs), which regulate gene expression through their function as transcription factors, reviewed in [144-146]. The super-family of NR, sharing more than 150 different members, can be sub-divided into three classes on the basis of sequence homology and functional properties: class I) the steroid hormone receptors (SHR), to which the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), estrogen receptor (ER), and AR belong; class II) the receptors for thyroid hormone (TR), vitamin D (VDR), and retinoids (RAR, RXR); class III) the so-called orphan receptors, for which no apparent ligands have been identified so far, and of which some may represent constitutive activators/repressors of transcription [145]. Within the SHR class, the ER is more distinct than the GR, MR, PR, and AR, in the sense that it shows less sequence homology and recognizes hormone response elements different from those of the latter group (**Chapter 1.3.2**). NR homologues are present in multiple species, ranging from plants and insects to vertebrates. Therefore, it is believed that they have a common evolutionary history dating back at least tens of millions of years [147].

The first SHRs to be cloned were the GR [148], ER [149], and PR [150], which was followed by the cloning of the human AR by several groups [151-154], and the cloning of the rat [151,155] and mouse AR [156-158]. Alternative splicing occurs for the GR, resulting in the functionally different receptor isoforms GRα, GRβ, GRγ, and GRP [159-161]. For the PR, two distinct promoters were identified, which give rise to two mRNA products resulting in the expression of PR-B and a shorter PR-A [162,163]. For the ER, two distinct genes were cloned encoding ERα and ERβ [164,165], which have distinct functions *in vivo*. The *AR* is encoded by a single-copy gene [166]. It was reported that alternative initiation of translation results in a shorter AR protein product in genital skin fibroblasts, constituting 7-15% of total AR [167]. However, no functional role *in vivo* has been described for such an alternatively initiated AR protein product.

The *AR* gene is located on the human X chromosome at q11-q12 [168], and contains 8 exons spanning more that 90 kilo base (kb) [166] (Figure 1.5). Two mRNA species of approximately 10.6 kb and 8.5 kb, respectively, arise from the *AR* gene [153,169]. Both mRNAs contain a 1.1 kb 5'-untranslated region (UTR), and a 2.7 kb open reading frame (ORF) [169,170]. While the 10.6 kb transcript contains a very long 3'-UTR of 6.8 kb, the 8.5 kb transcript lacks part of the 3'-UTR [170]. The promoter region does not have typical TATA-box and CAAT-box sequence motifs, but it contains a long homopurine stretch, and a short CG-rich region, which is a recognition site for the transcription factor SP1. Two transcription initiation sites TIS I and TIS II are present in a 13-base pair (bp) region. While SP1 binding

to the GC-box initiates transcription through TIS II, initiation through TIS I depends on sequences downstream the SP1 recognition site [170,171].

The protein encoded by the *AR* gene has a variable length of amino acid residues between individuals, due to the presence of a highly variable poly-glutamine stretch and a less variable poly-glycine stretch, located in the NH2-terminal part of the protein [172,173]. Therefore, the AR has been described as a protein with either 910 [173], 917 [154,166], 918 [174], or 919 amino acid residues [152] with a calculated molecular mass between 98 and 100 kilo Dalton (kDa) and an apparent molecular mass of 110 kDa. On SDS-PAGE, the AR can be detected in the presence of androgens as a 110-112-114 triplet, which represents several phosphorylated isoforms of the receptor [175-177]. The numbering of the AR described throughout this thesis is based on 919 amino acids (Figure 1.5), according to the AR database (www.mcgill.ca/androgendb/) [178]. The AR protein is expressed in the male and female reproductive tract, and in non-reproductive target tissues [179]. AR protein expression is regulated by androgens at the transcriptional and post-transcriptional levels, and by follicle-stimulating hormone (FSH) [180-182].

1.3.1 Functional domains of the androgen receptor

Like other NRs, the AR has a well characterized, unique modular structure, consisting of a DNA-binding domain (DBD), a ligand-binding domain (LBD), a hinge region, and an NH2-terminal domain (NTD), which harbors several activation functions (AFs) (Figure 1.5). Each domain has a specific role in the functioning of the receptor. The DBD of the AR shows high homology (around 80%) to the DBDs of PR, GR, and MR, and to a lesser extent to ER; the LBDs of steroid hormone receptors are moderately conserved (around 50%), whereas the NTDs show no obvious homology (less than 15%).

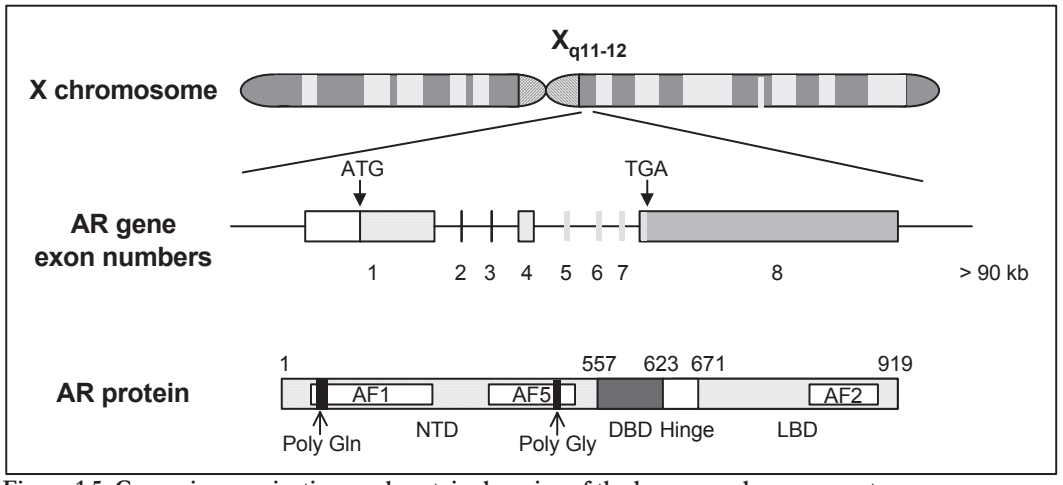

Figure 1.5. Genomic organization, and protein domains of the human androgen receptor
The AR gene is located on human chromosome X_{q11-12}, containing 8 exons, and spanning more that 90 kb. The gene encodes a protein of 919 amino acid residues and harbors several distinct domains, such as the NH2-terminal domain (NTD), the DNA-binding domain (DBD), the Hinge region, the ligand-binding domain (LBD), activation functions (AF), and a poly-glutamine and a poly–glycine stretch.

NH2-terminal domain

The NTD is encoded by exon 1 of the *AR* gene, which translates into the first 557 amino acid residues of the AR [166,173]. Besides the poly-glycine and poly-glutamine stretches, a large number of acidic amino acid residues are present in the NTD, between residues 100

and 325 [173,183]. In other proteins and transcription factors, acidic regions, which can form an amphipatic α-helical structure together with basic regions, have been shown to be involved in transcription regulation [184-186]. Deletion mapping of the AR NTD has revealed that amino acids 51-217 are involved in transcription activation, designated as activation function 1 (AF1) [187]. AF1 regions have been described for the other SHR, but their location within the NTD and amino acid composition varies. The AR NTD is the major determinant for androgen-specific regulation [188]. Furthermore, a COOH-terminally truncated AR displays constitutive transactivation activity which is regulated by a different region in the NTD, AF-5, located between residues 376-510 [189]. This indicates that the COOH-terminal domain has a repressive function in the full length AR in the absence of ligand, via AF5.

DNA binding domain

The DBD of the AR is encoded by exons 2 and 3, representing the central part of the receptor consisting of amino acids 557-623 [166,183]. The DBD is characterized by two zinc-cluster structures that are organized by three α-helices 1, 2, and 3 [190]. A zinc-cluster is formed by four conserved cysteine residues, which coordinate binding of a zinc-atom in the form of a tetrahedron, and facilitates binding to DNA and dimerization to a second AR [144,191]. Helix 1 in the first zinc-cluster contains the so-called proximal (P)-box and in addition residues that are involved in sequence recognition and specificity, and is inserted into the major groove of DNA [191]. The second zinc-cluster harbors helices 2 and 3, which contain the distal (D)-box and most of the residues involved in DNA-dependent dimerization [190,192]. Moreover, the DBD has been implicated in nucleo-cytoplasmic shuttling using a non-classical nuclear export signal, which is both sufficient and necessary for nuclear export [193].

Transcription factors in general are characterized by their ability to bind DNA at specific sites in promoter or enhancer regions of genes. The steroid hormone receptors AR, GR, PR, and MR are able to bind as homodimers to DNA at so-called steroid response elements (SRE), which are organized as inverted repeats of 5'-TGTTCT-3' like sequences with a three nucleotide spacer [194]. ER and other nuclear receptors recognize a specific estrogen response element (ERE) with the sequence 5'-AGGTCAnnnTGACCT-3' [195,196]. Since DBDs of steroid hormone receptors are highly conserved and recognize the same SREs, much attention has been focused on factors that determine receptor specificity. Using chimeric AR-GR DNA binding domains, it was postulated that AR specificity for response elements depends on the dimerization interface, which enables a head-to-tail dimerization of two AR proteins on a specific androgen response element (ARE) instead of a tail-to-tail dimerization on non-AR specific SREs [197] (Figure 1.6). Such alternative dimerization is only possible on direct repeat AREs. Furthermore, most SREs and AREs do not exactly fit the consensus, allowing individual sequence variations to increase specificity of gene regulation. Until now, numerous response elements in promoters of androgen-regulated genes have been analyzed, and based on these studies a classification of AREs has been proposed. Four different types of AREs represent: high affinity selective (A) and non-selective (B), and low affinity selective (C) and non-selective (D) elements [191]. Recently, it was determined that AR conformation varies depending on the bound response element, which in turn modulates AR function by adjusting responses to co-factors [198].

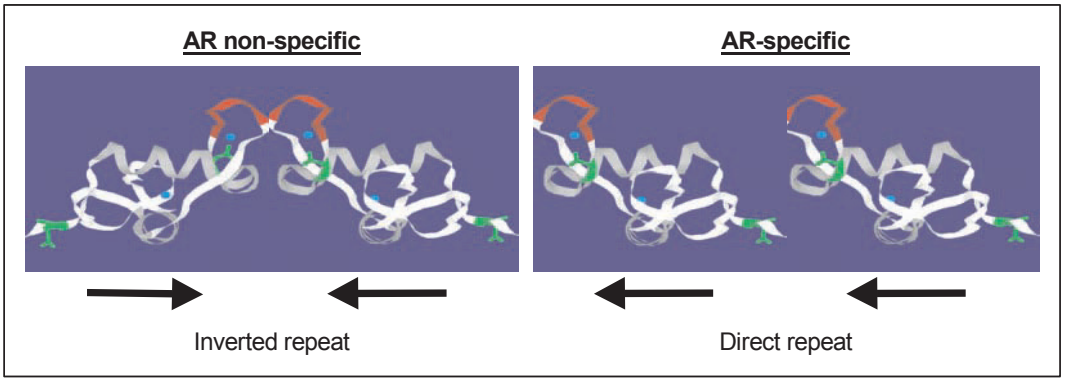

Figure 1.6. AR-DBD dimerization on direct and indirect repeat AREs
AR-DBD dimerization modeled as a head-to-head dimer on AR non-specific inverted repeat SREs, and as a head-to-tail dimer on AR-specific direct repeat AREs. Figure adapted from Claessens *et al.*, 2001 [191].

Ligand binding domain

The LBD is encoded by 5 exons, exons 4-7, and part of exon 8, and is located between amino acids 671-919 [166,183]. All nuclear receptor LBDs share the same 3-dimensional structure, which consists of 10-12 α-helices and 2 anti-parallel β-sheets that are folded into a triple layered α-helical sandwich, which is unique for the NR family. Crystallographic studies of several NR have revealed that the liganded receptor is more compact than the unliganded receptor. Upon ligand binding, a conformational change is induced, which causes helix 12 to fold back into the core of the LBD, thereby forming a lid of the ligand-binding pocket (LBP) [199]. The crystallographic structure of the AR LBD has been solved for the receptor complexed with the natural ligand DHT or with the synthetic androgen R1881 [200,201]. These crystal structures are very similar to previously proposed models of the AR LBD, with the exception that the COOH-terminal helix 12 is much longer than predicted from data obtained from other NRs, covering amino acids 892-908 [200-202]. Furthermore, the AR LBD consists of 9 α-helices, two 3_{10} helices, and four short β-strands associated in two anti-parallel β-sheets. One striking difference in the structure of AR LBD liganded with DHT or R1881 is the continuity of helix 12, which is split into two shorter helical segments in the presence of R1881 [200,201]. This shows that the AR LBD is a dynamic structure, whose conformation depends on the absence or presence of ligands and antagonists. Such dynamic conformations of the AR were already proposed based on limited proteolytic digestion experiments, in which digestion of AR complexed with either agonist or antagonist resulted in protection of protein fragments of different sizes [203].

An important function of the LBD is binding of androgen in the ligand binding pocket and transducing the signal via activation function 2 (AF2). As mentioned above, the conformational change induced by ligand binding repositions helix 12 onto the pocket as a lid, resulting in formation of an interaction surface for AR partner proteins and consequently in an active state of the receptor. If, however, the pocket is occupied by an antagonist, the AR LBD will have an altered conformation, which will be more suitable for recruitment of repressive co-regulators and consequently results in repression of activity. Most NRs have a strong ligand-dependent AF2 transactivation region, with a conserved sequence constituting an activation domain core region mapped to residues in helix 12 [199,204]. In contrast, the AR exhibits only a weak AF2 function, as was determined by deletion

studies [187]. However, the AF2 activation domain core region that contains the conserved sequence 893-Glu-Met-Met-Ala-Glu-Ile-Ile-Ser-900 appears to be essential for interaction with the NH2-terminal part of the receptor, and this interaction is necessary for maximum activation of the full-length receptor upon ligand binding [205-207].

The first implications for an interaction between the AR LBD and NH2-terminal domain came from the observation that AF1 becomes active in the presence of a ligand-bound LBD, whereas in a COOH-terminally truncated AR lacking the LBD, AF5 becomes active [189]. Subsequently, yeast two-hybrid interaction assays and functional transactivation assays in Chinese hamster ovary (CHO) cells showed that the AR NTD and LBD interact functionally in the presence of androgen, the so called N/C-interaction [208,209]. Deletion mapping and mutation analyses have revealed that the AF2 AD core domain interacts with a specific 23-Phe-X-X-Leu-Phe-27 (FXXLF) motif in the NTD [205,210,211]. This motif is part of a long amphipatic α-helix, which can bind in the hydrophobic interaction surface of the LBD by multiple charge interactions. Charged residues on either side of the hydrophobic cleft interact with oppositely charged residues flanking the FXXLF motif to mediate the ligand-dependent N/C-interaction [212]. Not only in *in vitro* transactivation assays is the N/C-interaction important for full-length AR activity, but it has been reported that N/C-interaction is a prerequisite for transcriptional activity on AR target gene promoters harboring non-specific AREs [213,214].

The AF2 region in the AR LBD is also involved in interaction with co-regulatory proteins (see 1.3.2), such as steroid receptor co-activators 1 and 3 (SRC1, SRC3), and transcription intermediary factor 2 (TIF2), which belong to the family of p160 co-activators [205,215,216]. Many co-activators possess a leucine-rich LXXLL signature motif, similar to but different from the FXXLF motif in the AR NTD, which facilitates binding to NRs [217,218], although some co-activators have been reported to contain an FXXLF motif with which they interact with the AR [219]. Interaction of LXXLL motif-containing co-activators with AF2 occurs in a similar fashion as for the FXXLF motif, using charged residues flanking the signature motif [212]. Co-activator binding facilitates an active state of the AR and enhances the ligand-dependent transcriptional activity through AF2 [216].

Hinge region

The LBD and DBD are connected by a flexible domain called the hinge-region, encoded in part by exons 3 and 4, which is poorly conserved between SHRs [144]. The hinge region of the AR contains a bipartite nuclear localization signal (NLS) located between residues 626-646, which directs nuclear transport of the receptor upon ligand binding [220,221]. Furthermore, an inhibitory domain between residues 628-646 has been identified that inhibits AF2 activity, either directly or indirectly, which can explain why AF2 function in the AR is rather weak compared to other SHR [222]. Mutations in this inhibitory region result in an enhancement of AF2 *trans*-activation by TIF2, suggesting that this domain exerts an inhibitory effect on co-activator-mediated AF2 function in the AR LBD [222].

1.3.2 Transcription regulation by the androgen receptor

The AR regulates transcription of androgen target genes, and the mechanism of regulation is in principle similar to that of other steroid hormone receptors, as illustrated in Figure 1.7. Testosterone enters the target cell by diffusion and binds, either directly or after reduction to DHT, to the intracellular AR. In the absence of androgen, the AR resides mostly in the cytoplasm [187,221], where it is held in an inactive state by an associated heat-

shock protein complex of HSP90, HSP70 [223], and FKBP52 [224], which mainly binds to the LBD . In the unliganded state, the receptor is constitutively phosphorylated on serine residue 94 [225]. Upon ligand binding, the HSP complex dissociates from the receptor [223], after which the AR becomes hyperphosphorylated which involves at least serine residues 16, 81, 256, 308, 424, and 650 [225]. The ligand-bound AR simultaneously translocates to the nucleus [187,220,221] where it dimerizes and binds to specific or non-specific AREs in promoter regions of target genes [216,226].

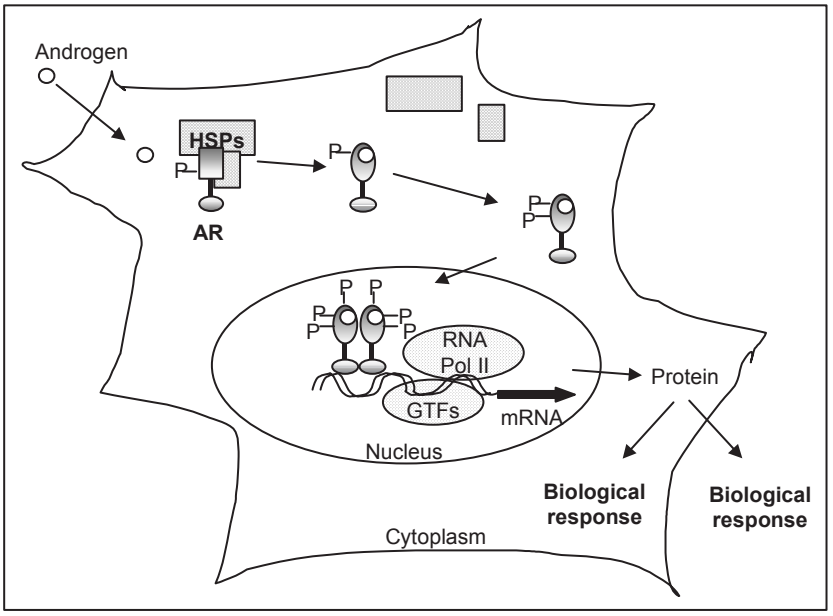

Figure 1.7. Mechanism of action of androgens
Circulating testosterone enters the cell via passive diffusion and binds either directly or after metabolism into the 5α-reduced DHT to the AR. Upon hormone binding, the HSP complex dissociates, the AR becomes phosphorylated and translocates to the nucleus. In the nucleus, dimerization and DNA binding takes place in the promoter region of target genes. In addition, general transcription factors, RNA polymerase II and other co-factors are recruited to initiate transcription activation.

Transcription initiation of androgen-regulated genes

One of the most studied androgen-regulated genes is the gene encoding prostate specific antigen (PSA). Its promoter has been characterized extensively [227-230], and the information obtained from studies performed on the *PSA* gene promoter has provided valuable clues as to how androgen regulates transcription via the AR.

Genetic studies have revealed that the *PSA* gene has a proximal promoter containing a TATA box and two AREs: ARE I and ARE II [227]. Furthermore, an enhancer element was identified, located approximately 4 kb upstream of the promoter, which harbors another ARE: ARE III [231]. Upon AR binding to ARE, general transcription factors constituting the transcription initiation complex, including RNA polymerase II (RNA Pol II), transcription factor II F (TFIIF), and the TATA box-binding protein are recruited, which interact with the AR NTD [232].

Using chromatin immunoprecipitation assays (ChIP), it has been recently described that AR recruits RNA Pol II preferentially to the *PSA* enhancer [229,230], which entirely depends on

the presence of androgen-liganded AR, and p160 and p300 cofactors (discussed below). These cofactors possess enzymatic activities, such as histone acetyl transferase (HAT) or methyltransferase that induce decompaction of local chromatin and thus allows for the transcription initiation complex to access DNA of the target gene. Histone acetylation has been correlated with transcriptionally active genes, whereas histone deacetylation is associated with a state of gene repression [233,234]. It has been postulated that, upon androgen-stimulated recruitment of the transcription initiation complex, the 4kb region between the enhancer and promoter is being "looped-out", facilitating an interaction between the enhancer and the promoter [228-230]. On the other hand, recent data by Louie et al (2003) suggest that the transcription initiation complex is cross-linked to the *PSA* enhancer, which facilitates tracking of the complex over the entire region to interact with the *PSA* promoter [230]. The occupancy of the *PSA* promoter and enhancer region by the AR transcription complex is transient and a cyclic process, and is mediated by protein degradation through the proteasome [228,229]. Using ChIP assays, it was demonstrated that the AR is recruited to the region within 15 minutes after hormone treatment, reaching a maximum at 45 minutes. A second cycle of AR recruitment started at 105 minutes after treatment, and lasted 45 minutes. RNA Pol II recruitment followed the same cyclic pattern but started approximately 15 minutes after AR binding [228].

Androgen receptor interacting proteins

Transcription regulation is dependent on the presence of multiple co-factors that interact either directly or indirectly with the androgen receptor. In the past few years a multitude of factors that modulate activity of the AR have been identified, reviewed in [216,235-237]. These AR interacting co-regulators are presented in Table 1.I according to the following categories: a) proliferation/apoptosis-associated proteins, b) RING proteins, c) LIM-domain proteins, d) co-activators, e) general transcription factors (GTFs), f) SET proteins, g) cytokine-associated proteins, h) chaperone-associated proteins, i) actin/cytoskeleton-binding proteins, j) membrane proteins, k) proteins involved in chromatin remodeling and acetylation, l) proteolysis/ubiquitination proteins, m) HMG proteins, n) RNA transcripts/protein interacting RNAs.

The different factors involved in regulating AR mediated transcription interact at all kinds of different levels within the AR signaling pathway: from chromatin remodeling to proteolysis of end products of gene expression. Most of the co-regulators identified so far interact with multiple NRs and only a small number shows specificity and selectivity for the AR, such as FHL2 [238]. Chromatin remodeling is achieved by factors that possess HAT or methyltransferase activity, or by factors that belong to ATP-dependent nucleosome remodeling (SWI/SNF) complexes, which unwind chromatin and make DNA more accessible for transcription factors [239-241]. p/CAF, CREB binding protein (CBP) and p300 have such intrinsic HAT activities and influence transcriptional activity of many classes of sequence-specific transcription factors [235], functioning in part by acting as molecular scaffolds, and in part by acetylating multiple substrates (Figure 1.8).

Table 1.I Androgen receptor co-regulators

Cofactor	Alternative name	Binding region	Comments	References
ANPK	PKY	DBD	Enhances AR protein stability.	242
ARA24 [a]	Ran	NTD	Interacts with the NTD polyglutamine repeat.	243
BRCA1 [a]	-	NTD, LBD	Interacts with CBP and enhances AR transcription synergistically with ARA70 and ARA55.	244
Cyclin E [a]	-	NTD	Enhances AR transcriptional activity independent of cell cycle progression.	245
pRb [a]	-	NTD, DBD	Tumor suppressor. Enhances transcription by AR and interacts with the TR co-activator Trip230 to repress TR transcription.	246
ARA54 [b]	-	LBD	Ligand dependent co-activator. Contains a RING finger. Also co-activates PR.	247
SNURF [b]	RNF4	DBD	RING finger protein; may recruit the chromatin remodeling factor HMGI(Y). Also interacts with ER and PR.	248
ARA55 [c]	Hic5	LBD	Ligand dependent co-activator. Contains a LIM domain. Also co-activates GR and PR.	249,250
FHL2 [c]	DRAL, Slim3	Requires intact AR	Prostate-specific and ligand-dependent co-activator of the AR.LIM only protein without LXXLL motif.	238
ARA70 [d]	RFG, ELE1	DBD, LBD	Ligand dependent co-activator. Bridging factor to p/CAF and TFIIB. Also co-activates PPARγ, and enhances ER and GR transactivation.	251,252
E6-AP [d,l]	-	Unknown	Contains separable co-activation and ubiquitin ligase domains. Also interacts with PR, GR, and ER.	253
Gelsolin [d,i]	-	LBD	Enhances AR transcription in prostate and muscle cells. Also functions as an actin filament severing and capping protein.	237
SRC-1 [d]	NcoA-1, p160	NTD, DBD, LBD	Enhances AR N /C- interactions, and interacts with CBP/p300. General nuclear receptor co-activator. Possesses weak acetyltransferase activity.	254-256
SRC-3 [d]	Rac3, ACTR, AIB1, p/CIP, TRAM1, NcoA-3	Unknown	Interacts with CBP/p300 and possesses acetyltransferase activity. Also enhances transcription by TR, PR, and RAR.	257-260
TIF2 [d]	GRIP1, NcoA-2, SRC2	NTD, DBD, LBD	General nuclear receptor co-activator. Facilitates N/C-interaction.	205,261-263
Ubc9 [d,l]	-	DBD, Hinge	Covalently links the ubiquitin-like molecule SUMO-1 to target proteins. This activity is separable from co-activation. Also interacts with GR.	264
Zac-1 [a,d,o]	-	LBD	Can function as a co-activator of AR in HeLa cells but as a co-repressor in 1471.1 cells. Also interacts with ER□, TR, and GR. In HeLa cells, co-activation is synergistic with TIF2.	265
ARA160 [e]	TMF	NTD	Functions synergistically with ARA70. Also enhances GR and PR.	266
ARA267 [f]	Nsd1	NTD, LBD	Contains SET and PHD domains. Also interacts with RAR, RXR, ER, TR.	267,268
ARIP3 [g]	PIASαx	DBD	Facilitates N/C-interaction. Represses trans-activation of the probasin promoter at high ratios.	269
PIAS1 [g]	-	DBD,LBD	Expression in the rat testes coincides with the onset of spermatogenesis. Also co-activates GR but functions as a co-repressor of PR.	270
BAG-1L [h]	-	Unknown	Enhances AR function, and also functions to regulate HSP70.	271
HSP40 [h]	dnaJ, ydj1p	LBD	Member of the chaperone heterocomplex. Mutation of hsp40 in yeast reduces AR transcriptional activation.	272
β-Catenin [i]	-	Unknown	Ligand dependent co-activator. Interacts also with FHL2 to activate Wnt-responsive genes, independent of AR trans-activation.	273,274
Filamin-A [i,o]	-	Hinge region	Involved in AR translocation to the nucleus. A Filamin-A fragment represses AR function by interfering with N/C-interaction and competing for TIF2.	275,276

Cofactor	Alternative name	Binding region	Comments	References
Supervilin [i]		NTD, LBD	Actin-binding protein. Also interacts with GR.	[277]
Caveolin-1 [j]	-	NTD, LBD	Membrane protein associated with caveoli membrane structures.	[278]
CBP [k]	p300	NTD, DBD	Facilitates N/C-interaction, possesses acetyltransferase activity, interacts with members of the SRC family. Co-activates multiple transcription factors.	[256,279,280]
RIP140 [k]	-	NTD, DBD, LBD	Functions as a co-activator at low receptor-co-activator ratios, but as a repressor at a high ratio. Influences the transcriptional activity of ER, PPARγ, and PPARα.	[281]
Tip60 [k]	-	Hinge, LBD	Member of the MYST/SAS family of histone acetyltransferases. Also co-activates PR and ER.	[282]
HMG-1/-2 [m]	-	Unknown	Enhances DNA binding of AR, PR, ER, and GR. Is found as an abundant chromatin-associated protein that does not bind a specific DNA recognition sequence.	[283]
PGC-1 [n]	LEM6	Unknown	General nuclear receptor co-activator.	[284]
SRA [n]	-	Unknown	Functions as an RNA transcript and associates with an SRC-1 containing co-regulator complex. Also enhances transcription by PR, GR, and ER.	[285]
Cyclin D1 [a,o]	-	Unknown	Reduces AR ligand-dependent transcription in a cell cycle-independent manner. Functions as a co-activator for ER.	[286,287]
HBO1 [o]	-	DBD, LBD	Ligand-dependent co-repressor. Member of the MYST/SAS family of proteins.	[288]
SMRT [o]	-	LBD	Ligand-dependent co-repressor by inhibiting the N/C-interaction and competing for p160 co-activators.	[289]
NcoR [o]	-	LBD	Antagonist-dependent co-repressor, competes with TIF2.	[205]
DAX1 [o,q]		LBD, AF2	Inhibits ligand-dependent trans-activation as well as N/C-interaction. Capable of relocalization of AR in cytoplasm and nucleus. Also inhibits SF1 and ERs.	[290]
Calreticulin [o,p]	-	DBD	Inhibits DNA binding and transcription. Also co-represses RAR, RXR, and, GR.	[291,292]
RAF [p]	IDE	NTD	Enhances AR and GR DNA binding.	[293]

Modified from Heinlein and Chang, 2002 [237] and Hermanson *et al*, 2002 [236].

[a] = proliferation/apoptosis
[b] = RING proteins
[c] = LIM-domain proteins
[d] = co-activators
[e] = GTF
[f] = SET protein
[g] = cytokine-associated protein
[h] = chaperone-associated protein
[i] = actin-binding cytoskeleton protein
[j] = membrane protein
[k] =chromatin remodeling and acetylation
[l] = proteolysis/ubiquitination
[m] = HMG protein
[n] = RNA/RNA-interacting protein
[o] = co-repressor
[p] = DNA-binding
[q] = orphan nuclear receptor

The p160 co-activators, to which steroid receptor co-activator 1 (SRC1 [254-256]), SRC3 [257-260], and TIF2 [261,263] belong, belong to the first family of proteins identified to interact with NRs and to associate with CBP. In addition, it has been reported that the COOH-terminal domains of SCR1 and SRC3 itself possess a weak HAT activity, which probably functions differently from CBP/p300 [294]. Furthermore, it has been suggested that p160 cofactors recruit other factors, such as methyltransferases to their COOH-terminus [207]. Based on studies performed on p160 proteins, it was proposed that *bona fide* co-activators should have the ability to interact directly with NR activation domains in an agonist-dependent manner, resulting in enhancement of receptor activation. Furthermore, they should interact with components of the basal transcription machinery and should not enhance basal transcription activity by their own, although they contain an autonomous activation function

and a NR interaction domain [263,295]. The p160 family members harbor the LXXLL signature motif in the NR interaction domain [217,218], which facilitates binding to the NR. Not only is the agonist-bound receptor conformation a prerequisite for co-activator binding, but for the *PSA* gene it was established that co-activator-receptor complexes are preferentially recruited to the enhancer region, indicating that the context of the response element is important as well [229].

Another class of co-activators consists of large multi-subunit mediator complexes. These mediator complexes were first identified as thyroid hormone receptor-associated proteins (TRAPs) [296] and vitamin D receptor-interacting proteins (DRIPs) [297], and are almost indistinguishable from compounds of the activator-recruited cofactor (ARC) complex [298]. The TRAP/DRIP/ARC complexes contain 9-12 proteins ranging in size between 33 and 250 kDa. The proteins in one mediator complexe are highly homologous or identical to the proteins in the other mediator complexes. The TRAP/DRIP complex was shown to interact with NR AF2 domain in a strictly ligand-dependent manner [299]. The high homology between TRAPs and DRIPs suggests that they function as mediators of transcription activation for all NR, albeit at slightly changed composition dependent on the NR they interact with [216,299]. Moreover, the ARC complex mediates transcription activation of other classes of transcription factors [298], and therefore it is thought that TRAP/DRIP/ARC mediators play a more general role in transcription regulation by directly contacting the basal transcription machinery [299,300]. Indeed, knocking-out a subunit of the TRAP/DRIP complex results in an embryonic lethal phenotype [301]. The promiscuous nature of p160 and p300 co-activators and the TRAP/DRIP/ARC mediators suggests that selectivity of transcriptional control of the different NRs lies in a combination of subtle differences in the particular composition of multi-protein complexes, the spatio-temporal expression of co-activators and receptors, and the context of enhancer/promoter sequences in the target genes. Thus, the transcriptional regulation of the AR and other NRs is extremely complex, and also very specific and delicately regulated [236,237,299,300].

While class I type NRs are transcriptionally silent in the absence of ligand because they are complexed to HSPs, the class II type receptors TR and RAR/RXR are capable of binding to DNA in the absence of ligand and be transcriptionally silenced [196]. Co-repressors were originally identified as class II type receptor-associated proteins mediating transcriptional repression in the absence of ligand, either by formation of a non-productive interaction with GTF [302], or by recruiting histone deacetylase complexes (HDAC), which compact the chromatin so that the DNA is not accessible for the transcription initiation complex [303-305] (Figure 1.8). The two best characterized co-repressors, N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor), are very large in size (molecular mass is ~ 270 kDa) and contain a highly related NH2-terminal region and at least four autonomous repressor domains [306,307]. N-CoR and SMRT do not interact with steroid hormone receptors in the absence of ligand but it was demonstrated that these co-repressors interact with ER and PR when complexed with an antagonist [308,309]. Similarly, AR transcriptional activity can be strongly repressed by N-CoR in the presence of a partial antagonist, but it is also partially repressed in the presence of agonist. N-CoR competes with TIF2 for AR binding sites, and therefore an equilibrium model has been proposed, in which the level of co-activator and co-repressor expression determines whether AR activity will be enhanced or repressed (Berrevoets *et al.*, unpublished observations). A similar competition has been described for SMRT, which inhibits the N/C-interaction and competes with TIF2 in a ligand-dependent fashion [289]. Thus, co-repressors

prefer an antagonist-bound conformation for AR repression, while co-activators prefer an agonist-bound conformation. Furthermore, it was shown by ChIP analysis that the co-repressor/HDAC complex is preferentially recruited to the promoter region of the *PSA* gene, and not to the enhancer region as is the case for co-activator complexes [229]. Since N-CoR and SMRT interact with multiple NRs, a general transcription regulatory role has been ascribed to these co-repressors, and indeed the N-CoR knockout mouse has an embryonic lethal phenotype [310]. It has been speculated that naturally occurring antagonist-like compounds must exist that exert N-CoR recruitment by SHRs.

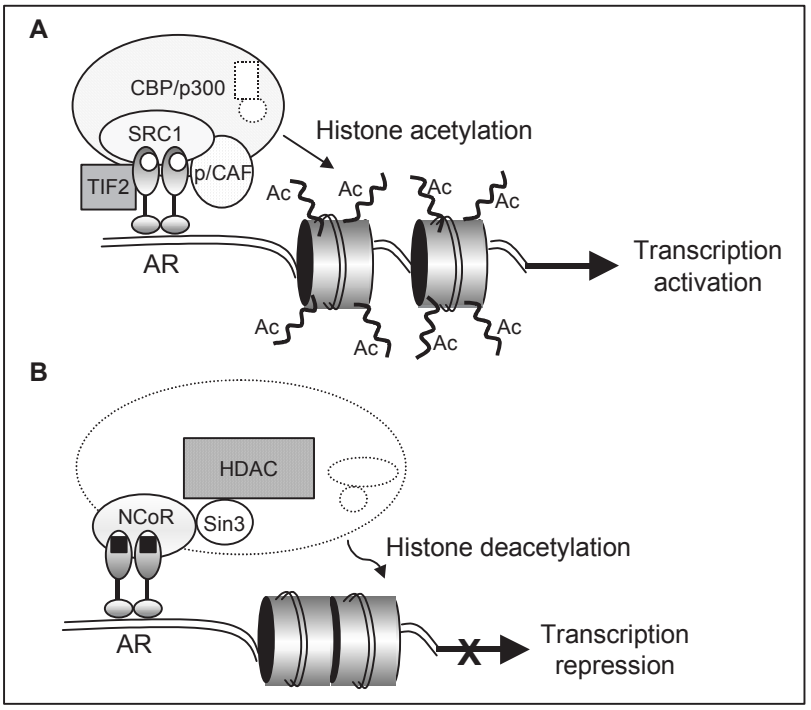

Figure 1.8. AR interacting protein complexes facilitating either activation or repression
A) Agonist-bound AR provides a conformation suitable for recruitment of p160 co-activators, p300/CBP, and P/CAF, which have histone acetyltransferase activity and enable transcription activation.
B) Antagonist-like compounds induce a conformation that is more suitable for the interaction with co-repressors and histone deacetylases, which repress transcription.
Dotted lines represent as yet unidentified factors taking part in larger complexes. Modified from Robyr *et al*, 2000 [295].

Androgen regulated genes

Since androgens play an essential role not only in fetal sex differentiation but also in pubertal and adult physiology and pathophysiology (**Chapter 1.4**), much effort has been undertaken to identify genes that are targets for regulation by the AR, in order to understand which players are involved in the androgen signaling pathway. The tissues studied have been mainly the testis, the epididymis, vas deferens, and the prostate. Castration and subsequent hormone replacement experiments in male rats and mice has resulted in identification of many androgen-regulated proteins in neonatal and adult epididymis and vas deferens, which are listed in Table 1.II. Many of these proteins appear to be involved in sperm cell

maturation, transport, and motility, and are secreted into the luminal fluid. Furthermore, they show a regional and temporal expression along the epididymis and vas deferens.

Table 1.II Androgen-regulated proteins identified in epididymis and vas deferens

Protein	Expression	Function	Reference
Acidic epididymal glycoprotein (AEG) or Protein DE	Secreted by the epididymal epithelium into the lumen	Product of the Crisp-1 gene. Glycoprotein that associates with maturing sperm. Expression is under the control of androgens and several AR binding sites are present in the promoter region.	311,312
AR	Mesenchyme and epithelium of epididymis, vas deferens, prostate gland, and many peripheral tissues	Transcription factor, up-regulated by androgens. Stromal cells in the epididymis are more sensitive to regulation of AR levels than epithelial cells.	313
Calcium binding proteins, CaBPs	All along the epididymis	CaBPs play an important role in maintaining cellular Ca^{2+} homeostasis. Circulating androgens are involved in regulation of intracellular Ca^{2+} in epididymal epithelium.	314
Carbonic anhydrase II, IV	Predominantly in corpus epididymis epithelium	Contribute to acidification of epididymal fluid necessary for regulation of sperm maturation and motility Androgen-dependent expression.	315
Clusterin/Sulfated glycoprotein-2, SGP	All along the epididymis	Secreted by principal cells of the caput epididymis and binds to spermatozoa. In corpus and cauda epididymis, clusterin is down-regulated by androgens.	316
Cyclooxygenase 1,and 2, Cox-1, and Cox-2	Epithelium of distal vas deferens and epididymis	Enzymes involved in prostaglandin synthesis. Androgen-dependent expression. Cox-1 is involved in anion secretion by epididymal epithelia.	317,318
Epididymal protein 2, EP2	Specific expression in the proximal caput epididymis	Epididymis-specific secretory protein. Expression dependent on circulating androgens.	319
EPI-EPS	Predominantly in mouse epididymis and vas deferens, low expression in seminal vesicle, kidney, and submandibular gland	Defective, endogenous murine leukemia proviral sequence. Highly androgen-regulated in both reproductive and non-reproductive tissues, due to its integration near a highly tissue-specific and androgen-regulated genetic locus.	320
Estrogen sulfotransferase, EST	Leydig cells of testis, epithelium of corpus and cauda epididymis, and luminal epithelium of vas deferens	Catalizes sulfoconjunction and inactivation of estrogen. Androgen-dependent expression in epididymis and vas deferens. Plays a physiological role in maintaining functional integrity of epididymis by regulating luminal estrogen homeostasis.	321

Protein	Expression	Function	Reference
Germ cell nuclear factor, GCNF	High in testis, low in epididymis, brain, liver, kidney. Expressed by corpus principal cells	Orphan nuclear receptor, involved in spermatogenesis, neurogenesis, differentiation, embryo development. Down-regulated by androgens.	322
Glutathione peroxidase 1, GPx1	All along the epididymis and nearly all other tissues in a broad range of species	The family of GPx enzymes catalyze the reduction of organic hydroperoxidases and hydrogen peroxide, using gluthatione as a reducing agent. GPx-1 is cytosolic and androgen-repressed in the epididymis.	314
Glutathione peroxidase 1, GPx3	Epididymis, vas deferens, kidney, and many other organs	Secretory enzyme, has a putative role in protecting sperm from the deleterious effect of reactive oxygen. In the epididymis, GPx3 is spatially and temporally regulated by androgens.	323,324
Glutathione peroxidase 1, GPx5	Caput epididymis specific	Secretory enzyme, androgen-dependent expression.	323
Glutathione S-transferases	Principle and basal cells of epididymal epithelium	Androgen-dependent expression. Forms part of the epididymal antioxidant system that protects luminal spermatozoa from oxidative damage.	314,325
Mouse epididymal retinoic acid binding protein, mE-RABP	Secreted into the luminal fluid of the mid/distal caput epididymis	Lipocalin involved in retinoic trafficking. Contains two AR binding sites in the promoter region, and is regulated by circular androgens.	326
Metallothionein I, II, III	Basal cells of all epididymal regions, testis	Cytosolic proteins involved in cellular stress response. Androgen-dependent expression.	327
Mouse vas deferens protein, MFDP	High levels in vas deferens, adrenal glands, ovary, and low expression in other tissues	Aldo-keto reductase1-B7 protein, responsible for detoxification of steroidogenesis byproducts. Expression in the adrenal cortex under control of ACTH, whereas it is strongly androgen-dependent in the mouse vas deferens.	328-330
Pem	Testis and epididymis	Homeodomain transcription factor. Androgen is necessary but not sufficient for Pem expression. Regulatory sequences in the Pem gene drive expression in different subregions of the epididymis.	331,332
Prostaglandin D2 synthase, PGDS	Predominantly in corpus and cauda epididymis, and testis	Secreted lipocalin, androgen-dependent expression. Increasing luminal concentration from distal caput to cauda.	333,334
Transforming growth fector β (TGF-β) 1, 2, 3	Epididymis and ventral prostate	Androgen-repressed gene. Androgen modulates expression in an isoform- and tissue-specific manner.	335

Androgen-regulated gene expression in the prostate has been the focus of much research, because of its involvement in prostate cancer (**Chapter 1.4**). Mostly, prostate cancer cell lines, and in some cases microdissected prostate tissue, have been used to identify androgen-regulated proteins [336-340]. Large-scale cDNA microarray studies (**Chapter 3**) covering ~ 20.000 distinct human genes showed that ~ 8.000 of these genes are expressed in the human prostate cancer cell line LNCaP, and approximately 2% of these genes are regulated by androgen [338,340]. However, the identified androgen-regulated genes in LNCaP cells are quite different from the genes that are regulated by androgens in epididymis and vas deferens, suggesting that androgens regulate the expression of different proteins depending on the target tissue and the pathway that needs to be turned on. Therefore, it is conceivable that androgen-regulated proteins involved in genital tract development are expressed in the epididymis and vas deferens primarily during a particular developmental time window, and may be different from the androgen-regulated proteins expressed in the adult epididymis and vas deferens.

Non-genomic actions of androgens

Apart from the AR signaling pathway that leads to transcription regulation, an increasing body of evidence suggests that androgens, like progestins and estrogens, can excert rapid, non-genomic effects, which are mediated by steroid receptors [341,342]. In prostate and breast cancer cells, it has been shown that androgens, progestins, and estrogens trigger the association of AR, PR, ERα, or ERβ with Src and stimulate the Src/RAF1/ERK (extracellular signal-regulated kinase) signal transduction pathway [343-346]. Src is normally targeted to the inner surface of the plasma membrane, and has a tyrosine kinase activity that is auto-inhibited by interaction of the tyrosine kinase domain and the Src homology 2 (SH2) and SH3 domains. Proteins that bind to these SH2 and SH3 domains disrupt the intramolecular interaction of Src, which results in activation of the Src kinase. Src, Raf and ERK are upstream regulators of the mitogen-activated protein kinase (MAPK) signaling pathway, which is involved in multiple cellular processes, such as proliferation and differentiation [347]. It has been reported that AR binds to the SH3 domain of Src through its proline-rich sequences in the NTD, in response to androgen. Simultaneously with AR binding, either ERα or ERβ can bind to the SH2 domain of Src, through interation of phosphotyrosine 537 (or Y443 for ERβ) [343]. This AR-ER-Src ternary complex is stimulated by androgen and estradiol and couples steroid hormone receptors to the cytosolic signaling pathway, inducing cell proliferation. Cross-talk between ERα and PRB has also been described. Progestins can activate the Src-Raf-ERK cascade by binding to PRB [348], which in turn binds to ERα via two interacting domains [346]. Also, a ternary complex can be formed with ERα and c-Src through direct binding of the proline-rich sequence of PRB to the SH3 domain of c-Src, although this interaction is much weaker than that of AR [349]. This kind of cross-talk regulates the non-genomic proliferative pathways of different steroid hormones, though there is also evidence that the non-genomic actions of steroid hormone receptors eventually influence transcriptional activity, since the activity of both receptors and co-activators is enhanced by direct phophorylation by MAPK [350,351].

In addition to non-genomic effects induced by steroid hormones and their intracellular receptors, some reports also indicate that membrane-bound receptors may be involved [342], which could be sex hormone-binding globulin [352,353] or G protein-coupled 7 transmembrane receptors [354].

1.4 Androgen receptor disorders

Androgen insensitivity syndrome

One of the causes of male pseudohermaphroditism is a defect in androgen exposure. The profound importance of androgen action in development is evident from observations on individuals who develop male pseudohermaphroditism. Male pseudohermaphroditism is characterized by partial or complete sex reversal of internal and/or external genitalia, except for the testes, in XY individuals. Disorders of androgen action can be caused by a defective AR, resulting in insensitivity of the target tissue, or by defective androgen synthesis or testosterone metabolism. Androgen insensitivity syndrome (AIS) is the clinical entity of a non-functioning AR leading to feminization of a 46,XY individual, described extensively in several reviews [179,355-359].

AIS is a relatively rare disorder caused by a heterogeneous group of defects in the *AR* gene and the incidence is probably between 1:40,800 and 1:99,000 male births [360]. Mutational defects in the *AR* gene result in either a partial or a complete non-functioning receptor protein, or a low expression level or complete absence of the protein, giving rise to a wide spectrum of defective masculinization in 46,XY individuals. The complete form of AIS (CAIS) is characterized by a seemingly completely feminized phenotype with normal breast development, but with absence of axillary and pubic hair, and with presence of testes in the abdomen. Due to the production of anti-Müllerian hormone, the Müllerian duct derived tissues are absent, and due to the defective action of androgens development of the Wolffian ducts does not occur. On the other end of the AIS spectrum mildly affected individuals show subtle undervirilization or infertility [361]. In between are individuals with partial AIS (PAIS) who show different degrees of virilization, ranging from a predominantly female external phenotype with clitoromegaly and labial fusion, and a phenotype with ambiguous genitalia, to a male phenotype with a micropenis or hypospadias, and gynecomastia. PAIS phenotypes can have either remnants of WDs or fully developed WD derived structures, depending on the degree of insensitivity. If the testes remain *in situ* in individuals with PAIS, virilization and/or feminization may occur at puberty, depending upon the hormonal milieu of the individual [179]. Furthermore, testicular tumors of germ cell like carcinoma *in situ* and occasionally seminomas, and non-germ cell precursors (Leydig- and Sertoli cells), occur with an increased frequency of 6-9% [362]. In cases of CAIS, testosterone, which is circulating at high levels, is aromatized into estradiol, resulting in feminization of breasts and body contours.

Most defects in the *AR* gene are the consequence of single nucleotide substitution, although single nucleotide insertions and deletions as well as larger deletions have been reported [355,363]. A few hundred different mutations in the *AR* gene have been reported so far, and most of these are compiled and described in the AR database (http://www.mcgill.ca/androgendb) [178,364]. Nearly 90% of the mutations are found in the DBD and LBD of the AR, and roughly 10% of mutations are reported in the NTD [365]. Mutations in the LBD can either completely abolish ligand binding, resulting in CAIS, or affect ligand binding in such a way that some residual AR activity remains, which causes PAIS. Mutations in the DBD are associated with normal ligand binding but result in defective DNA binding or dimerization, and consequently in defective activation of androgen-responsive genes. Furthermore, mutations in the DBD can result in premature stopcodons. The phenotype of individuals with AR-DBD mutations can be both CAIS and PAIS. Mutations detected in the NTD have been predominantly associated with premature

stop codons, which results in either a truncated AR protein or in the absence of AR protein [179,355,357,365].

Although it was initially believed that the wide spectrum of phenotypic variability in individuals with PAIS was the consequence of high variety of mutations in the *AR* gene, it is now clear that also identical mutations can result in significantly different phenotypes, even within one family [178,360,366]. One possible explanation for phenotypic variability is the occurrence of a somatic mutation in the *AR* gene in specific androgen sensitive tissues. Somatic mosaicism was first described in a PAIS patient who revealed a mutation in exon 1 leading to a premature translational stop, which was previously described to result in CAIS due to the absence of AR protein [367]. Apparently, in somatic mosaicism, both wild-type AR and mutated AR are present, which leads to a higher degree of virilization than would be expected from the mutation. The presence of the wild-type allele in somatic mosaicism can be due to *de novo* post-zygotic mutation in the *AR* gene [368-370]. A somatic mosaicism may be the cause of 30% of PAIS cases. Additional defects in steroid biosynthesis or steroid metabolism, or varying levels of androgen during fetal development may also account for phenotypic variation [360]. In families with CAIS, phenotypic variation is rarely observed, and to date only one family with both CAIS and PAIS resulting from one mutation has been reported [366]. It can be concluded that molecular defects of the *AR* are not the sole indicators for an AIS phenotype, and that careful study and documentation of every mutation provides important information about the role of a specific amino acid residue in AR function.

Another syndrome in which the AR is involved, is the X-linked spinal and bulbar muscular atrophy, or Kennedy's disease, a neurodegenerative disease [371]. Patients with SBMA have ARs with an extension of the polymorphic trinucleotide CAG repeat segment, present in the NTD of the AR, of 40 or more as compared to a maximum of 38 in healthy individuals. The role of AR is not very clear, but it has been proposed that extended CAG repeats in general are toxic for motor neurons.

Syndromes caused by defective steroid synthesis and metabolism

Mutations causing male pseudohermaphroditism have been described that impair each of the six enzymatic reactions involved in the conversion of cholesterol into testosterone and subsequently DHT [372].

One such disorder leading to male pseudohermaphroditism is the inherited steroid 5α-reductase type 2 deficiency. As described in **Chapter 1.2**, 46,XY individuals with steroid 5α-reductase type 2 deficiency present with female external genitalia, bilateral testes, normally virilized WD structures, and a blind-ending vagina (reviewed by [107,108,358]). This phenotype is the result of defective DHT production, as was first established by studies in two families in Dallas, USA and the Dominican Republic [373,374]. The syndrome of steroid 5α-reductase type 2 deficiency was found to be due to mutations in the steroid 5α-reductase type 2 gene [101]. Like in AIS, there is a high phenotypic variability between affected individuals with 5α-reductase type 2 deficiency, ranging from female sex assignment at birth to isolated hypospadias [107]. Due to the presence of testes, some affected individuals show a variable degree of masculinization during puberty when the testosterone level is increased, which can result in the acquisition of male gender in patients that were initially raised as girls. Gynecomastia as well as growth of beard and pubic hair does not occur in 5α-reductase deficient patients, which offers the possibility to distinguish this syndrome from PAIS.

5α-Reductase isozymes are NADPH-dependent enzymes that reduce testosterone into DHT. The COOH-terminal domain is the cofactor-binding domain, while both NH2-and COOH-terminal domains are involved in substrate binding. More than 40 mutations in the type 2 isozyme have been described, located through all 5 exons and affecting either cofactor binding, substrate binding, blocked formation of a functional isozyme, or complete loss of enzymatic activity [375,376].

Yet another autosomal recessive disorder leading to male pseudohermaphroditism is the deficiency of 17β-hydroxysteroid dehydrogenase-type 3 (17β-HSD3). This disorder is very rare in the general population but frequent among highly inbred Arab populations in the Gaza strip [377]. 17β-HSD3 is the enzyme that converts the precursor steroid androstenedione into the active androgen testosterone in the testis. Several other, extragonadal, isozymes of 17β-HSD have been identified, of which type 5 also catalyzes the conversion of androstenedione into testosterone, although with much lower efficiency [378]. Mutations in the 17β-HSD3 gene severely reduce testosterone production in the testis, but the unaffected or enhanced extragonadal type 5 isozyme, together with 5α-reductase can compensate to some extent for this loss of androgen after puberty [378,379]. The clinical appearance of affected 17β-HSD3 individuals is predominantly female, but also more male phenotypes with micropenis and hypospadias have been reported. Testes are always present and WD structures are normally developed [375,378,379]. When testes are still present at puberty, the activation of residual androgen production capacity causes partial virilization, which may sometimes result in gender change. In prepubertal patients the 17β-HSD3 deficiency is clinically indistinguishable from AIS, but measurement of elevated serum androstenedione and reduced testosterone levels can give a correct diagnosis [380]. As with AIS, mutations found in the 17β-HSD3 do not necessarily correlate with one particular phenotype, but may be influenced by residual or extragonadal testosterone formation, or by a somatic mosaicism [381].

The above-described deficiencies are only examples of the many known disorders leading to incorrect sex differentiation. It clearly demonstrates that many factors are involved in androgen-mediated sex differentiation and development, and that disruption of one such factor may already have a tremendous effect on phenotypic sex.

Other pathologies involving the AR pathway

Apart from their role in fetal sex differentiation and maintenance of male physiology in the adult, androgens and the AR also play in role in prostate cancer (PC) progression [382-384]. The incidence of PC varies widely between ethnic populations and countries, being highest in the United States, Australia and the European countries, where it is the second leading cause of death by cancer in men [385]. The majority of prostate cancers expresses the AR, and regresses in size upon withdrawal of androgen, demonstrating their dependence on androgens for growth. Therefore, hormonal therapy consisting of androgen suppression, AR blockage, or both, is widely used in the treatment of prostate cancer. However, growth of PC is only temporarily affected by inhibition of androgen action, and eventually the tumor relapses in an androgen-independent state (AIPC), thereby becoming untreatable. The underlying mechanism of the transition of androgen-dependent prostate tumor cells to tumor cells growing independently of androgens is largely unknown, but it is assumed that it is selected for by androgen inhibition. Much attention is focused on unraveling the

mechanism of the transition to AIPC, so that future therapies will be more effective against PC.

Several possible mechanisms are assumed to be involved in AIPC development, reviewed in [386,387]. Tumors that continue to depend on both androgens and AR, may increase their sensitivity to low circulating androgens by amplification of the *AR* gene, increased levels of co-activators, or increased production of DHT. Another mechanism would involve ligand-independent activation of the AR, bypassing the need for androgens. This is achieved by "cross-talk" with other signaling pathways, or by mutations in the AR causing a broadened specificity to include binding and activation of AR by other steroid hormones and even by anti-androgens that are used for androgen-inhibition therapy [388-390].

During prostate cancer progression, androgens regulate expression of diverse molecules involved in the processes of invasion and metastasis, that may lead to positive and negative effects on PC cell invasion [391]. Eventually, multiple factors play a role in the selection of cells with higher aggressive potential, leading to an invasive behavior of prostate cancer cells. Comparison of PC with other hormone-sensitive cancers, such as breast and endometrium cancer, indicates that development of less differentiated tumors with a poor prognosis is often associated with the loss of functional steroid hormone receptors [391].

1.5 Aim and scope of this thesis

It is clear that androgens play a pivotal role in male sex differentiation, and that this role is mediated through the androgen receptor, which regulates expression of specific target genes. While the epithelial cells in the embryonic Wolffian duct and urogenital sinus are dependent on androgens for their growth and development, they do not express the AR during that critical time window. Rather, the AR is found in surrounding mesenchymal cells, and many studies have indicated the importance of the mesenchymal cells and their paracrine potential to transmit androgenic signals to epithelial cells. To study the effect of androgens on gene regulation in androgen target cells and tissues, proper model systems such as cell lines derived from target tissue are required. Although many androgen-regulated genes have been identified in prostate cancer (cell lines) and adult epididymis, not much is known about target genes that are regulated during embryonic development. Identification of such genes would help unraveling the androgenic pathway underlying male sexual development. In this respect, the following question was addressed in this thesis:

1. Is it possible to develop an androgen-responsive cell line derived from Wolffian duct mesenchymal cells, and use it as a model system for studying androgen-induced regulation of gene expression?

Lack of androgenic signals during embryogenesis leads to defective male sexual development, causing male pseudohermaphroditism. In individuals with partial androgen insensitivity syndrome, the phenotype is only partly determined by the nature of the defect of the androgen receptor. Analysis of the effect of mutations in the AR on gene transactivation has already provided important clues on the molecular mechanism of AR transcriptional activity, but a correlation with the AIS phenotype is not always consistent. Therefore, the effect of a mutation in the AR was characterized to address the following question:

2. Can detailed functional analyses provide insight into the effect of a novel mutation in the AR gene in an individual with PAIS, and do the results of these analyses correlate with the observed phenotype?

Regulation of gene expression can be studied at the mRNA and/or protein levels. While one mRNA species corresponds with one gene, it does not necessarily correspond to one protein product, due to the fact that proteins undergo modifications upon each stimulus the cell receives. Each change in gene regulation has the potential to result in a multitude of changes at the protein level. To monitor changes in protein expression, the technique of 2-dimensional gel electrophoresis (2DE) was introduced in 1975 [392]. In the last decade, proteomics has evolved as a research field investigating the total protein complement of the genome: the proteome [393]. To date 2DE is still commonly used in proteome research in combination with advanced technology based on mass spectrometry. In this respect we addressed the questions:

3. *What are the changes in protein expression patterns of androgen target cells upon treatment with androgen as detected by means of 2DE analysis, and can differentially expressed androgen-regulated proteins be identified by mass spectrometry?*

4. *Do the androgen-regulated proteins identified in a cell line play a part in the androgenic signaling pathway of a whole organ?*

In **Chapter 2** the effect of the Q902K mutation in the AR protein on its transactivation properties is described, using two different and sensitive functional assays.

In **Chapter 3** the term proteomics is introduced and the evolution from genome to proteome research is described. Different techniques used in proteomics are described and proteomics research in reproductive endocrinology is highlighted.

In **Chapter 4** the development of a mouse fetal vas deferens (MFVD) cell line is described. The MFVD cells have been used to identify proteins that show a differential protein expression pattern upon androgen treatment.

In **Chapter 5** proteomic changes in expression profiles of fetal epididymis and vas deferens were monitored *in vivo* during a continual developmental time window, and *in vitro* upon androgen treatment.

The results described in this thesis will be discussed in a broader context in **Chapter 6**, and answers will be proposed to the questions addressed in **Chapter 1**.

Chapter 2

Functional analysis of a novel androgen receptor mutation, Q902K, in an individual with partial androgen insensitivity

Arzu Umar[1], Cor A. Berrevoets[1], N. Mai Van[1], Marije van Leeuwen[1], Michael Verbiest[1], Wim J. Kleijer[2], Dennis Dooijes[2], J. Anton Grootegoed[1], Stenvert L. S. Drop[3], and Albert O. Brinkmann[1]

[1] Department of Reproduction and Development,
[2] Department of Clinical Genetics, Erasmus MC
[3]Division of Endocrinology, Sophia Children's Hospital, Rotterdam, The Netherlands

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Abstract

Androgen insensitivity syndrome (AIS) is caused by defects in the androgen receptor (AR) that render the AR partially or completely inactive. As a result, embryonic sex differentiation is impaired. Here we describe a novel mutation in the AR found in a patient with partial AIS (PAIS). The mutation results in a substitution of a glutamine (Q) by a lysine (K) residue at position 902, Q902K. The AR Q902K mutation was investigated *in vitro* with respect to its functional properties. Although the Kd of AR Q902K was not severely elevated, transcriptional activity was decreased to 80% of wild type. The response to hormone was shifted to a ten-fold higher concentration due to the mutation. Furthermore, the 114 kDa androgen-induced phosphorylated AR protein band was not detectable in genital skin fibroblasts in the presence of either 1, 10 or 100 nM R1881, but could be detected in transfected COS-1 cells overexpressing the mutant receptor and at a hormone concentration of 100 nM R1881. Functional assays showed that the interaction between the NH2- and COOH-terminus of AR Q902K was reduced to 50% of wild type, and that the trans-activation by the co-activator TIF2 (transcriptional intermediary factor 2) was decreased 2-3 fold. The half-maximal response in both assays was shifted to a ten-fold higher hormone concentration as compared to wild type. These results indicate that residue Q902 is involved in TIF2 and NH2/COOH interaction and that the Q to K mutation results in a mild impairment of AR function, which can explain the PAIS phenotype of the patient.

Introduction

Androgen insensitivity syndrome (AIS) is a disorder that results from mutations in the X-linked androgen receptor (AR) gene [179,357]. Several hundreds of different mutations in the AR gene have been reported (www.mcgill.ca/androgendb), which result in a wide spectrum of clinical phenotypes [179,356,394].

The AR belongs to the superfamily of nuclear receptors that is well characterized and conserved in structure, and function as ligand-inducible transcription factors [145,146,226,235]. Like other steroid hormone receptors belonging to the same family, the AR is composed of distinct domains [363]. The variable NH2-terminal domain (NTD) is mainly involved in transcription activation through the activation function-1 (AF-1) region. The activation of AF-1 is ligand dependent. The COOH-terminal region harbors the moderately conserved ligand binding domain (LBD), which is involved in ligand-dependent transcription activation through AF-2, and functional interaction with nuclear receptor co-activators and co-repressors [187,237,397].

Crystal structures of AR LBD revealed that the LBD is constituted of 11 α-helices and one β turn, arranged as an antiparallel α-helical sandwich [200,397]. An AF-2 activation domain (AD) core region, conserved throughout the nuclear receptor family, was identified in helix 12, at the C-terminal end of the LBD between residues 893 and 900 [199]. Upon ligand binding, helix 12 repositions, providing an interaction surface that is suitable for co-activator interaction and thereby generating transcriptional activity of AF-2 [205,215,263,397]. Various mutations introduced in the AF-2 AD core decrease transcription activation but not necessarily ligand-binding [205,215,398]. Co-activators like TIF2 and SRC1 have been shown to functionally interact with AF-2 AD core via their conserved LXXLL leucine motifs [205,215,217,398] and enhance AR transactivation. Furthermore, the NTD also functionally interacts with AF-2 (the so-called N/C interaction) in a ligand-dependent manner, and mutational analysis showed that AF-2 AD core plays an important role in this interaction

[205,206,208,262]. The N/C interaction with AF-2 occurs via an FXXLF motif present in the AR N-terminus [210,211]. Several mutations found in the AR of AIS patients appear to affect either co-activator binding or N/C interaction or both, and therefore such functional analyses are very useful in determining AR function after mutation and provide detailed information on the mechanism of AR transcription activation.

Herein we report a novel AR mutation resulting in a Q to K substitution at position 902 in a patient with PAIS. We have performed functional analyses on the AR to determine the effect of the Q902K mutation on TIF2 enhanced transcription activation and N/C interaction, in order to explain the phenotype and potential functional activity.

Materials and methods

Clinical data

The patient was referred to the clinic at the age of two years because of hypospadias. He is the first child of unrelated parents born after an uneventful term pregnancy. The 46,XY boy had proximal shaft hypospadias and a bifid scrotum. Because of a positive family history of genital malformations (uncle and nephew of the mother were known with hypospadias, gynecomastia and infertility), the AR gene was screened for a mutation. Single-strand conformation polymorphism (SSCP) and DNA sequencing analysis identified a point mutation in exon 8 of the *AR* gene (CAA → AAA) at amino acid residue 902 (according to http://www.mcgill.ca/androgendb/) [178] leading to an amino acid substitution of glutamine (Q) to lysine (K). This Q902K mutation was also identified in the mother, grandmother, and great grandmother of the patient (Figure 2.1). The boy's uncle and great-uncle have a reported positive history of genital malformation. At the age of three years, the boy underwent a chordectomy and hypospadias repair. During this procedure, a genital skin biopsy was obtained for genital skin fibroblasts (GSF) culture.

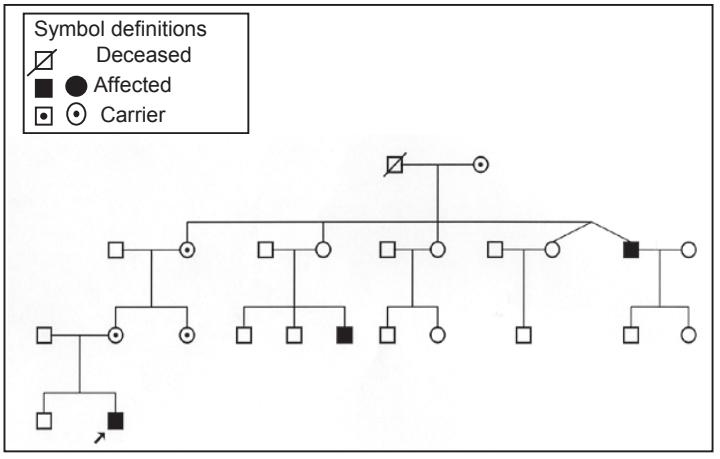

Figure 2.1. Pedigree of family with AR Q902K mutation
A square symbol represents a male, a circle represents a female. The arrow indicates the boy described in this study.

Scatchard analysis

GSFs were cultured in minimal essential medium containing 1% non-essential amino acids (Gibco, Life Technologies, Gaithersburg, MD), 10% fetal calf serum (Hyclone, Logan, UT), 100 IU/ml penicillin and 100μg/ml streptomycin (BioWhittickar, Vervier, Belgium). For ligand binding characteristics, a whole cell assay was performed as previously described [399]. Briefly, the genital skin fibroblasts were cultured to confluency and subsequently placed on medium without serum overnight. The cells were than incubated for 1 hour at 37 C with

increasing concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 nM) of ^{3}H-R1881 (New England Nuclear, Boston, MA) in the absence or presence of 100-fold non-radioactive steroid, and ^{3}H-activity was measured in a scintillation counter. Scatchard analysis was carried out to determine the Kd using the Kell software package (Radlig, Biosoft).

Site-directed mutagenesis and construction of AR expression vectors

The Q902K mutation was introduced into the AR using site-directed mutagenesis. The human wildtype AR cDNA expression plasmid pAR0[183] was used to generate pARQ902K in two separate PCR amplification steps, using Pfu polymerase. Sense and antisense Q902K primers containing the mutation, which is depicted in lower case lettering, were combined with the sense EQ1 and antisense EQ4 primers previously described by Berrevoets *et al.*[205]: Q902K sense (5'-ATCTCTGTGaAAGTGCCCA-3') was combined with EQ4 antisense (5'-CAAGGGGCTTCATGATGTCC-3'), and Q902K antisense (5'-TGGGCACTTtCACAGAGAT-3') was combined with EQ1 sense (5'-ACAGCCAGTGTGTCCGAATG-3') primer. The generated EQ1 PCR product was used as a template for the second PCR using the EQ1 and EQ4 as sense and antisense primers. The resulting PCR fragment was directly ligated into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO Cloning kit (Invitrogen, Life Technologies, Gaithersburg, MD). The mutated AR fragment was digested with *Eco*R1 and exchanged for the corresponding wildtype fragment in pAR0.

AR.N1 and AR.C that were used for the N/C interaction and TIF2 activation studies, were previously described[205]. The Q902K mutation was introduced into the AR.C construct via the *Eco*R1 restriction fragment of pARQ902K.

Luciferase assay

For transcription activation studies, CHO (Chinese hamster ovary) cells were cultured in DMEM/F12 medium, supplemented with 5% dextran-coated charcoal-treated FCS (Gibco). For all transcription activation studies, CHO cells were plated in 24-well plates (Nunc, Brand Products, Denmark) at a density of $2x10^{4}$ cells per well. After 24 hours, cells were transfected using FuGENE reagent (Roche Diagnostics, Basel, Switserland), according to the instructions of the manufacturer, at a ratio of 1:2 DNA:FuGENE reagent. The DNA mixture was composed of 50 ng/well MMTV-LUC (mouse mammary tumor virus-luciferase) reporter plasmid, increasing concentrations of wild type AR or AR Q902K (0.3 ng – 30 ng/well), and carrier plasmid pTZ19 to adjust to a total amount of 250 ng DNA per well. 5 hours after transfection, 1 nM synthetic androgen R1881 or vehicle (0.1% ethanol) was added to the cells, or in the case of the dose-response curves a range of 1 pM – 100 nM R1881 was added. After overnight incubation, cells were lysed in 50 µl lysis buffer (25 mM Trisphosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT)), and 25 µl of the lysate was used to measure luciferase activity using Steady-Glo Luciferase substrate (Promega, Madison, WI). The data shown are the mean of three independent experiments (average +/- SEM).

N/C interaction assay, TIF2 activation assay

The functional N/C interaction assay and TIF2 activation assay were performed in essentially the same way as the trans-activation assay described above, except for the constructs that were used. For N/C interaction, 100 ng/well AR.N1 in combination with increasing concentrations AR.C or AR.C-Q902K (0.3 ng –30 ng/well) was used. For TIF2

activation, 100 ng/well TIF_2 in combination with increasing concentrations AR.C or AR.C-Q902K (0.3 ng – 30 ng/well) was used, in both cases together with 50 ng/well MMTV-LUC reporter and pTZ carrier to adjust to 250 ng/ well.

Western blot analysis

For AR Western blot analysis, GSFs containing wild type AR and the Q902K AR were cultured in the presence of FCS for 7 days, as described above. When grown to confluency, medium was replaced by medium containing 10% dextran-coated charchoal-stripped-FCS in the presence of increasing concentrations of R1881 (0.1-100 nM) or vehicle (0.1% ethanol) for 24 hours. Additionally, COS-1 cells were seeded in 80 cm^2 culture flasks at a density of $2x10^6$ cells per well. The next day, cells were transfected with 4 μg pAR0 or pARQ902K DNA, mixed with 12 μl FuGENE reagent. After 5 hours, increasing concentrations of R1881 (0.1-100 nM) or vehicle (0.1% ethanol) were added to the culture medium. The next day, both CHO cells and GSF were washed with PBS, collected in ice-cold PBS and centrifuged for 5 minutes at 1200 rpm (Hereaus Christ). The cell pellet was resuspended in 200 μl ice-cold RIPA buffer (40 mM Tris-HCl pH 7.4, 5 mM EDTA, 10% glycerol, 10 nM sodiumphosphate, 10 mM sodiummolybdate, 50 mM NaF, 0.5 mM sodium orthovanadate, 10 mM DTT, 1% triton X-100, 0.08% SDS, 0.5% deosoxycholate) containing Complete™ protease inhibitors (Roche Diagnostics), and centrifuged for 10 min at 40,000xg. Protein concentration was determined using the RCDC Protein Assay™ (Bio-Rad, Hercules, CA). From the COS-1 cell lysate, 30 μg protein was used, and from the GSF cell lysate 50 μg protein was used to load onto a 7% SDS-polyacrylamide gel. Proteins were separated and blotted to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Western immunoblotting was performed using polyclonal antibody SP197 [400] and proteins were visualized by Western Lightning chemiluminescence detection (Perkin Elmer, Boston, MA).

Protein structure

The 3-dimensional crystal structure of the AR ligand binding pocket complexed with R1881 was obtained from the NCBI structure data bank, accession number 1E3G, deposited in the data bank by Matias *et al.*, 2000 [200]. The stereo diagram showing the ligand binding pocket, and selected residues that were subject to mutation, was created using the DeepView/Swiss-PDB Viewer 3.7 program.

Results

The AR Q902K mutation displays decreased transcription activation

The Q902K mutation is located within the LBD of the AR, within helix 12 and next to the reported AF-2 AD core domain [199,200]. From its position in the LBD, it can be predicted that residue Q902 is not part of the LBP, but may play a role in co-activator mediated activation of transcription and in the interaction of the LBD with the NTD. Therefore, functional studies were performed to address this question.

First of all, it was determined whether the mutated AR is functionally expressed in GSFs obtained from a patient with PAIS. The mutated AR Q902K was detectable at the protein level in GSF cells and, in the absence of R1881, showed a similar expression compared to the wild type AR in normal GSF cells (Figure 2.2A, lanes 1 and 2). It is well known that the AR protein shows a hormone-induced phosphorylated isoform of 114 kDa[176]. However, while GSFs expressing wild type AR respond to hormone by expressing the hyperphosphorylated 114 kDa protein band (Figure 2.2A, lanes 3, 5, 7), Q902K AR did not show the 114 kDa band upon addition of either 1, 10, or 100 nM R1881 (Figure 2.2A, lanes 4, 6, 8). Thus, although the mutated residue is not a direct target for phosphorylation, it seems that the substitution by a lysine residue at position 902 affects the hormone-induced phosphorylation status of the AR.

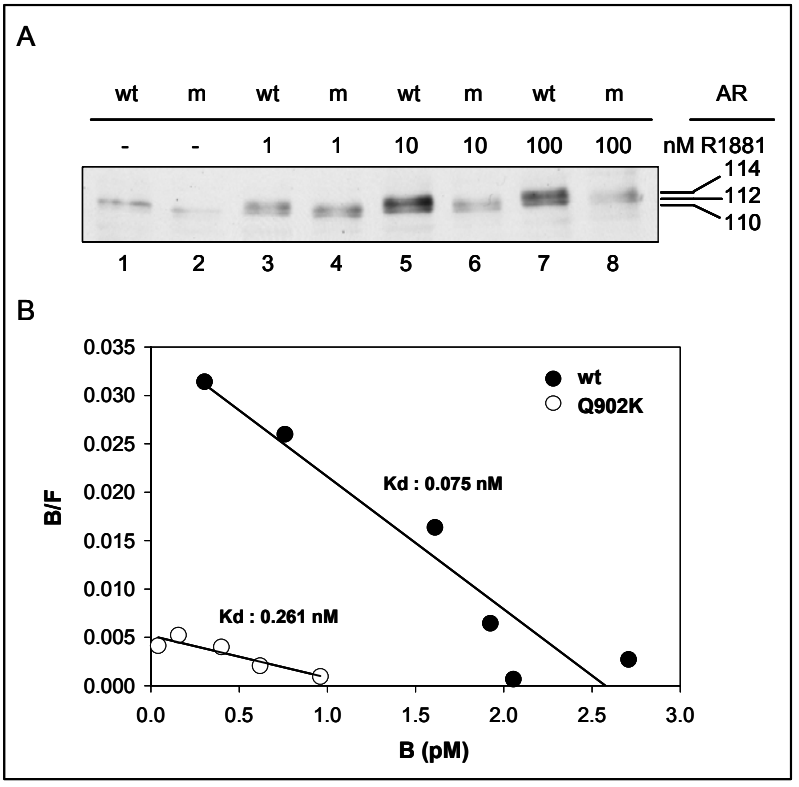

Figure 2.2. Expression and ligand-binding properties of AR Q902K in genital skin fibroblasts
A) Protein expression pattern of wild type AR (wt) and AR Q902K (m) in GSFs in the absence or presence of 1, 10, and 100 nM R1881. Note the absence of the 114 kDa protein band in the mutated AR.
B) Scatchard analysis of whole cell binding data obtained from genital skin fibroblasts harboring wt AR or AR Q902K.

It was then tested whether the Q902K mutation in the AR had an effect on ligand binding. Scatchard plot analysis performed on GSFs revealed a Kd value of 0.26 nM (normal range is 0.03 – 0.13 nM), suggesting that hormone binding was slightly but not severely impaired (Figure 2.2B). To determine whether the Q902K mutation affects the transcription activation potential of the AR, the mutation was created in a mammalian expression vector, AR Q902K. Transcription activation was determined in a luciferase assay using the MMTV-LUC reporter plasmid. Transfections with increasing amounts (0.3, 1, 3, 10, 30 ng/well) of wt or mutant AR showed that maximum transcription activation was reached with 3 ng/well DNA (Figure 2.3A). Higher concentrations of DNA resulted in squelching of the signal. Wild type AR showed a 27-fold induction of activity, whereas the mutated AR induced activity to maximally 23-fold, which is 85% of the wt value (Figure 2.3A). This difference in transactivation was small but significant ($p<0.05$). Furthermore, the dose-response curve showed that the half maximum response of AR Q902K to hormone had shifted to a ten-fold higher hormone concentration as compared to wild type. Both receptors showed a maximum activity at 1 nM R1881, with the difference that the mutant did not reach maximum wild type activity, but varied between 75-85% (Figure 2.3B). Thus, the Q to K mutation mildly affects AR transcription activation potential.

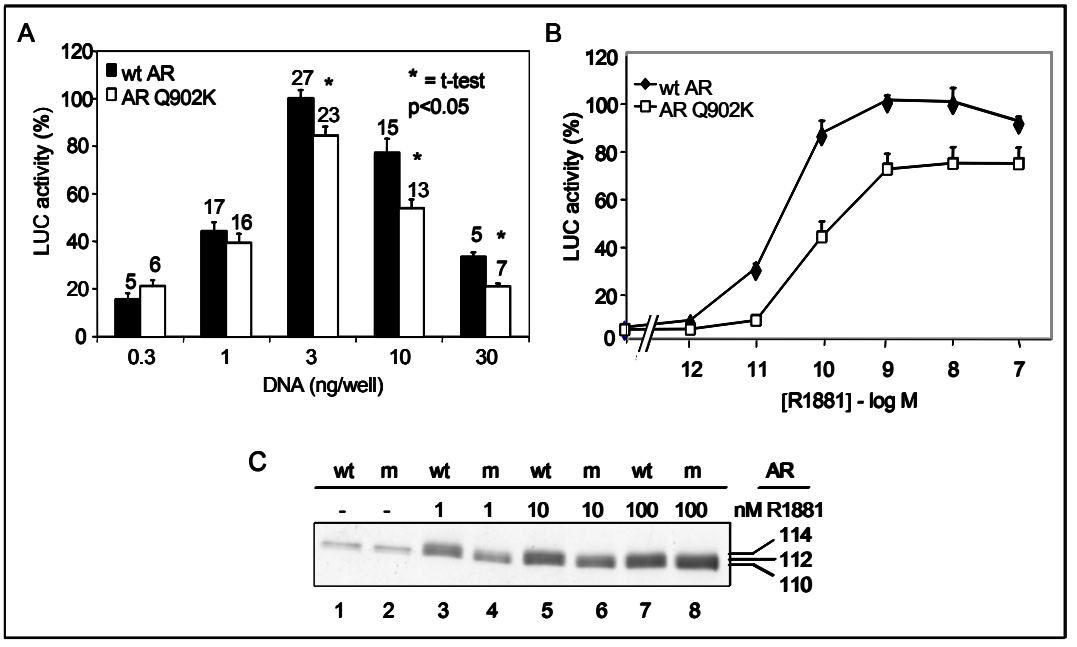

Figure 2.3. Transcription activation assay in CHO cells

A) Increasing amounts of wild type AR (wt AR) and mutated AR (AR Q902K) were transfected into CHO cells. Luciferase activity measured for wt AR at 3 ng/well was set at 100% and all the other points were calculated relative to that. The activities measured in the hormone treated cells are displayed, together with the fold induction, which represents the ratio between the activities measured in the absence and presence of hormone. Statistical significance was calculated using a Student's t-test with $p<0.05$.

B) Dose-response curves of wt AR and AR Q902K in the presence of increasing amounts of R1881. The activity of wt AR measured at 1 nM R1881 was set at 100% and the other points were calculated relative to that.

C) Protein expression of wt AR and AR Q902K in COS-1 cells. Note the precence of the 114 kDa protein band in AR Q902K only when stimulated with 100 nM R1881.

Protein expression of AR Q902K in COS-1 cells revealed a slightly different pattern than in GSFs. While 1 and 10 nM R1881 only partially stimulated the upshift to the 114 kDa phospho-protein band, 100 nM R1881 obviously restored the expression pattern (Figure 2.3C), although this restoration did not result in higher transcription activation in CHO cells (Figure 2.3A). Thus, the Q902K mutation in the AR results in a 15-25% reduced hormone-induced transcriptional activity, a ten-fold decrease in hormone response and a defective ligand-induced phosphorylation.

Decreased N/C interaction due to the Q902K mutation

It was previously determined that the NH2-terminal domain (NTD) and ligand binding domain (LBD) of the AR interact in a ligand-dependent manner (N/C interaction) [208], and that the AF-2 AD core domain is important for this functional interaction [205]. Since the Q902K mutation lies in close proximity of the AF-2 AD core region, we have performed a functional N/C interaction assay to study in more detail the effect of the Q902K mutation on transcription activation. For the N/C interaction, AR.N1, AR.C, and AR.C-Q902K constructs were used (Figure 2.4A). In the presence of 1 nM R1881, AR.N1 and AR.C co-expression resulted in transactivation of the MMTV-LUC promoter with a 50-fold induction. Maximum activity was reached at 10 ng DNA/well. AR.C-Q902K showed a 50% reduced interaction with AR.N at 10 ng DNA/well, and is thus severely affected (Figure 2.4B).

The dose-response curve of AR.C showed that functional interaction with AR.N1 is first measurable in the presence of 10^{-10} M R1881, and that the activity is approximately 40% of the maximum activity, which is reached at 10^{-8} M R1881. Interaction between AR.C-Q902K and AR.N1 was shifted ten-fold in the dose-response curve and maximum activity, which was reached at 10^{-8} M, was 50% of wild type (Figure 2.4C). These results indicate that the N/C interaction in the AR is severely impaired by the Q902K mutation. Although 100 nM R1881 can rescue the impaired N/C-interaction to some extent, wild type activities are not reached using supra-physiological doses, since a maximum response is reached at 10 nM R1881.

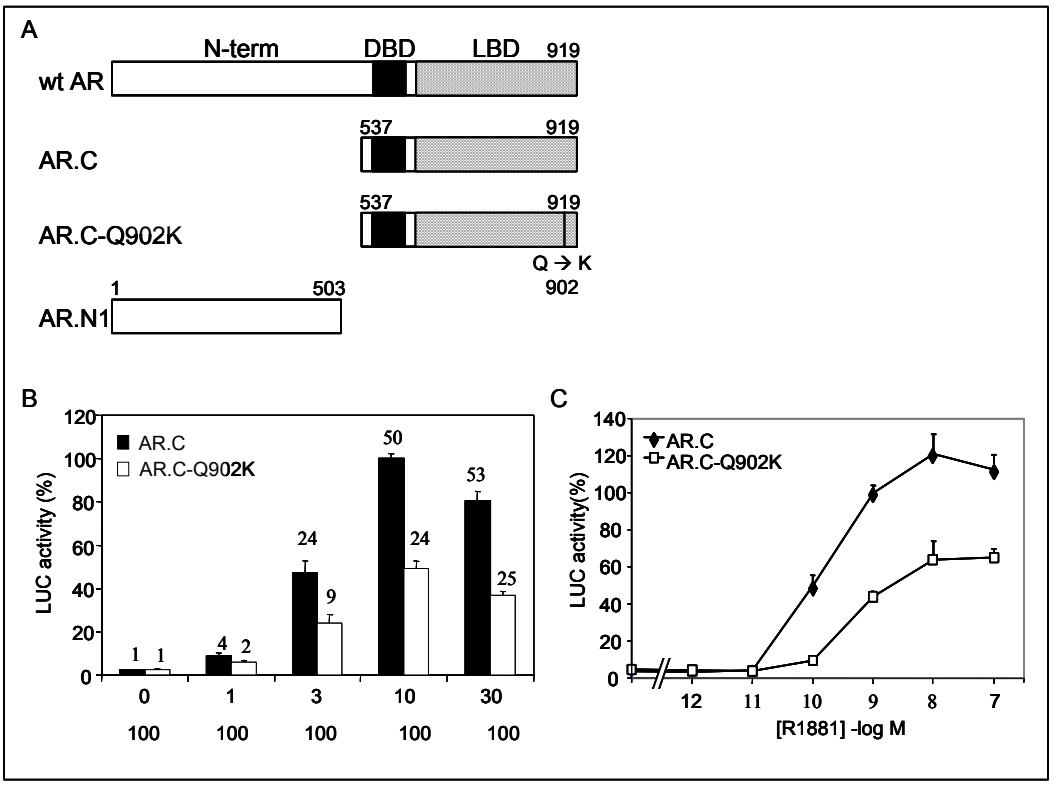

Figure 2.4. Functional N/C interaction

A) Constructs used for the functional N/C interactions. AR.C, N-terminally truncated AR; AR.C-Q902K, N-terminally truncated AR with Q902K mutation; AR.N1, C-terminally truncated AR.

B) Functional N/C interaction as measured in CHO cells transfected with 100 ng/well AR.N1 and increasing amounts of either AR.C or AR.C-Q902K. The activities measured in the hormone treated cells are displayed, with the fold induction indicated above the bars. The activity of AR.C at 10 ng DNA/well was set at 100% and the other points were calculated relative to that.

C) Dose-response curve of the N/C interaction at increasing concentrations of R1881. The activity of AR.C measured at 1 nM R1881 was set at 100% and the other points were calculated relative to that.

TIF2 transactivation is impaired by the Q902K mutation

The AF-2 AD core domain is not only involved in N/C interaction but is also part of an interaction surface for the binding of nuclear receptor co-activators [199,205]. In order to further characterize the effect of the Q902K mutation on AR transcription activation, we have studied the transactivation potential of TIF2. Because the effect of TIF2 transactivation is more pronounced on the NH2-terminally truncated AR [205], we have used the AR.C construct for TIF2 experiments. A constant amount of TIF2 was co-transfected with increasing amounts of either AR.C of AR.C-Q902K (Figure 2.5A). The maximum transactivation enhanced by TIF2 was reached at 10 ng DNA per well, resulting in a 34-fold induction of transcription with AR.C and a 13-fold induction with AR.C-Q902K. At higher concentrations DNA, auto-squelching reduced the transcription activation, but the difference between wild type and mutant AR.C remained 2-3-fold.

TIF2 activation of AR.C and AR.C-Q902K was tested at increasing hormone concentrations (Figure 2.5B). Wild type AR.C was activated by TIF2 initially in the presence of 10^{-10} M R1881, which was 30% of the maximum activity measured at 10^{-7} M.

In contrast to the dose-response curves of full length AR and the N/C interaction (Figures 2.3S and 2.4C, respectively), no maximum activity was reached in the TIF2 activation assay, even in the presence of 100 nM R1881. Activation of AR.C-Q902K by TIF2 was not detectable at hormone concentrations below 10^{-9} M R1881, and could be maximally induced to 50% of wild type activity. Furthermore, alike AR.C, AR.C-Q902K response to hormone was not saturated at 10^{-7} M. In conclusion, TIF2 activation of Q902K AR was impaired by approximately 50%. It can be speculated that higher, supra-physiological concentration of androgen may partly compensate for the decreased TIF2 transactivation due to the Q902K mutation.

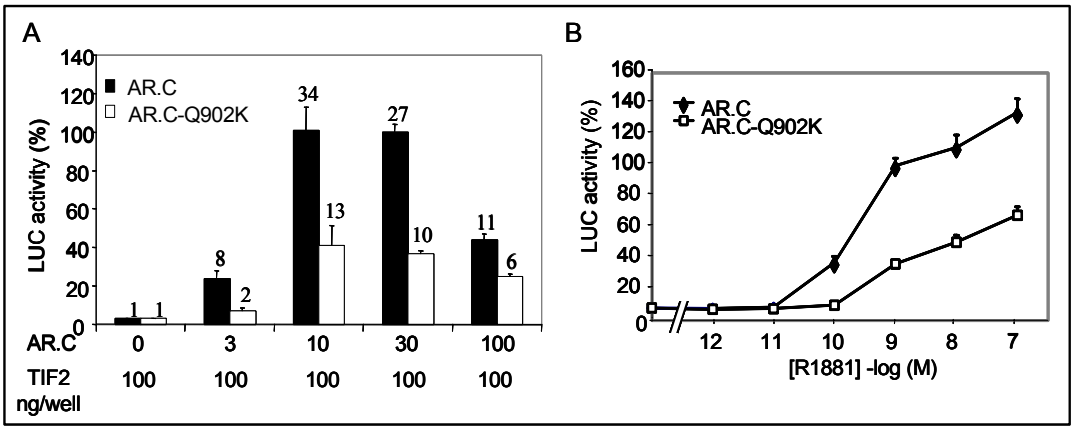

Figure 2.5. TIF2 activation of wild type and mutated AR
A) Transactivation of increasing amounts of AR.C and AR.C-Q902K by TIF2 (100 ng/well). The activity of AR.C at 10 ng DNA/well was set at 100% and the other points were calculated relative to that.
B) Dose-response curve of TIF2 activation assay in the presence of increasing concentrations of R1881 (1 pM-100 nM). The activity of AR.C measured at 1 nM R1881 was set at 100% and the other points were calculated relative to that.

Positioning Q902K in the AR LBD 3D model structure

We have used the AR LBD model structure structure, deposited in the structure data bank by Matias *et al.*, 2000 [200], to locate the position of Q902 (Figure 2.6). The 18 amino acid residues described to constitute the ligand binding pocket (LBP) are shown in white, whereas residues located in and next to the AF-2 AD core domain in helix 12 that have been reported to be mutated in AIS patients (V889, I898, V903, and P904) are shown in purple. The Q902 residue is shown in green. It is obvious from the 3D model that residue Q902 is not located close to the 18 residues that interact with the bound ligand, but may be part of the AF-2 AD core domain interacting surface.

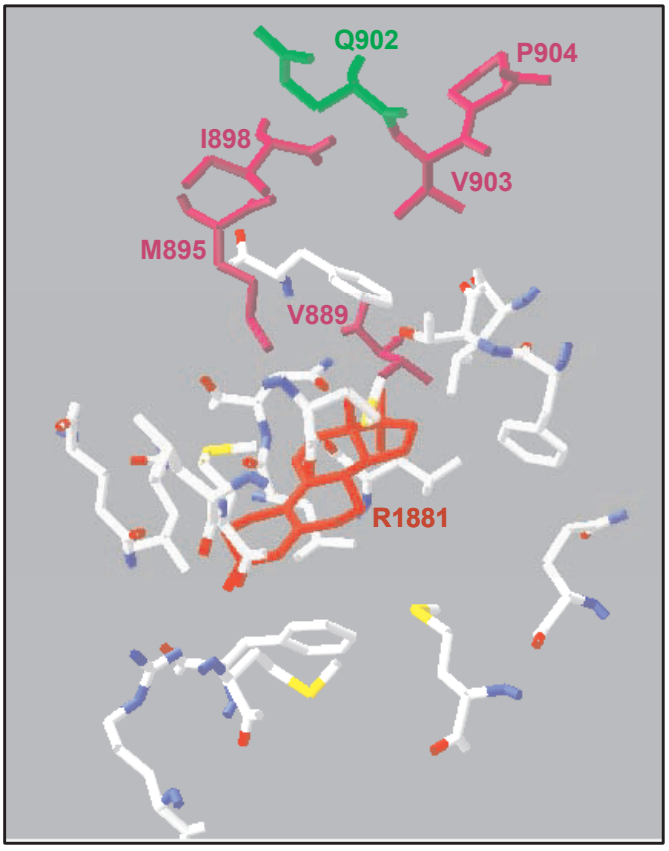

Figure 2.6. Model structure of the AR ligand binding pocket and the AF-2 AD core domain
Amino acid residues constituting the LBP are represented in white, residues in and close to the AF-2 AD core reported to be mutated in AIS are represented in purple, the Q902 residue is shown in green, and the ligand R1881 is shown in red. Blue, red, and yellow side chains stand for represent the backbone side chains. The crystal structure was retrieved from the NCBI structure data bank (accession number 1E3G) and created using the DeepView/Swiss-PDB Viewer 3.7 program.

Discussion

The Q902K mutation, described in this study, has not been associated with AIS before, but a somatic Q902R mutation has been reported in a patient with androgen-independent prostate cancer [401], of which no functional analysis has been described so far. Therefore we have studied the effect of the Q902K mutation on AR transcription activation to see whether the mutation could be correlated with the PAIS phenotype.

It was determined that the Q902K mutation leads to a mild impairment of transcription activation (15-25%) and to defective hormone-induced hyperphosphorylation of the AR protein, leading to the absence of the 114 kDa androgen-induced phospho-protein band on Western blots. The phosphorylation pattern of the mutant AR in genital skin fibroblasts seems to be more affected, than that of the overexpressed mutant AR in transfected COS-1 cells. So far, no conclusive role has been ascribed to AR phosphorylation. Some studies performed *in vitro* report that AR phosphorylation is necessary for full transcription activation and ligand binding [176,363,402-404], while a more recent study does not find such a correlation [405]. AR Q902K expressed in COS-1 cells results in hyperphosphorylation upon stimulation with a high (100 nM) concentration R1881, but does not coincide with a rescue of transcription activation. This finding suggests that, for the Q902K mutation, there is no clear correlation between AR phosphorylation and transactivation potential.

Detailed functional analyses revealed that the Q902 residue plays a substantial role in the functional interaction between the LBD and NTD, and in co-activator mediated enhancement of transactivation. These functional interactions are not necessarily linked to ligand binding, since the Kd of the AR Q902K receptor is only slightly elevated, but N/C interaction and TIF2 transactivation are more severely affected. Similar results were obtained by Thompson *et al.*, 2001, who described mutations located between helices 3-11 within the LBD of the AR in PAIS patients that displayed normal or slightly reduced androgen-binding activity, but severely impaired N/C interaction, decreased co-activator response, and decreased transactivation [406]. Recently, another study described that the decrease in N/C interaction in mutated ARs correlated with the severity of the AIS phenotype, while the Kd was not or only slightly affected by the mutation [407]. Thus, loss of a functional N/C interaction in the AR may be an important molecular defect in AIS patients and screening for such a functional interaction, in addition to hormone-binding studies, may be a valuable tool to determine the effect of novel mutations in more detail.

In addition to inter-domain interactions, disrupted co-activator interactions have also been implicated with several forms of AIS. Mild forms of AIS leading to sub-fertility or oligospermic infertility in men have been associated with mutations in the AR LBD leading to disrupted inter-domain interactions (LBD with LBD, or LBD with NTD) in combination with defective co-activator TIF2 activation [408,409].

Furthermore, CAIS has been reported in an individual where no mutations in the AR were found, but where the transmission of the activation signal from AF-1 was disrupted, possibly due to the absence of a functional co-activator [410]. Thus, a minor disruption of co-activator binding or a complete loss of co-activator function can result in varying degrees of AIS. Our finding that the Q902K mutation leads to 50% decreased TIF2 transcription activation in a patient with a PAIS phenotype, is in line with these reports.

Since the 3D crystal structure of the liganded AR LBD is known [200,411], it is possible to make assumptions about the role of certain amino acid residues in AR function. From the model structure, it can be predicted that the Q902 residue is in relative close vicinity of the ligand binding pocket and the AF-2 activation domain core motif, and that Q902 is

surrounded by residues that have been found mutated in individuals with AIS, such as V889, M895, I898, V903, P904. Except for V903, which is only conserved between different AR species, residues V889, M895, I898, Q902, and P904 are either highly conserved or identical between AR, progesterone receptor, glucocorticoid receptor and mineralocorticoid receptor, which are other members of the steroid hormone receptor family [412]. V889, which is located just before the AF-2 AD core domain, has been reported by several groups to be mutated into a methionine residue, resulting in either PAIS or CAIS [413-415]. Two mutations within the AD core domain, M895T and I898T, have been reported in CAIS [368,416]; the V903M substitution was identified in a patient with PAIS [417], and P904S and P904H mutation have been reported to cause CAIS [413,417]. Based on these reports, it can be concluded that the position of residue Q902 in the model structure is informative but cannot be easily correlated to an AIS phenotype.

The V889M and I898T mutations have been characterized at the molecular level, and it was found that both mutations do not affect ligand-binding affinity, although dissociation half times were decreased. Strikingly, the N/C interaction was completely abolished by the I898T mutation (leading to CAIS) and severely hampered by the V889M mutation (leading to either CAIS or PAIS), which could be rescued only at high ligand concentrations [206,262]. The TIF2 interaction seems to be unaffected by these mutations. Detailed functional analysis of all the above-mentioned mutations in the AR would be needed to determine the role of each amino acid residue in inter-/intra-molecular domain interaction, co-activator binding, and the relation to AIS. Our results, together with previous reports, indicate that residues outside the AF-2 AD core domain can play an important role in functional N/C interaction and the severity of this disruption can be correlated with an AIS phenotype. Furthermore, although not every residue in and around the core domain is involved in TIF2 induced activation, amino acid Q902 appears to play an important role in this interaction.

With respect to clinical relevance, the TIF2 activation study showed that a high level of androgen could partially rescue the negative effect induced by the Q902K mutation. Since the dose-response curve with TIF2 did not show a saturation level, it can be speculated that even higher, supraphysiological concentrations of hormone could have a greater effect on activation and thus would be of use as clinical therapy. This study provides further support, that detailed functional analyses on mutated ARs not only provide insight in AR functioning, but can also show their relevance for clinical therapy.

Acknowledgements

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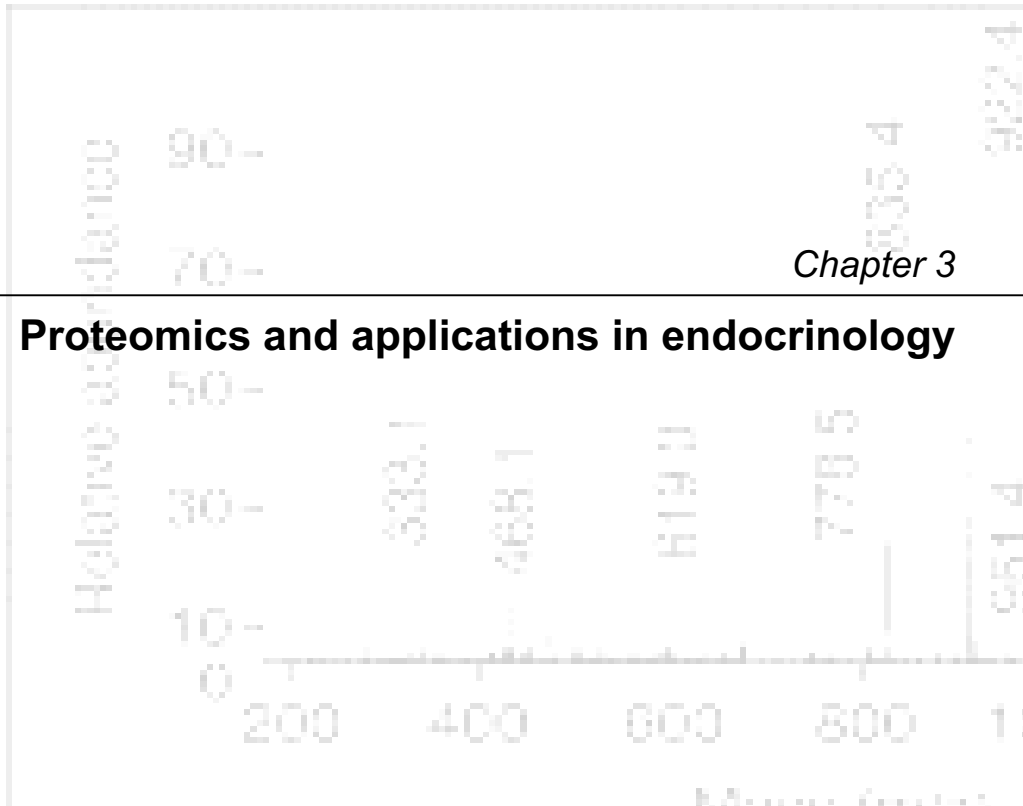

Chapter 3

Proteomics and applications in endocrinology

Arzu Umar[1], Theo M. Luider[2], J. Anton Grootegoed[1], and Albert O. Brinkmann[1]

[1]Department of Reproduction & Development,
[2]Department of Neurology and Erasmus Center for Biomics,
Erasmus MC, Rotterdam, The Netherlands

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Introduction

The proteome is a term that was first introduced in the mid 1990s to define "the total **pro**tein complement of a gen**ome**" [418,419]. Proteomics covers all the technology currently available to analyze the proteome, the global patterns of gene expression at the protein level [420]. The emergence of proteome research was based on of the worldwide rapidly proceeding genome projects, as was foreseen in the original proposal of the Human Genome Project. The generation of large amounts of sequence data from genomes provided a start for the scientific community, to look beyond genes and genomes and to focus on proteins.

Reproductive endocrinology is a field, in which research was, and still is typically focused on identification of proteins and their functions. For example, in search for new and better therapies for hormone-controlled cancers in reproductive endocrinology (prostate, breast, ovary, and endometrial cancer), large-scale screens of expressed genes and the encoded proteins are now widely used.

As research is constantly evolving by the introduction of new techniques, this chapter will describe the scientific evolution from genomics to proteomics research in relation to reproductive endocrinology.

3.1 Genomics

3.1.1 Introducing the genome

Mendel's description of crucial hallmarks of heredity in 1866 and the subsequent rediscovery of his findings in 1900 started an era of scientific quest for understanding the nature and content of genetic information [421,422]. The cellular and molecular basis of heredity was defined with the discovery of chromosomes [423] and the DNA double helix [424], followed by the elucidation of the genetic code [425]. The introduction of the DNA sequencing technique [426], together with the technique of cloning, soon led to the cloning and sequencing of the first human gene [427], and further automation of DNA sequencing [428] opened the road for the first genome to be fully sequenced, the 1.8 megabase pair genome of the bacterium *Haemophilus influenzae* [429].

The genome can be described as the complete set of genetic material (DNA) present in an organism. Sequencing the genomes of small unicellular organisms such as bacteriophages [430] and other viruses [431] quickly led to the idea that global views of genomes could greatly accelerate biomedical research. Backed by programs to create physical maps of the yeast *Saccharomyces cerevisiae* [432] and the worm *Caenorhabditis elegans* [433] genome, and a program to create a human genetical map to locate disease genes of unknown function [434], scientists proposed the idea to sequence the entire human genome in 1985 [435,436]. As a consequence, the Human Genome Project (HGP) was launched in 1990 as a 15-year program supporting a worldwide joint effort to create a genetic, physical, and sequence map of the human genome, in parallel with such efforts in key model organisms such as bacteria, yeast, worms, flies, and mice, together with the development of technology to support the objectives. Initially, the goal of the HGP was to generate high-quality, long-range finished sequence that was freely available for public purposes by the year 2005. However, the announcement in 1998 by the private initiative Celera Genomics Corporation to compete with the public HGP [437,438], shifted the goal of HGP to produce a draft genome sequence first [439]. Celera planned to finish the sequence of the human genome by the end of 2001, market their database system and sell it for profit [437]. As a consequence, HGP speeded up the process of sequencing that resulted in the publication of the HGP and the Celera draft human genome

sequences in February 2001 [440,441]. Although the draft sequences were acquired using different methods, both showed gaps in the physical maps and needed to be further sequenced to produce a fully finished sequence of the human genome [440,442]. Recently, a 99.9% finished draft sequence has been published, 50 years after the discovery of the DNA double helix [443].

The completion of the working draft of the human genome is a milestone in biomedical research, providing information about genes, evolution, and genetic variation in humans, but more importantly it serves as a foundation for understanding the function of genes and their products.

3.1.2 From genome to transcriptome

The size of the sequenced euchromatic region of the human genome is between 2.9 [441] – 3.2 [440] billion base pairs. Based on the draft sequence, the estimated number of protein-coding genes lies between 29,000-36,000 [443]. Earlier estimates ranged between 30,000 to more than 140,000 genes [444-447]. The number of protein-coding genes in an organism provides a relatively simple, although incomplete, measure of its molecular complexity. The unicellular prokaryote *E. coli* and the eukaryote S. *cerevisiae* have 4,300 [448] and 6,000 genes [449], respectively. Comparison of unicellular genomes with multicellular ones leads to the conclusion that evolution of multicellularity is apparently accompanied by an increase in gene number, since the worm *C. elegans* has 19,000 genes [450] and the fruitfly *D. melanogaster* has 13,600 genes [451]. Therefore, it came as a surprise that humans have only twice as many genes. However, the relatively low number of genes can be compensated for by a higher complexity of the genes, with more alternative splicing (at least 50% of the genes [440,441,452-455]), transcriptional and translational control, leading to more protein products that are in turn subject to post-translational modifications (Figure 3.1).

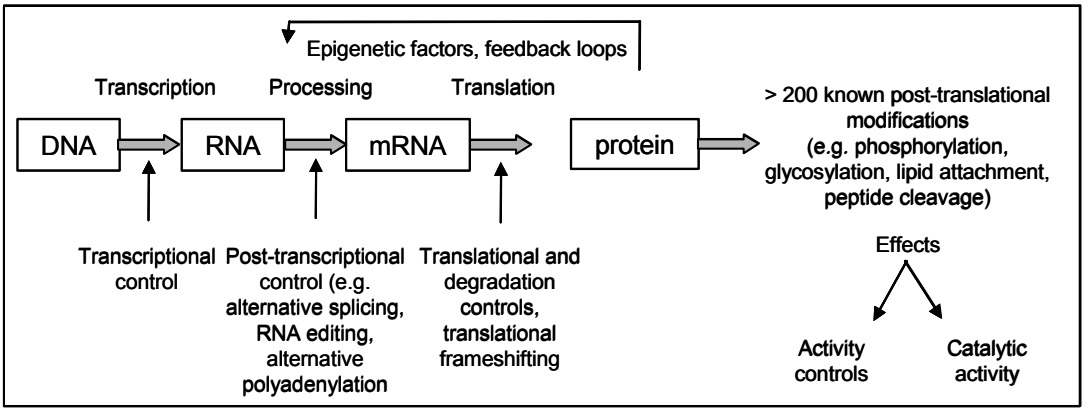


Figure 3.1 From genome to proteome
Expression of proteins starts with transcription of DNA into RNA that is processed into mRNA and subsequently translated into proteins. Each step is tightly controlled and results in a large diversity of gene products. Modified from Banks *et al.*, 2000 [456].

With the completion of large amounts of genome sequences came the realization that sequence information alone was not sufficient to identify the complete set of genes. Therefore, description of the complete transcriptome helps to define genes and gene

functions. The transcriptome includes all RNAs synthesized in a given system (cell, tissue, or organism), including protein-coding, non-protein-coding, alternatively spliced, alternatively polyadenylated, alternatively initiated, sense, antisense, and RNA-edited transcripts [457]. Cataloguing the mammalian transcriptome is based on the assembly of sequences of expressed-sequence-tags (ESTs) [458], complemented by the approaches of Serial Analysis of Gene Expression (SAGE) [459], open reading frame ESTs (ORESTES) [460], and microarray based strategies [461]. ESTs represent either 3' or 5' short cDNA (reversed transcribed mRNA) fragments, SAGE produces short sequence tags that are located adjacent to defined restriction sites near the 3'end of the cDNA, and ORESTES produces a high proportion of sequence tags that are in the coding regions of the transcripts. Microarray based strategies use "exon-scanning" to test sets of annotated or predicted exons across a range of tissues, and "tiling arrays" to refine the precise exon structures of genes in a genomic region [461,462]. Combining the data obtained by all of these approaches provides a closer description of the transcriptome and has largely contributed to the annotation of the human genome.

Which genes are transcribed into mRNA is cell type and tissue specific, and furthermore dependent on a multitude of regulatory events within cells. While the genome is a static entity (with few exceptions, every cell type and tissue of one organism has the same genome), the transcriptome is very dynamic: the expression profile within a certain cell type changes upon signals from different stimuli. Therefore, analysis of the mRNA expression profiles of cells under different states or comparison of diseased *versus* normal tissue reveals insights into the dynamics of the transcriptome. Recent advances in technology have provided micro-array based tools for high-throughput mRNA expression profiling. In this way thousands of cDNAs can be monitored under different conditions. However, several studies have indicated that mRNA abundance does not correlate very well with the corresponding protein levels [463,464]. Since proteins, rather than genes or mRNAs, are the key players in the cell, they determine the cellular phenotype and response to signals. Thus, a poor correlation between protein and transcript might lead to misinterpretation of mRNA profiling experiments. The discrepancy between mRNA and protein levels can be partly explained by the fact that translation of a large fraction of mRNA is controlled at the translational level [465]. mRNAs that are fully loaded with ribosomes, so-called polysomes, represent actively translated transcripts and can therefore be directly correlated with protein synthesis [465]. High-throughput expression profiling of mRNA represents more precisely changes occurring at the protein level when polysome-bound mRNA is being analyzed, although it does not provide any information about post-translational modifications, subcellular localization or protein degradation.

3.1.3 Functional genomics

Deciphering the transcriptome goes hand in hand with identification of gene function. Computational analysis is widely used to predict the function of the protein set in humans by comparing the human genome with other mammalian (mouse [466]) or other eukaryotic (fly [451] and worm [450]) genomes. Methods based on analyses at the level of protein families and at the level of protein domains revealed that 40% of the predicted genes did not match any ascribed protein function [440,441]. This percentage contains new genes not previously identified, but also largely represents pseudogenes (inactive gene copies), and genes generating non-protein coding RNAs, including rRNAs and tRNAs [466]. Based on a recent re-evaluation of the human gene annotation, it was estimated that the genome contains 21,300 pseudogenes, and 1,500 genes coding for antisense RNAs [443]. Algorithms that do not

rely on amino acid similarities have the potential to assign functions to previously unknown proteins by detecting functional linkages between proteins [467]. One such approach is the phylogenetic profile method, which scores the presence or absence of a given protein in all sequenced genomes. If two or more proteins have identical or similar phylogenetic profiles, they are likely to be engaged in a common pathway or complex [468,469]. The Rosetta Stone method analyzes fusion patterns of protein domains, based on the observation that many proteins consist of two domains in one organism, whereas the domains are two separate proteins in another organism [470]. A third computational approach uses the gene neighbor method, implying that each set of encoded proteins that is located next to each other on the chromosome in multiple genomes tends to be functionally linked [471].

It is evident that the availability of multiple genome sequences, the enormous amount of mRNA expression data, and several computational analyses methods provide a basis for understanding the complexity and functioning of cells. However, genomics research does not directly provide information about protein function, and therefore the basis of such understanding lies within the proteome. As is the case for the transcriptome, the proteome is not static but its expression state depends on cellular and environmental factors. Unraveling expression level, sub-cellular localization, post-translational modification, and interacting protein partners is necessary for complete understanding of function of a given protein. Therefore, without extensive proteomics research the benefits of genomics will be largely unused.

3.2 Proteomics

Introduction

Proteomics usually involves separation of proteins from cells or cell fractions in 2-dimensional polyacrylamide gels and identification by mass spectrometry. The technique of 2-dimensional electrophoresis (2DE) finds its roots back in 1975, when it was first independently introduced by O'Farrell and Klose [472,473] as a powerful analytical tool providing high resolution, sensitivity, and reproducibility, which could, as O'Farrell stated, potentially find use in a wide range of investigations [472]. The revival of 2DE in the "post-genomic era" surely found its use in a wide range of investigations, and to date it is still a corner-stone technique, although highly potential alternative techniques are under development. It was the introduction of mass spectrometry into the field of biology that changed proteome research, for it can generate very sensitive structural and analytical information on proteins. These data are used to search sequence databases, providing a relatively quick and easy way of protein identification [474].

3.2.1 Classical proteomics

2D gel electrophoresis

Proteomics research can be divided into a "classical" 2DE driven approach and recently rapidly evolving non-2DE approaches (discussed in paragraph 3.2.3). 2DE is still largely the basis for proteome research, because of the high-resolving power of 2D-gels for the separation of complex mixtures of proteins. High-resolution 2DE is based on two independent properties and is conducted in two distinct steps. In the first step, proteins are separated according to their isoelectric point (pI) using isoelectric focusing (IEF). In the second step, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their molecular mass (M_r, relative molecular mass), resulting in a 2-dimensional profile of protein spots. When the technique was first

introduced, IEF was performed in carrier ampholyte (artificial protein) generated pH gradients using self-cast tubing gels [472], typically covering pH ranges from 3 to 10 over a distance of 13 cm. Such pH gradients tend to become unstable in time and suffer from batch-to-batch manufacturing variations, and as a result are prone to irreproducible results. The introduction of immobilized pH gradients (IPG) and pre-cast gels on plastic backings greatly enhanced resolution and reproducibility of 2D gels [475,476]. The separation of proteins in a pH gradient is based on the amphoteric property of proteins; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The isoelectric point is the specific pH at which the net charge of a protein is zero. IEF is performed at high voltages (up to 8000 V) providing an electric field in which proteins migrate to the position in the pH gradient where their net charge is zero.

After 2DE, the gels can be stained using Coomassie Brilliant Blue (detection limit of 25-50 ng), colloidal Coomassie (detection limit of 5-10 ng), silver nitrate stain (detection limit of 1 ng), or fluorescent dyes (detection limit of 1-5 ng). Coomassie and fluorescent dyes have a linearity over multiple orders of magnitude, whereas silver stain is less linear for higher abundance proteins. Using these staining methods, typically 1,000-3,000 protein spots are visualized, representing a few hundred distinct proteins. Considering that the human genome contains 30,000 genes that yield 3-6 times more proteins [477], which are in turn subject to all kinds of post-translational modifications, it is estimated that the human body may contain at least 500,000 protein isoforms [456]. At the cellular level, where 5,000-10,000 genes may be expressed, this will still yield an enormous amount of modified proteins. The dynamic range of all these proteins is very large, considering that actin, which is the most abundant protein in almost all human cells, is present at 10^8 molecules per cell, whereas some cellular receptors or transcription factors may be present at 100-1,000 molecules per cell [478,479]. As a consequence, analysis of a total proteome in a regular 2D gel will result in the visualization of highly (abundant) or moderately expressed proteins. Several ways to overcome this problem have been introduced. Improvements in sample preparation such as the addition of strong denaturant chaotropes (thiourea in addition to urea) and zwitterionic detergents that have zero net charge (like CHAPS) to the lysis buffer result in better solubilization of proteins [480]. Also, multiple protein extraction steps using different lysis buffers provide a broader range of solubilized proteins. Furthermore, subcellular fractionation enriches for proteins from specific organelles (Figure 3.2), thereby reducing the complexity and diversity of the sample, and allowing for the detection of proteins of lower abundance [481]. Technical improvements of IEF include higher sample loading (several mg of total protein) using IPG gel strips [482-484], increasing the possibility of detecting low-abundance proteins. The use of large format (up to 24 cm) and narrow-range (covering 0.5-3 pH units) IPG gels strips allows for higher resolution [478,485], which further increases the total amount of visualized proteins and the detection of low-abundance proteins. The advantage of overlapping narrow-range IPG strips is that the obtained 2D gels can be annealed, resulting in a proteome profile that is resolved in a multitude of 24 cm gels. The technical drawback of these improvements is that relative large amounts of sample are needed (mg instead of μg), and that the fractionation of the sample in combination with the use of multiple overlapping narrow-range IPG strips results in handling of multiple gels representing the protein content of one sample. In cases were multiple samples need to be compared in order to find differentially expressed proteins, the handling of a large number of gels will be difficult. Furthermore, introducing more steps during sample preparation

increases protein loses. This is particularly a problem if the sample is unique and not available in large amounts.

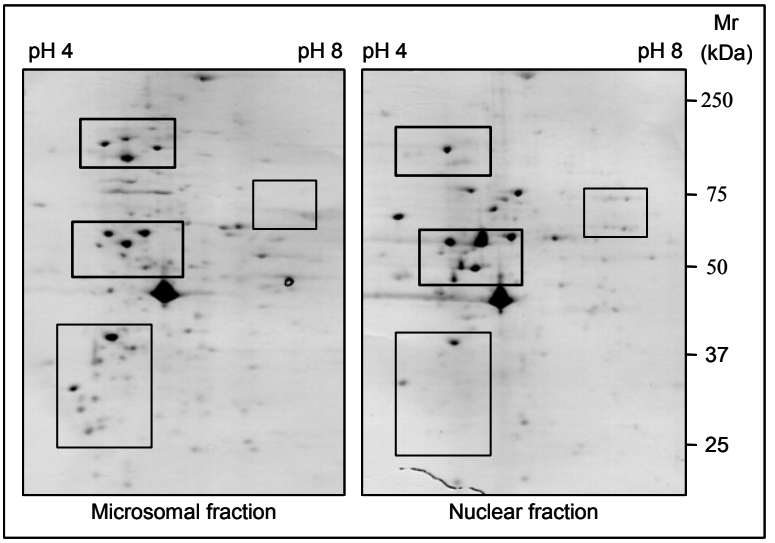


Figure 3.2. Subcellular prefractionation of MFVD cells
A microsomal and a nuclear fraction was prepared from the cell lysate and subsequently subjected to 2D-PAGE. Note the enrichment of specific proteins in the boxed regions.

Other limitations of 2DE include, that large proteins (> 200 kDa) do not enter the gel, that small proteins (< 10 kDa) run too far, and that very acidic or basic proteins (pH< 4 and > 8) and hydrophobic and insoluble proteins (such as membrane proteins) are not or rarely resolved. Another limitation is that 2DE cannot be automated for high-throughput analysis, and thus remains tedious and time consuming. In addition, 2DE is biased to high abundant proteins [486]. Thus, the usefulness of 2DE for proteome research depends on the question that needs to be answered, and the choice of sample preparation and pH range to be used will depend on the starting material used. Undoubtedly, some properties of 2DE make the technique very strong and unique. For example, 2D gels can very easily distinguish different post-translationally modified protein isoforms. Furthermore, protein quantities can be determined, either manually or by computer analysis, provided that a linear staining method is used. Since post-translational modifications determine the activity of a protein, and protein quantity influences the effect of protein function, 2DE is still a powerful tool for proteome analysis.

Mass spectrometry

After 2DE separation, gel plugs are taken containing the proteins of interest, and cleaved into smaller peptide fragments using enzymes that recognize a specific restriction sequence, such as trypsin, which cleaves C-terminally of lysine and arginine residues unless they are followed by a proline. Because of this specific cleavage pattern, which is unique for every protein, the masses of tryptic digests can be predicted theoretically for any entry in a protein sequence database. Subsequently, the peptide fragments are extracted from the gel and analyzed by a mass spectrometer (Figure 3.3). Different types of mass spectrometers are available, but they all consist of three essential parts - an ion source, a mass analyzer, and an

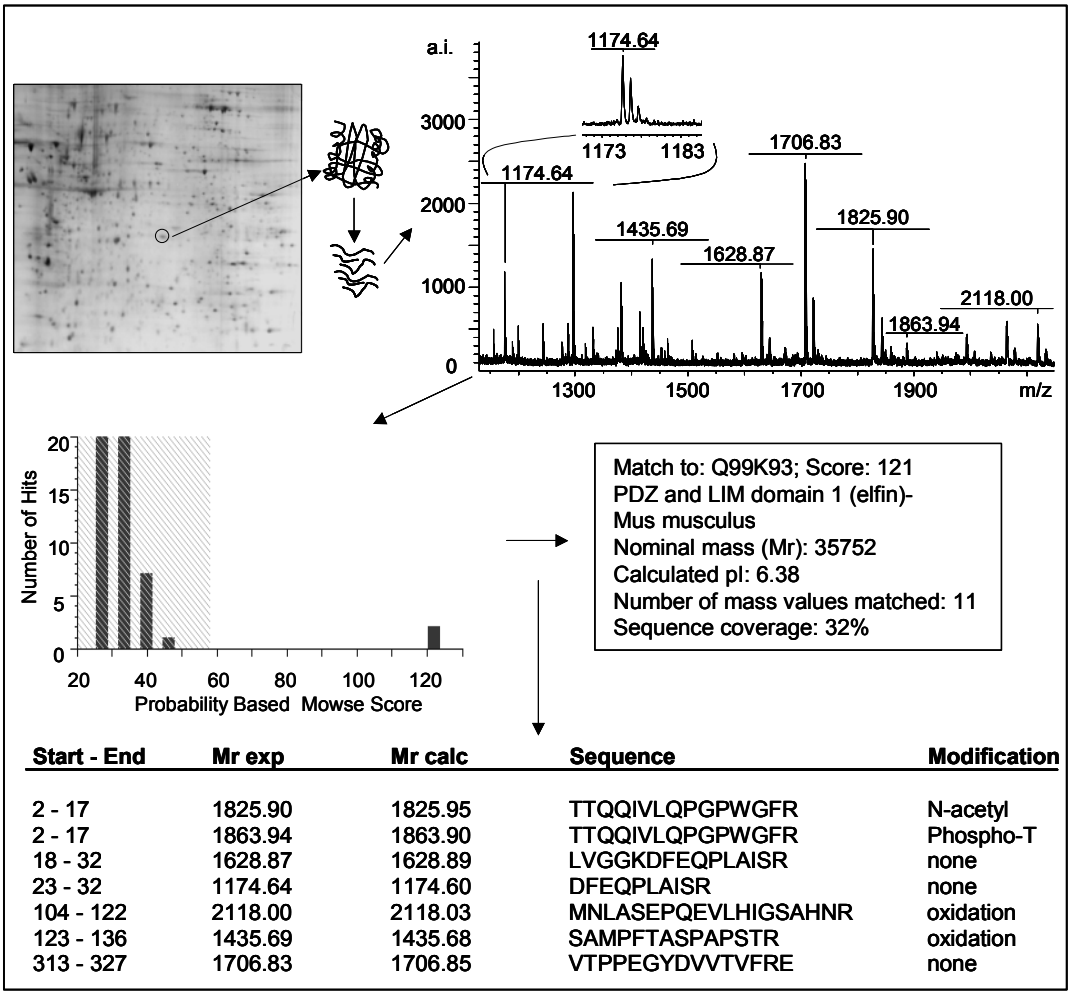
ion detector. In the ion source, molecules are converted into gas-phase ions. The created ions are separated into individual mass-to-charge ratios (m/z; m=mass; z=charge state) in the mass analyzer, and subsequently transferred to the ion detector [487]. The mass analyzer uses a physical property, such as an electric or magnetic field, time-of-flight (TOF), quadrupole, ion trap, or Fourier-transform ion cyclotron resonance (FTICR) [488-491], to separate ions of a particular m/z value, which are then detected by the ion detector. An important innovation for proteomics has been the development of two robust techniques to create ions of large molecules:

MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry. In a MALDI ion source, the peptide samples to be analyzed are embedded in an energy absorbing crystalline matrix (α-cyano-4-hydroxycinnamic acid is a frequently used matrix for peptide analysis), which is excited by the energy of a laser (337 nm nitrogen or infra-red). The excited matrix vaporizes and ionizes by addition of one proton to analyte ions (peptides) that in turn convert into gas-phase ions [488-492]. The MALDI ion source uses a high voltage gradient, which causes the ionized peptides to accelerate into a flight tube. Small ions fly quicker to the detector than large ions and therefore time required to reach the detector is correlated with the m/z value, which is determined by measuring the time it takes ions to move from the ion source to the detector (time-of-flight). Deconvolution of the m/z value results in the exact mass of the compound analyzed. The accuracy of such measurements is given in ppm (parts per million). In MALDI, peptides are generally single charged.

ESI-MS: electrospray ionization mass spectrometry. ESI creates a continuous ionization source by application of a potential to a flowing liquid causing the liquid to charge and subsequently spray. The electric field generates small, charged droplets of solvent-containing analyte. The solvent is removed by heat or drying gas, resulting in the formation of multiply-charged ions [493]. The introduction of nano-electrospray has made it possible to analyze very small amounts of analyte, e.g. 1 µl can give a stable spray for 40-60 min [494,495]. ESI can be easily coupled to all kinds of mass analyzers.

The acquired peptide mass maps by TOF-MS are used for peptide mass fingerprinting, in which the experimentally observed m/z values are compared with the theoretical m/z values predicted after *in silico* digestion of each protein in the database with the site specific enzyme used (e.g. trypsin) [496,497]. Furthermore, database search programs can allow for protein modifications during the search, such as oxidation, methylation, or phosphorylation, which change the m/z value of a peptide (Figure 3.3).

The quality of the mass spectrum, which is determined by mass accuracy and resolution, determines the number of peptide matches in the protein sequence and the positive identification of a protein [498]. Peptide mass fingerprinting requires that the full-length sequences be present in the protein sequence databases. Therefore, the completeness of a database determines the success rate for protein identification using peptide mass fingerprinting.



Start - End	Mr exp	Mr calc	Sequence	Modification
2 - 17	1825.90	1825.95	TTQQIVLQPGPWGFR	N-acetyl
2 - 17	1863.94	1863.90	TTQQIVLQPGPWGFR	Phospho-T
18 - 32	1628.87	1628.89	LVGGKDFEQPLAISR	none
23 - 32	1174.64	1174.60	DFEQPLAISR	none
104 - 122	2118.00	2118.03	MNLASEPQEVLHIGSAHNR	oxidation
123 - 136	1435.69	1435.68	SAMPFTASPAPSTR	oxidation
313 - 327	1706.83	1706.85	VTPPEGYDVVTVFRE	none

Figure 3.3 2DE driven proteomics
Proteins are separated by 2D-PAGE, a protein of interest is cut from the gel and cleaved into peptide fragments. The peptides are analyzed by MALDI-TOF-MS resulting in a peptide mass fingerprint. Selected peptide masses are submitted to the protein database and the results are represented as a statistically determined Probability Based Mowse Score. When the score is above the threshold (shaded area), the identification is significant. The database search provides information about the accession number, mass, pI, number of matched mass values, and sequence coverage of the identified protein. Detailed information is given in the table concerning the matched peptide experimental mass (Mr exp) and calculated mass (Mr calc), the sequence, position, and possible modification.

Peptide mass maps are created by single-stage mass spectrometers, such as a MALDI-TOF or ESI-TOF (Figure 3.4). However, several different mass analyzers, such as a quadrupole or hexapole, can be coupled to an ion source, separated by a collision cell. In general, such mass spectrometers are called tandem mass spectrometers, because they generate peptide mass maps as well as peptide fragmentation patters (MS/MS spectra). The MS/MS analysis is performed on an individual ion with a particular m/z value that was selected from the peptide mass map, which dissociates in a collision cell. The dissociated products are subsequently analyzed in the second mass analyzer, generating peptide fragmentation data of that specific peptide, from which amino acid sequence data can be derived [487,499,500] (Figure 3.4). Tandem mass spectrometry (MS/MS) is technically more complex and less scalable than peptide mass fingerprinting, but the sequence information is more specific for one particular protein. In addition, fragmentation data can be used to search protein sequence databases as well as nucleotide, EST, and raw genomic sequence databases.

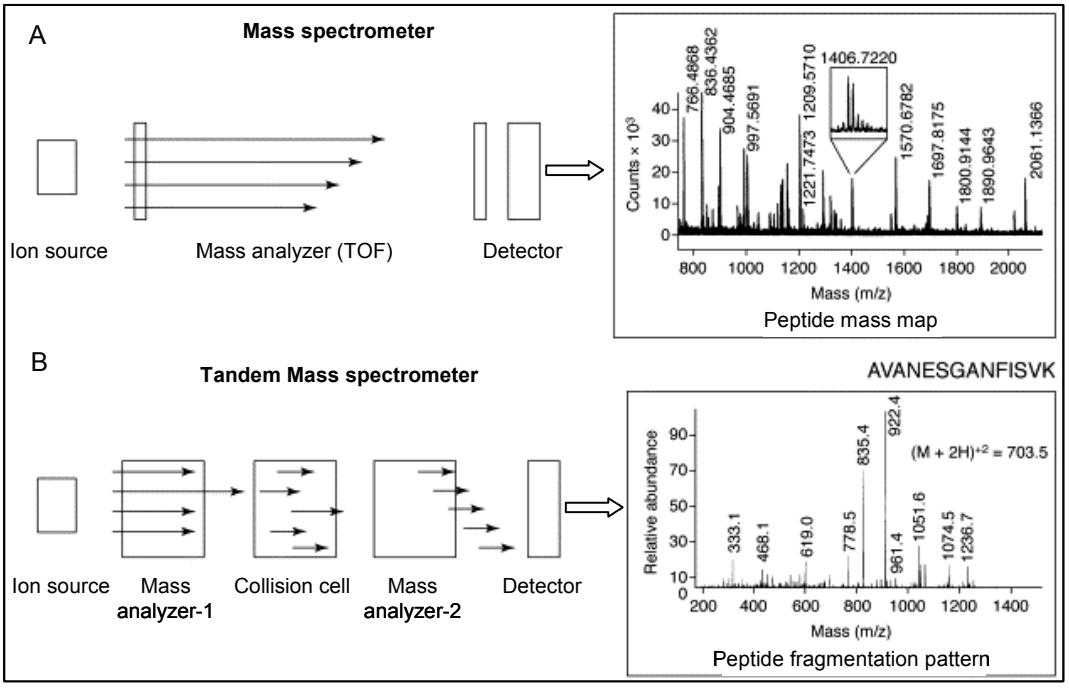


Figure 3.4 Principle of mass spectrometry and tandem mass spectrometry
A) The sample analyte is ionized in the ion source, transferred to the mass analyzer and detected on the detector according to the mass to charge ratio (m/z). This results in a peptide mass fingerprint.
B) The tandem mass spectrometer first creates a peptide mass fingerprint, and subsequently a peptide with a particular m/z value is dissociated in a collision cell and further analyzed in the second mass analyzer. This provides a peptide fragmentation patterns from which the amino acid sequence can be deduced. Adapted from Yates, 2000 [487].

Mass spectrometric identification of gel-separated proteins (either 1D of 2D) has been a major breakthrough in proteomics and has found its applications in a whole variety of research fields.

3.2.2 Proteomics in reproductive endocrinology

Protein identification in the male genital tract

After the introduction of 2DE, this technique was quickly adapted in endocrine research. In the context of the present thesis, proteins in epididymis and vas deferens have been studied in detail with respect to their role in tissue function. The developmental process of epididymis and vas deferens is under control of androgens, and also maintenance of function of the organs in the adult [4,501]. Epididymis, vas deferens, and luminal fluid secreted by the epididymis and vas deferens provide a microenvironment, which is suitable for sperm maturation and storage [502]. It has been reported that epididymal fluid proteins interact with spermatozoa and bind to the sperm membrane [503]. 2DE has been used to investigate the effect of androgens on protein synthesis and secretion in various regions of the epididymis and vas deferens [504-506]. These early studies characterized proteins of specific pI and Mr, established their regional expression and dependence or independence of androgens, but could not reveal the identity of these proteins. Later studies have used measurements of enzyme activity [501], immunoblotting [507], or Edman degradation amino acid sequencing [508,509] for the identification of specific proteins. Enzyme activity measurements and immunoblotting can only be used in cases where the proteins of interest are already known. Edman degradation, on the other hand, can be applied for sequence information on any unknown protein. It does, however, require larger amounts of pure peptide, skill and time before a sequence can be retrieved [510,511].

Technological developments on mass spectrometers lead to an enormous progress of identification of epididymal proteins. Prostaglandin D2 synthase was identified as an epididymal fluid protein using MALDI-MS and nanoESI-MS/MS. 2DE showed multiple protein isoforms of Prostaglandin D2 synthase in the gel (pI 6.5-8.8 and M_r 20-25 kDa) and treatment with N-glycanase resulted in one protein isoform, demonstrating post-translational N-glycosylation of the protein [334]. MALDI-TOF-MS identification of rat sperm membrane proteins separated by 2DE, revealed the presence of heat shock protein 70, disulfide isomerase ER60, and subunits of the proteasome on the membrane (Ooms and Vreeburg, unpublished observations). Surprisingly, these proteins were also identified as sperm membrane antigens, against which antisperm antibodies are directed, causing human immunological infertility [512]. Furthermore, one major rat sperm membrane protein, synthesized in late spermatogenesis, was recently extracted from 2D gels that were processed 10 years ago and was identified as hexokinase (Ooms and Vreeburg, unpublished observations). This finding demonstrates that proteins can be identified from 2D gels even after long-term preservation and storage.

An interesting approach that is currently being undertaken to determine protein profiles of regions of the epididymis, is the use of imaging MALDI-TOF-MS. In a recent study, caput and cauda epididymis cryostat tissue sections were either blotted on a PE membrane and spotted with matrix, or directly mounted on a target plate and subsequently spotted or coated with matrix. MALDI-TOF-MS was performed on these tissue sections providing protein profiles and optical images of the epididymis [513]. Comparing the protein profiles of caput and cauda epididymis revealed that specific m/z signals were present, providing information about protein distribution within the epididymis. Furthermore, optical imaging of the different m/z values in the epididymis sections shows the spatial distribution of that specific protein corresponding to the m/z value [513].

As described in **Chapter 1**, androgens play an essential role in embryonic development of genital tract tissues. 2DE driven proteomics was also used to identify androgen-regulated

proteins in embryonic epididymis and vas deferens tissues (**Chapter 4**) and the vas deferens cell line MFVD (**Chapter 5**). In MFVD cells, androgens induced post-translational modification of mElfin and CBF-A (Figure 3.5), two actin-binding proteins that are possibly involved in recruitment of signaling molecules [514]. Embryonic epididymides and vasa deferentia that were cultured in the presence of androgen showed post-translational modification of triosephosphate isomerase, a glycolytic enzyme, heterogeneous nuclear ribonucleoproteins hnRNP A2/B1 and hnRNP A1, and RhoGDI, a signaling protein (**Chapter 5**). To understand the role of these proteins in genital tract development, more detailed proteome analysis and functional analysis will be needed.

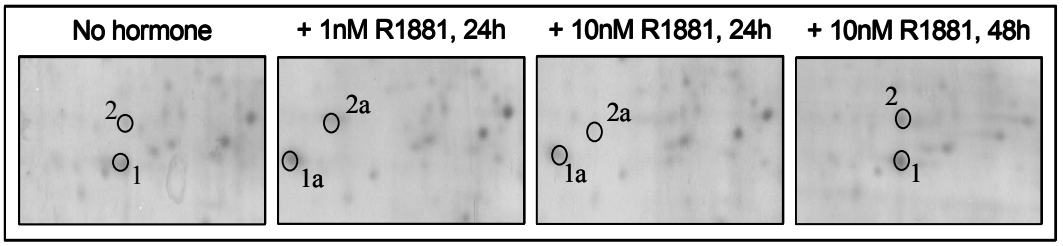


Figure 3.5. Post-translational modification of proteins upon androgen treatment
MFVD cells show regulation of mElfin (1) and CBF-A (2) upon androgen treatment. Both proteins shift towards the acidic part of the gel (1a and 2a) upon R1881treatment for 24 hours but this effect is reversed within 48 hours.

2D gels have already greatly contributed to the field of reproductive endocrinology, but the emergence of state-of-the-art proteomics techniques (paragraph 3.2.3) will provide new possibilities for large-scale studies on androgen-regulated proteins that are expressed in the genital tract and sperm, in order to better understand the physiology of fertility.

Expression profiling in health and disease

While androgens and androgen-regulated proteins are involved in growth, development and maintenance of male physiology, they also play a role in the development of malignancies, such as prostate cancer. Not only for prostate cancer, but also for a whole variety of diseases, much effort is now being put in understanding pathogenesis at the protein level, and in the development of effective strategies for early diagnosis and treatment, using applications in disease proteomics [515]. For prostate cancer several different approaches were undertaken to identify tumor specific marker proteins involved in transition from androgen-dependence to androgen-independence, and marker proteins representing different stages of tumor progression.

In one such study, 2D maps of normal prostate tissue were compared with 2D maps of malignant prostate, and 20 proteins were identified that were lost during the transition to malignancy. These proteins included PSA (prostate specific antigen) and other proteins known to be involved in prostate cancer, but also proteins not previously described in this process, like NEDD 8, calponin, and follistatin-related protein [516]. Nelson *et al.*, 2000 used the prostate cancer cell lines LNCaP and M12AR to study androgen-regulated protein expression. Androgen-stimulation resulted in the up-regulation of human nucleoside diphosphate kinase A, which is a tumor metastasis suppressor protein [336]. This finding coincides with the observed decrease in invasion and metastasis of LNCaP and M12AR cells compared to the prostate cancer cell line M12 that does not express the androgen receptor and is highly tumorigenic and metastatic.

Although studies in cell lines can be very informative, it is becoming increasingly clear that protein expression patterns of tumor cell lines do not accurately correspond to expression patterns of primary tumors. While 2D maps of prostate cancer cell lines are rather similar, they differ substantially from microdissected prostate tumor cells. Even cell lines that were derived from a tumor showed altered protein expression profiles when compared to the primary tumor [517]. Thus, one should be careful in drawing conclusions from studies performed on cell lines, since the results may not precisely represent the expression profile of the tumor. However, analysis of tumor samples is difficult to interpret because of their heterogeneity. Solid tumors may represent tumor cells, stromal cells, normal epithelial cells, endothelial cells, inflammatory cells, including macrophages, and extracellular matrix, which complicates the biological interpretation of the analysis. To overcome these problems, several approaches are now being used to obtain pure populations of cells, such as antibody-based flow-cytometric sorting, and laser microdissection (LCM) [518]. It appears that microdissected prostate tumor cells have a different protein expression pattern as compared to microdissected normal or stromal cells [517]. Although LCM microdissected tumor material does not yield high amounts of protein (µgs of protein from 100,000 cells), it seems feasible to perform 2DE and subsequently MS/MS to identify numerous proteins that are differentially regulated between tumor cells and normal prostatic epithelial cells [339]. LCM purified cancer cells have also been analyzed using a new type of affinity-based mass spectrometer, surface-enhanced laser desorption/ionization (SELDI) mass spectrometry. The underlying principal of SELDI is surface-enhanced affinity capture through the use of protein chips consisting of chemical characteristics (hydrophobic, hydrophilic, cationic, anionic, etc.) or biological ligands (proteins, receptors, antibodies, DNA oligonucleotides). A complex protein mixture (cell lysate or body fluid) is directly applied on the surface, non-specific binding partners are washed away, and the retained proteins are subsequently analyzed by time-of-flight MS [519].

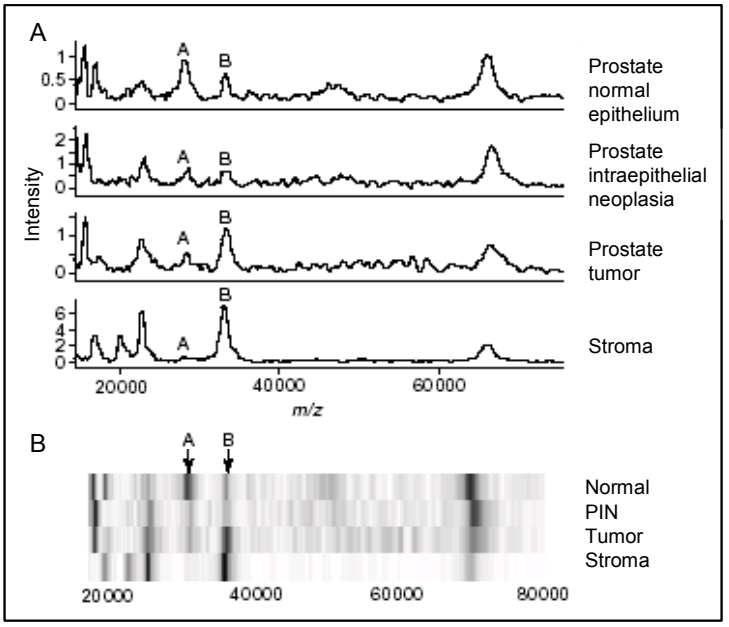


Figure 3.6 SELDI expression profile of different stages of prostate cancer samples
A) Mass profile of prostate normal epithelium, prostatic intraepithelial neoplasia, tumor, and stroma.
B) A gel-like image of the raw mass data shown in a). Note the changes in expression level of proteins A (28 kDa) and B (32 kDa) between the different samples. Adapted from Petricoin and Liotta, 2000 [520].

Comparison of prostate tumor cell lysates from different stages (benign hyperplasia, intraepithelial neoplasia, and prostate cancer) showed altered protein expression profiles between the stages of disease and can be distinguished from each other according to their profile [521-523] (Figure 3.6). SELDI-TOF mass spectra have also been generated for breast [524], ovarian [519,525], and several other cancers [526]. Such SELDI expression profiles can be used in screens for biomarker discoveries, because it is very suitable for high-throughput analyses in a relatively short time, which may lead to the development of new diagnostic tests for early detection of cancer. However, the proteomic patterns that are generated have a low resolution and thus low mass accuracy, and the biomarkers cannot be routinely identified because of the lack of TOF-MS/MS possibilities. Furthermore, although biomarker discovery seems successful in the low-molecular weight range of 2-20 kDa, reproducibility is a problem with high-molecular weight proteins [526]. Thus, the SELDI approach does not identify specific proteins that are unique for a particular disease state but merely provides information about protein patterns that belong to a certain disease state.

Screens for biomarker discovery for many disease types, including prostate cancer, have already been performed on a large-scale using SAGE and cDNA microarrays [336,527,528]. However, considering the poor quantitative correlation between mRNA and protein expression [337], and the importance of post-translational modifications, there is a growing need for other profiling technologies, such as protein arrays and biochips. Pathogenesis will be best understood when mRNA expression data can be combined with data on proteome alterations, such as protein expression levels, interacting biomolecules, subcellular localization, and post-translational modifications. Further technological innovations in proteomics are underway to increase sensitivity, reduce sample requirement, increase throughput, and more effectively uncover various types of protein alterations [515].

3.2.3 State-of-the-art applications

Non-2DE driven proteomics

The rapid accumulation of genomic sequences in public databases is putting pressure on proteomics research to establish large-scale high-throughput analysis methods for the functional annotation of all these genomes [474,487]. Although 2DE based protein identifications have already provided valuable information, this method is labor intensive and difficult to automate, in addition to other limitations mentioned in paragraph 3.2.1 (e.g. not all proteins focus in the gel and there is a bias towards high-abundant proteins). Therefore, a great effort is being put in developing non-2DE driven proteomics tools that separate, quantitate and analyze complex protein mixtures and identify the proteins of interest in one analysis run, using only a small amount of sample. Such approaches use complex mass spectrometers and require strong bioinformatics tools and data mining. Some of the recent advances in quantitative proteomics will be outlined.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS): LC-MS/MS is a method to directly identify proteins from complex mixtures. Mixtures of proteins such as cell lysates or body fluids are digested with an enzyme (e.g. trypsin) producing a large collection of peptides. Such peptide mixtures can be separated by microcolumn reversed-phase liquid chromatography (RPLC), which is directly coupled to the tandem mass spectrometer, resulting in a large number of mass spectra [529]. Several variations on this theme have been presented, such as multidimensional chromatography-coupled tandem mass spectrometry (LC/LC-MS/MS), which combines a strong cation exchange with RPLC providing a two-dimensional separation prior to MS/MS. Database searches on the MS/MS spectra using special algorithms provides a multidimensional protein identification technology, named MudPIT [530]. MudPIT performed on the yeast *S. cerevisiae* proteome revealed the identification of 1,500 proteins covering proteins of low-abundance, proteins with extremes in pI and M_r, and integral membrane proteins, in an analysis time of 90 hours using 1.32 mg protein [530,531]. A MudPIT approach was also used for a comprehensive proteomic view of the malaria parasite *P. falciparum* life cycle. Over 2,400 proteins were identified from four different stages of the life cycle, and functional profiling of the proteins agrees with the physiology of each stage [532]. Other non-gel based 2D-separations include the coupling of high performance liquid chromatography (HPLC) with RPLC [533], or capillary isoelectric focusing integrated with capillary RPLC [534]. Transferring isoelectric focussing from gel to capillary format contributes to a high-resolution protein separation with pI differences as small as 0.005 pH units, and analysis of low-abundance proteins with a concentration factor of 50-100 times [534]. The combination of capillary IEF with capillary RPLC not only results in high-resolution protein/peptide separation but also provides information on pI.

Quantitative isotope labeling: a significant advance in quantitative proteomics is differential isotope labeling. Two sets of proteomes to be compared can be labeled at different points in the sample preparation pathway. First of all, metabolic labeling can be performed during e.g. cell growth, using ^{14}N containing medium in one condition and ^{15}N enriched medium in the other condition [535,536]. Labeling can also be performed after protein extraction using a commercially available isotope-coded affinity tag (ICAT™) reagent. The ICAT™ reagent consists of a biotin affinity tag, a reactive group with specificity towards thiol groups (cysteines), and a linker that can incorporate stable isotopes. The ICAT™ light reagent contains 8 hydrogen atoms, whereas the heavy reagent contains 8 deuterium atoms, creating a mass difference on the mass spectrometer of 8 Da for singly charged ions and 4

Da for doubly charged ions. ICAT™ light and heavy reagents can be used to label cysteine residues in two separate protein pools. The two samples are subsequently combined and enzymatically cleaved into peptide fragments, the tagged peptides are isolated by avidin chromatography, and finally separated and identified using multidimensional LC-MS/MS. The relative intensities of the signals of two differentially isotopically labeled peptides are measured to determine the relative peptide concentration in the two mixtures [537-540]. The combination of these methods and techniques allows for the detection and quantification of proteins of low-abundance over an effective dynamic range of 10^4 by reducing the complexity of the mixture [539]. While isotope-tagged peptide mixtures separated by multidimensional LC can be identified in a robust, sensitive, and automated fashion by ESI-MS/MS, it requires continuous sample application, and much time for data collection and analysis. The use of MALDI-MS/MS (such as MALDI-quadrupole time-of-flight) reduces the experimental time and increases the efficiency of global proteomic comparisons of biological cell states, because MS/MS can directly be focused on labeled peptide pairs showing interesting changes in relative abundance [540,541]. Although reducing the complexity of a sample by labeling results in the detection of low-abundance proteins, it also discards information on proteins that are not labeled. Future labeling strategies should ideally focus on all proteins isoforms present in a mixture.

Fourier transform ion cyclotron resonance (FTICR): relatively new to proteomics is the FTICR mass analyzer, which has an extremely high sensitivity and resolution. A detection limit at the low attomole level can be achieved as compared with the low femtomole limits with other instrumentation, with a mass accuracy of <1 ppm and a resolution of more than 100,000. FTICR-MS has the potential to identify and quantify hundreds to a few thousand different proteins in one analysis using the accurate mass of one single peptide and sequence database searching [542-544]. The accurate mass and time (AMT) tag approach is a two-stage stategy that provides increased sensitivity, coverage, and throughput. In the first stage a proteome sample is analyzed by high-resolution capillary LC-MS/MS to identify "potential mass and time tags", which are subsequently used to validate the AMT tag obtained by analyzing the same samples by FTICR. By analyzing a whole variety of samples, an AMT tag database is constructed, in which the peptides function as markers to identify their corresponding proteins in all subsequent, second stage, experiments [545] (Figure 3.7).

In a single 2.5 hour LC-FTICR-MS analysis an average of 1500 AMT tags are identifiable [543]. Although the generation of an AMT tag database for a specific organism, tissue or cell type will require large amounts of sample, once generated AMT tags will allow for peptide identification using only pg amounts of protein [543]. Furthermore, combining the AMT tag strategy with isotope labeling provides quantitative information of protein abundance. Although FTICR-MS has a sub-attomole detection limit, the dynamic range of a single mass spectrum is about 10^3. To further increase the dynamic range in FTICR-MS measurements, a dynamic range enhancement applied to MS (DREAMS) methodology is applied. DREAMS involves an alternate acquisition of a normal mass spectrum and a subsequent spectrum of the first spectrum from which the most abundant ion peaks are removed. This approach extends the dynamic range of FTICR by 10 to 100 fold and allows for the detection of 35% more peptides in a single analysis run [546,547].

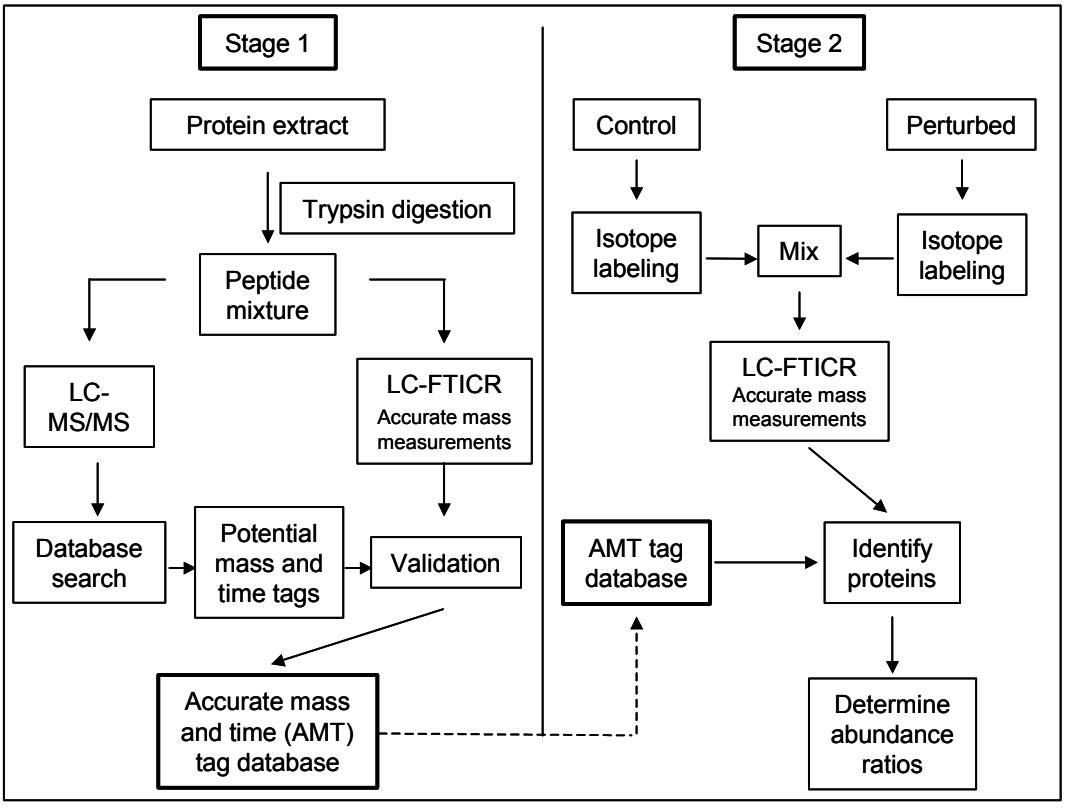


Figure 3.7. AMT tag based proteomics
Stage 1: A proteome sample is analyzed by LC-MS/MS and peptides are identified by the resulting sequence information as potential mass and time (PMT) tags. The same proteome sample is than analyzes under the same conditions using an FTICR-MS. An AMT tag is established when a peptide elutes at the same time and the corresponding mass of the PMT tag initially identified. An AMT database is constructed from a large number of different samples.
Stage 2: For any given proteome that is subsequently analyzed, the peptides in the AMT tag database function as markers to identify the corresponding protein. When two samples have been isotopically labels, protein abundance rations can be determined. Modified from Pasa-Tolic et al, (2002) [543]

It is evident that the current state-of-the-art technologies already offer great sensitivity to analyze small amounts of cells (e.g. obtained by laser microdissection) yielding less than 1 μg of proteins with a dynamic range covering four to six orders of magnitude. The rapid evolution of technologies will further increase the applicability of proteomics into medical research.

Functional proteomics

While most of the proteomics efforts are focusing on cataloguing large sets of proteins expressed under certain conditions in specific cell types or organisms, it also brings back the question of protein function. Protein function is determined by a variety of factors, such as sub-cellular localization, stability, post-translational modification, binding (protein) partners, and participation in complexes. Furthermore, a single protein may be present in multiple complexes and as a consequence be involved in more than one process. As proteins contain

many types of information, each of their properties need to be analyzed and put in a proteome-wide context in order to understand the physiology of a cell, tissue, or organism [548].

One way to determine a protein's function is the loss-of-function approach, by generating knockout mice or using RNA interference. At the cellular level, green fluorescent protein (GFP)-tagged proteins and confocal microscopy enable the investigation of specific proteins of interest in living cells, providing information on sub-cellular localization, dynamics, and interactions with other proteins under investigation, using techniques such as fluorescence resonance energy transfer (FRET) or fluorescence redistribution after photobleaching (FRAP) [549]. Recently, it has been postulated that post-translational modifications might be detected by FRET, using anti-phosphotyrosine antibodies coupled to a FRET acceptor molecule and measuring tyrosine phorphorylation of the GFP-fusion protein [550,551].

A commonly used method to identify interacting protein partners of a protein under investigation is the yeast two-hybrid system, in which a particular library can be screened for novel interacting proteins or in which the properties of binding of two known interactors can be assessed by mapping the precise region of binding [552,553]. As yeast two-hybrid can generate false-positive interactions, it is crucial to validate the putative protein-protein interaction. One condition of protein-protein interaction that has to be met is the presence of both proteins in the same sub-cellular compartment, and this interaction can be further verified by immunoprecipitation and Western blot analysis, using specific antibodies directed against the two proteins of interest.

The functional analysis of proteins using GFP-tags, yeast two-hybrid, or immunoprecipitation and Western blot is based on a one-protein-at-the-time approach. However, these techniques are now being put in array format and are combined with mass spectrometry, which provides a means of analyzing and identifying multiple proteins at a time, determine their localization and interacting partners, in a high-throughput manner. For example, large-scale analysis of GFP-tagged proteins was performed using a transfected cell microarray. This method involves transfection of cells on a glass slide, which contains different cDNAs on defined areas. By screening transfected cell microarrays, proteins involved in tyrosine kinase signaling, apoptosis, and cell adhesion were identified with the simultaneous analysis of their distinct sub-cellular distribution [554]. Protein-protein interactions can also be studied on a large-scale by performing FRET on transfected cell microarrays [551].

Another high-throughput way to study protein-protein interactions is the yeast two-hybrid array approach [555], or the high-throughput mass spectrometric protein complex identification (HMS-PCI) method [556]. HMS-PCI was performed on the yeast proteome using 10% of predicted yeast proteins as baits (725 proteins), and identified 3,617 protein interactions. Using this approach, many new, previously undescribed, interactions in signaling pathways and DNA damage response were identified [556].

Protein-protein interactions can also be studied, by purification of an entire multi-protein complex using affinity-based methods, using for example glutathione S-transferase fusion proteins, antibodies, peptides, or small binding molecules. The purified protein complex can either first be separated on 1D or 2D gels or can be directly identified by mass spectrometry [557]. Using an affinity-based-MS approach, several large protein complexes have been identified [558,559].

Relatively new in functional proteomics is the development of protein microarrays, which can be used for either analytical of functional analyses. Analytical arrays are typically coated with antibodies, antigens, carbohydrates or small molecules, with high affinity and specificity, which can be used for monitoring protein expression levels, profiling, and diagnostics. Functional arrays are spotted with purified native proteins or peptides, and can be used to analyze protein activities, binding properties, post-translational modifications, and building biological pathways [551,560,561]. As a consequence, functional arrays may be very useful in drug discoveries. One of the first large-scale protein arrays reported the purification of nearly 6,000 tagged yeast proteins and the identification of interacting proteins [562]. Antibody arrays were created to screen more than 18,000 recombinant antibody clones against 15 different antigens [563]. The hurdles to overcome for protein arrays, are the production of sensitive and non-cross reacting antibodies, the large-scale purification of proteins, and possible changes in protein conformation induced by immobilization, to mention just a few [564]. Nevertheless, the advantages of protein arrays being scalable, flexible, automatable, and easy to perform, will facilitate the high-throughput functional protein analysis on a genome-wide level.

Future applications in proteomics will benefit from the combination of several techniques brought together into one, like imaging mass spectrometry and transfected cell arrays. As mass spectrometers are being designed for higher sensitivity, resolution, accuracy, and automation, the ultimate goal would be to follow the dynamics of the proteome of living cells, from protein identification to sub-cellular localization or even post-translational modifications. In order to achieve such goals, different disciplines such as physics, bioinformatics, chemistry, and biology would have to work together, even more than they are doing right now.

Concluding remarks

The wealth of new technologies emerging in proteomics research goes hand in hand with high costs, limited technical expertise, and a high threshold for researchers in the field to access state-of-the-art facilities. In order for biomedical research to fully benefit from the advances made in proteomics, knowledge and expertise must be joined in institutional or national proteomics centers that are easy to access [565]. Furthermore, to coordinate large-scale proteomics efforts, the Human Proteomics Organization (HUPO) was founded in 2002 as a worldwide organization [566]. The goals of HUPO include developing an infrastructure in proteomics that facilitate unraveling the complexity of the human proteome and that of model organisms [515].

The impact of proteomics on endocrinology and all other fields has just started, but will certainly change the way research was conducted to date to tackle biological mechanisms [567].

Chapter 4

Proteomic analysis of androgen-regulated protein expression in a mouse fetal vas deferens cell line

Arzu Umar[1], Theo M. Luider[2], Cor A. Berrevoets[1], J. Anton Grootegoed[1], Albert O. Brinkmann[1]

[1]Department of Reproduction & Development
[2]Department of Neurology and Erasmus Center for Biomics, Erasmus MC, Rotterdam, The Netherlands

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Summary

During sex differentiation, androgens are essential for development of the male genital tract. The Wolffian duct is an androgen sensitive target tissue that develops into the epididymis, vas deferens and seminal vesicle. The present study aimed to identify androgen-regulated proteins that are involved in development of Wolffian duct-derived structures. We have used male mouse embryos transgenic for temperature sensitive Simian virus 40 large T at 18 days of gestation, to generate immortalized mouse fetal vas deferens (MFVD) parental and clonal cell lines. The MFVD parental and clonal cell lines express androgen receptor protein and show features of Wolffian duct mesenchymal cells. Clonal cell line MFVD A6 was selected for proteomic analysis and cultured in the absence or presence of androgens. Subsequently, two dimensional gel electrophoresis (2DE) was performed on total cell lysates. Differentially expressed proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and two androgen-regulated proteins were identified as mElfin and CArG-binding factor-A (CBF-A). CBF-A and mElfin are known to bind to cytoskeletal F-actin. Both proteins appeared to be regulated by androgens at the post-translational level, possibly involving phosphorylation. Post-translational modification of mElfin and CBF-A by androgens may be associated with a cytoskeletal change that is involved in androgen-regulated gene expression.

Introduction

Androgens are essential for development and differentiation of the male genital tract. Androgen action is mediated by the androgen receptor (AR) that functions as a transcription factor by binding to specific DNA sequences in promoter and enhancer regions of target genes, thereby regulating their transcriptional activity [237]. During male embryonic development, testicular testosterone stimulates the Wolffian ducts to develop into epididymides, vasa deferentia and seminal vesicles. In the urogenital sinus, 5α-reductase converts testosterone into the more potent 5α-dihydrotestosterone, which stimulates the urogenital sinus to develop into the prostate gland and external genitalia [568]. The importance of AR signaling during male sexual differentiation is demonstrated in certain pathological situations, in particular androgen insensitivity syndrome (reviewed in [179]).

In the mouse Wolffian duct, AR expression can be detected in mesenchymal cells from day 12.5 of gestation (E12.5) onwards [117,118] shortly before testicular testosterone secretion starts [569]. In contrast, epithelial AR expression first appears in a temporal, cranial to caudal fashion, starting in the efferent ductules at E16, immediately after morphological differentiation of the Wolffian duct has started. AR protein is not expressed in epididymis and vas deferens epithelium before E19, and in seminal vesicle epithelium before postnatal day 1 [117,118]. The transition from AR negative to AR positive epithelium between E18 and E19 in the mouse is similar to what is found for fetal reproductive tract development in the rat [570]. Since androgen dependent development of the genital tract occurs before epithelial cells express AR, it was postulated that androgen action in epithelium is mediated by paracrine influences from mesenchymal cells [571], suggesting an important role for AR expressing mesenchymal cells in the development of the genital tract. Thus, androgen action in the developing Wolffian duct is mediated solely by the AR positive mesenchymal cells until E18, and from E19 onwards by both the mesenchymal and the epithelial cells. Tissue recombination experiments have proven to be successful model systems for investigating mesenchymal-epithelial cell interactions in the reproductive tract, and were used to identify

growth factors as androgen-induced paracrine factors [126,571]. However, it is largely unknown which target genes, or other processes, in the mesenchymal cells are regulated by androgens.

To study androgen regulation of protein expression in mesenchymal cells, we developed androgen target cell lines derived from E18 Wolffian duct mesenchymal tissue. We used a transgenic mouse line that harbors the DNA sequence encoding thermolabile Simian virus 40 large T under the direct control of an β-actin promoter. A clonal mouse fetal vas deferens (MFVD) cell line was used for proteomic analysis. Comparative two dimensional gel electrophoresis (2DE) was performed and androgen-regulated proteins were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Materials and methods

Materials

All media (Glutamax-supplemented DMEM/F12 nutrient mix and Hanks balanced salt solution [HBSS]), HEPES, collagenase, trypsin, TRIzol reagent, multiwell tissue culture plates (Nunc), and filtertop culture flasks (Nunc) were purchased from Gibco-BRL, Life Technologies (Gaithersburg, MD, USA). Fetal calf serum (FCS) was obtained from Greiner (Frickenhausen, Germany). A mixture of penicillin, streptomycin and fungizone was obtained from BioWhittacker (Walkersville, MD, USA). DNase and Complete protease inhibitors were from Roche (Basel, Zwitserland). New England Nuclear (Boston, MA, USA) supplied R1881 (methyltrienolone). Schering (Bloomfield, NJ, USA) provided hydroxyflutamide (OHF). Antibodies used were SV40 T Ag (Pab 101; Santa Cruz, CA, USA), anti-vimentin (clone VIM 3B4; Boehringer Mannheim, Germany), and anti-calponin (clone hCP; Sigma, St. Louis, MO, USA). PROTRAN nitrocellulose transfer membranes were from Schleicher & Schuell (Keene, NH, USA). West-Pico chemiluminescence substrate was purchased from Pierce (Rockford, IL, USA). Sequencing grade trypsin was from Promega (Madison, WI, USA). 10x Tris-Glycine-SDS electrophoresis buffer, TEMED (N,N,N,N'-tetra-methyl-ethylenediamine), RC DC Protein assay and Prestained precision protein standard were from Bio-Rad laboratories (Hercules, CA, USA). Immobiline dry immobilized pH gradient (IPG) strips (pH 3-10 NL, 18 and 24 cm), IPG buffer pH 3-10, and PlusOne chemicals for isoelectric focussing (IEF) and 2DE and 12.5% w/v acrylamide SDS precast gels were purchased from Amersham Biosciences (Uppsala, Sweden). Duracryl (30% v/v acrylamide/0.8% v/v BIS) was obtained from Genomic Solutions (Chelmsford, MA, USA). Colloidal Blue Staining Kit was from Novex / Invitrogen (Carlsbad, CA, USA). Alpha-cyano-4-hydroxycinnamic acid and peptide calibration mix were from Bruker-Daltonica (Bremen, Germany).

Animals

Young adult mice, age 10-12 weeks, (B10/CBA strain) were purchased from Harlan Winkelmann GmbH (Bohren, Germany). Dr. E. Dzierzak (Department of Cell biology, Erasmus MC Rotterdam, The Netherlands) kindly provided the SV 40 large T transgenic Tag 5 mice [572]. Both mice strains were housed under standard animal housing conditions in accordance with the NIH Guide for the Care and Use of Laboratory Animals. This study was approved by the Dutch experimental animal committee, protocol number 124-98-03.

Preparation of primary cell cultures

Timed pregnancies were performed by placing Tag 5 transgenic male mice in individual cages overnight with B10/CBA females. Day 0 (E0) of gestation was determined by the presence of a vaginal plug in the morning. On day E18, females were sacrificed, uteri were removed and fetuses were dissected in a sterile hood in DMEM/F12 medium containing 25 mM Hepes, 5% v/v dextran-coated charcoal (DCC)-treated FCS and a mixture of penicillin/streptomycin/fungizone (PSF). Wolffian duct-derived tissues were isolated and vasa deferentia from six animals were pooled and washed twice in Ca^{2+}-and Mg^{2+}-free PBS. The tissues were incubated in HBSS with 150 Units/ml collagenase and 1 μg/ml DNase at 37 C for 30 min, and were further dislodged into a single-cell suspension by pipeting up and down. This cell suspension was washed once with DMEM/F12 medium, supplemented with 5% (v/v) DCC-FCS and PSF, and the cell number was determined. Cells were subsequently seeded into 24 well plates at a density of $5x10^5$ cells per well and cultured at 33 C in DMEM/F12 supplemented with 5% DCC-FCS, PSF, and 10 nM synthetic androgen R1881.

Establishment of vas deferens cell lines

After one week of culture, medium from the cells was collected, centrifuged and sterile filtered through a 0.22 μm filter. This conditioned medium was obtained freshly every week. Cells were washed with Ca^{2+}-, Mg^{2+}-free PBS, detached with 0.25% (v/v) trypsin / 0.05% (w/v) EDTA by incubating at 33 C for 5 minutes and washed once with medium. Subsequently, cells were seeded at a lower density of $5x10^4$ cells/cm^2 in medium as described above containing 20% (v/v) conditioned medium, until cell crisis was reached. During cell crisis, cell density was kept high, $2.5x10^5$ cells/cm^2, in order to promote cell proliferation. Once the primary culture started proliferating again, it was considered immortal. These cells were seeded at a density of $5x10^5$ cells/80 cm^2 culture flask. The immortalized cell line was designated parental mouse fetal vas deferens cell line (MFVD). In order to obtain clonal cell lines, limited dilution cloning was performed on the MFVD cell line (passage 10) in a 48-well plate at an average density of 1 cell/well. After one week of culture, colony formation had started. Medium was replaced weekly, until the cell number in the well was high enough to be passaged to larger plates. From this point on, addition of conditioned medium was omitted and the R1881 concentration was lowered to 1 nM. The immortalized cell cultures were passaged once a week. Three clonal cell lines were obtained from the parental MFVD cell line, designated clones A5, A6, and E2.

Growth studies

Growth studies were performed on the MFVD clone A6. At day 0 cells were seeded, in duplicate, into 24-well plates at a density of $2x10^4$ cells per well. The cells were grown for 7 days in the presence of vehicle (0.1% v/v ethanol), 1 nM R1881, or 100 nM anti-androgen OHF, at 33 C and 39 C. On day 2, 5, and 7, cells were trypsinized and cell numbers were determined using a hemocytometer.

Western blotting and immunoprecipitation

MFVD parental and clonal cell lines were grown in 80-cm^2 culture flasks for 7 days in the presence of vehicle or 1nM R1881 at 33 C and 37 C or 39 C. On day 4, medium and hormone were replaced. On day 7, the confluent cell layer was washed twice with PBS and cells were subsequently collected by scraping on ice in 0.5 ml lysis buffer [40 mM Tris-HCl,

1 mM EDTA, pH 7.4, 10% glycerol (v/v), 1% Triton X-100 (v/v), 0.08% SDS (w/v), 0.5% sodium desoxycholate (w/v), 10 mM DTT, and Complete protease inhibitors]. The obtained lysate was sonicated for 5 seconds to decrease viscosity and centrifuged 10 min at 4 C, 50,000 rpm. Total protein lysate (25 μg) was subjected to SDS-PAGE, and the proteins were blotted onto a nitrocellulose membrane. The AR protein was detected with monoclonal antibody F39.4.1 [573]. Marker proteins were detected using monoclonal antibodies against SV40 T Ag, vimentin, and calponin. In addition, AR protein was immunoprecipitated from 100 μg total cell lysate with F39.4.1, and detected on blot with polyclonal antiserum against synthetic peptide SP 213 [573]. Proteins were visualized by chemiluminescence detection, according to the instructions of the manufacturer.

Northern blot analysis

MFVD parental and clonal cell lines were cultured in 80-cm² culture flasks for 7 days at 33 C. Total RNA was isolated using TRIZOL reagent according to instructions of the manufacturer. 15 μg RNA of each sample was applied on a formamide gel and blotted overnight to a PROTRAN nitrocellulose membrane. The Hoxa11 probe was obtained using a 300 bp BamHI-BglII fragment of the mouse Hoxa11 cDNA [574] and was labeled with α^{32}P dATP. Hybridization was performed at 63 C overnight. RNA was visualized by autoradiography.

2DE

2DE was performed on protein lysates of MFVD A6 cells between passages 12 and 18, which were cultured in 80-cm² culture flasks as described above in the presence of either vehicle (0.1% ethanol), 1 nM R1881 or 10 nM testosterone for 24 hours. After 7 days of culture, cells were washed twice with PBS and collected in PBS. Cells from five 80-cm2 culture flasks from the same passage were pooled and centrifuged. Pellets were solubilized with 2D lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS (3-[3-(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), 40 mM Tris-HCl, 10 mM dithiothreitol and Complete protease inhibitors. This lysate was sonicated and centrifuged, and protein concentration was determined as described above. For analytical gels 100 μg, and for preparative gels 500 μg protein lysate was mixed with rehydration buffer (8M urea, 2% w/v CHAPS, 0.5% v/v IPG buffer pH 3-10, and a trace of bromophenol blue). Immobiline dry IPG gel strips of 18 cm for analytical (n=4) and 24 cm for preparative gels (n=1) with a non-linear pH range of 3-10 were actively rehydrated overnight with sample containing rehydrationbuffer applying a voltage of 30V per hour (Vh). IEF was performed with an IPGphor system (Amersham Biosciences) according to the manufacturer's instructions, until 92 kVh was reached. After IEF, proteins in the gel strips were reduced in equilibration buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.8) with 60 mM DTT for 20 min, and subsequently carbamidomethylated in the same buffer, containing 280 mM iodoacetamide, for 2 min. Resolution in the second dimension was carried out in an Ettan-Dalt II system (Amersham Biosciences) on 10% w/v Duracryl gels (analytical) or 12.5% w/v SDS precast gels (preparative). The gels were run 20 min with a constant power of 3W and subsequently 3,5 hours with a constant power of 180W. During the run the temperature was kept constant at 25 C. Prestained protein standards were run together with the strips. The gels were fixed overnight in 5% v/v phosphoric acid / 40% v/v methanol, and protein spots were visualized by colloidal Coomassie stain according to

the manufacturer's instructions. Analytical gels were subsequently silver stained according to Morrissey et al [575].

In-gel trypsin digestion and mass spectrometry

Protein spots of interest were excised out of the gel, were washed briefly in MilliQ water, de-stained twice for 20 minutes in 37% acetonitrile (ACN)/2.5 mM ammonium hydrogen carbonate, washed again, vacuum dried for 30 minutes, digested overnight with sequencing grade trypsin (0.7 U per protein spot) and re-hydrated in 50% v/v ACN / 0.1% v/v trifluoro acetic acid. The sample was mixed with a saturated alpha-cyano-4-hydroxycinnamic acid matrix solution in 100% ACN (one part sample and four parts matrix) and 0.5 µl of this mixture was spotted on an anchorchip plate (Bruker-Daltonica, Bremen, Germany). Digested peptide fragments were analyzed in a matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF-MS) in a positive reflectron ion mode using a Biflex III apparatus (Bruker-Daltonica). The obtained peptide mass fingerprint spectra were analysed searching the MSDB nonredundant protein database (NCBI, USA) with MASCOT software (http://www.matrixscience.com), allowing one missed cleavage of trypsin per peptide. After protein identification, the database was further searched allowing the peptides to be modified for carbamidomethylation, oxidation of methionine and phosphorylation. Peptide mass accuracy was set to a maximum of 200 ppm. Upon protein identification, mass spectra of different isoforms of one and the same protein were compared to investigate the presence of modified peptides.

Results

Development of vas deferens cell lines and establishment of their growth characteristics

An immortalized mouse fetal vas deferens (MFVD) cell line was developed from vasa deferentia from E18 mouse fetuses as described in Materials and Methods. From this parental cell line, clonal cell lines designated A5, A6 and E2 were derived. All cell lines were continuously cultured at 33 C, the permissive temperature of large T. Higher temperatures (37 C-39 C) cause degradation of the large T protein and are therefore non-permissive. It was described for other immortalized cell lines that cell proliferation at large T permissive conditions will stop, whereas cell differentiation will be initiated [576,577].

Initially, the MFVD parental and clonal cell lines were continuously cultured under permissive conditions in the presence of methyltrienolone (R1881), a synthetic androgen. Growth studies were performed under variable culture. For this purpose, the MFVD clonal cell line A6 was cultured for 7 days at 33 C and 39 C in the absence of AR ligand, in the presence of 1 nM R1881, or in the presence of 100 nM of the anti-androgen hydroxyflutamide (OHF). Microscopic analysis revealed a difference in morphology between cells cultured at 33 C and 39 C (Figure 4.1A and 1B). At 33 C, MFVD A6 cells were found to grow to a high density, reaching confluency (Figure 4.1A). At 39 C, the cells developed a much larger and flatter appearance and entered a more quiescent stage, although cell growth had not completely stopped (Figure 1B). Quantification of cell growth showed a difference in growth between cells cultured at 33 C and 39 C (Figure 4.1C). Culturing under large T permissive conditions provided sufficient stimuli for cell growth (Figure 4.1C, open symbols). However, raising the culture temperature to 39 C resulted in a dramatic reduction of cell proliferation (Figure 1C, closed symbols). A reduction in cell growth was observed to a lesser extent at 37 C (data not shown). Cell growth at 33 C and 39 C was neither stimulated by R1881, nor inhibited by OHF. Western blot analysis showed a

correlation between growth and large T protein expression. As expected, large T protein was highly expressed at 33 C in all cell lines (Figure 4.1D, lanes 1,3,5,7). Shifting the temperature to 39 C (or 37 C; data not shown) resulted in a decline of large T protein expression in the MFVD parental cell line, in MFVD clone A5, and most prominent in MFVD clone A6 (Figure 4.1D, lanes 2,4,6). MFVD clone E2, however, did not show any large T regulation, as the protein level remained high under non-permissive conditions (Figure 4.1D, lane 8). This may be due to a varying basal level of large T protein between the cell lines and to the turnover rate of the large T protein. It can be concluded that differentiation of the MFVD A6 clone in particular is dependent on large T protein expression.

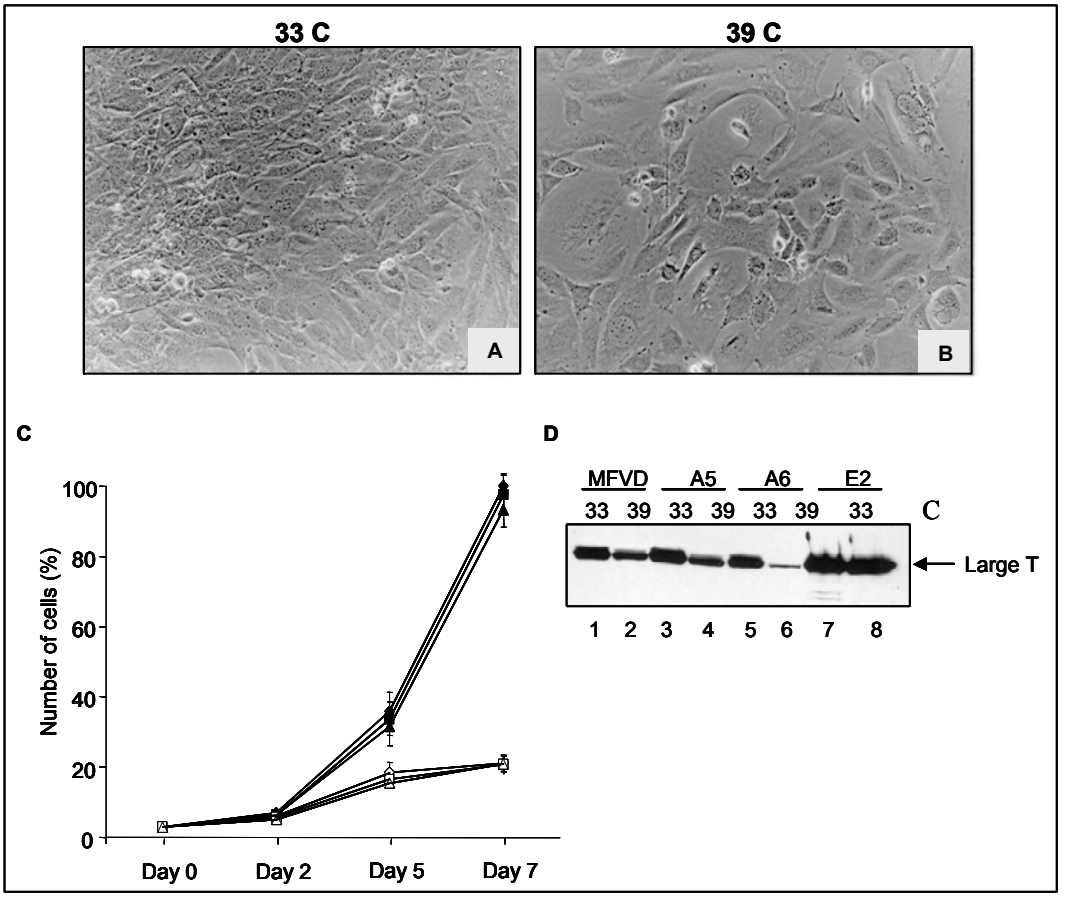

Figure 4.1 Phase-contrast microscopy photographs of MFVD clonal cell line A6 at passage 20
The cells were cultured for 7 days at 33 C (**A**) and 39 C (**B**). Magnification x200. Note the difference in morphology between cells cultured at 33 C and 39 C.
C) Growth curve of MFVD A6 clonal cell line cultured at 33 C (closed symbols) and 39 C (open symbols) in the presence of vehicle (0.1% ethanol) (squares), 1 nM R1881 (rectangles), or 100 nM OHF (triangles). Each point represents the standard error of the mean (+/- SEM) of three individual experiments performed in duplicate. The number of cells grown for 7 days in the absence of hormone at 33 C was set at 100%, and all individual points were calculated relative to this value.
D) Western blot of large T protein expression in MFVD parental and clonal cell lines.

MFVD cells are of wolffian duct stromal origin

To select for the stromal cells in our vas deferens cultures, the MFVD parental and clonal cells were cultured in serum-containing medium. To confirm the stromal origin of MFVD cells, some marker proteins were analysed. On Western blots, all clonal cell lines were positive for two mesenchymal cell markers, vimentin and calponin (Figure 4.2A, lanes 1-3) [578,579]. In contrast, the cells were negative for the epithelial cell marker pan-keratin (a mixture of all cytokeratins) [580]. Chinese hamster ovary cells (CHO) were used as a positive control for mesenchymal cells (Figure 4.2A, lane 4). The prostate cancer cell line LNCaP was used as a control cell line for epithelial cells expressing pan-keratin (Figure 4.2A, lane 5).

To determine the vas deferens origin of these stromal cells, Northern blot analysis was performed with a Hoxa11 probe. This member of the Hoxa complex of homeotic genes is expressed mainly in developing limbs and the embryonic kidney (not in adult kidney), and in stromal cells surrounding the müllerian and wolffian ducts [574]. MFVD parental and clonal cell lines expressed Hoxa11 mRNA (Figure 2B, lanes 1-4), as did the embryonic kidney (positive control; Figure 4.2B, lane 5), but not the adult kidney (negative control; Figure 4.2B, lane 6). From these experiments, we conclude that the MFVD parental and clonal cell lines have features of fetal vas deferens mesenchyme.

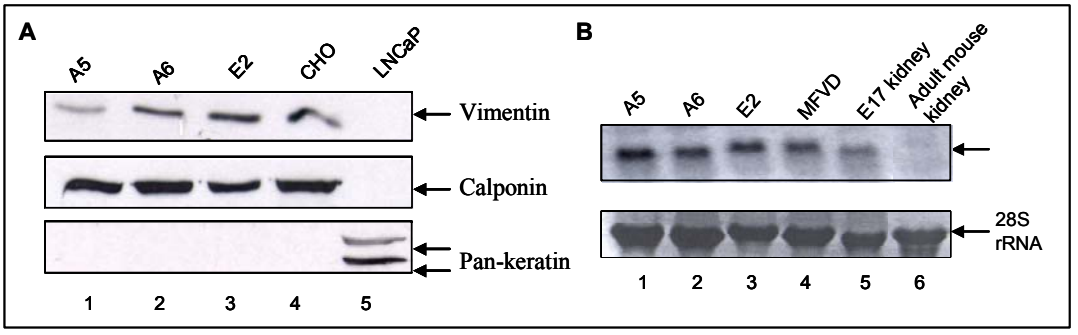

Figure 4.2 Characterization of MFVD parental and clonal cell lines

A) Protein expression of two mesenchymal cell markers (vimentin and calponin) and one epithelial cell marker (pan-keratin). CHO cells were used as mesenchymal control cells. LNCaP cells were used as epithelial control cells.

B) Hoxa11 mRNA expression in MFVD clonal cell lines and mouse fetal kidney. Mouse adult kidney was used as a negative control tissue.

AR protein is expressed in MFVD parental and clonal cell lines

Since cell growth in MFVD cell lines was not stimulated by androgens, we investigated the expression level of the AR protein. For that purpose, immunoprecipitation of AR protein from MFVD parental and clonal cell lines, cultured at 33 C and 39 C, was performed (Figure 4.3A). The parental cell line showed AR protein expression at both large T permissive and non-permissive temperatures (Figure 3A, lanes 1,2). All three clonal cell lines showed AR expression. While AR expression at 33 C was rather low (Figure 4.3A, lanes 3,5,7), it was clearly higher at 39 C (Figure 4.3A, lanes 4,6,8) when cells were not under the control of large T and thus in a differentiating state. In all our further studies MFVD cells were cultured at 37 C instead of 39 C, to provide a more physiological temperature. AR expression was followed in the MFVD A6 clone during a culture period of 20 passages, by performing Western blots on whole cell lysates at passages 10 and 20. Figure 3B shows a relatively low AR expression at 33 C and 37 C in the absence of R1881 (lanes 1,3,5,7).

However, addition of R1881 to the cells resulted in a marked increase in AR protein level (Figure 4.3B, lanes 2,4,6,8). This increase is most probably due to stabilization of the receptor protein in the presence of ligand [581]. In cells cultured at 37 C, AR protein expression in the presence of R1881 was more pronounced than at 33 C. The AR protein expression was reduced at passage 20 (Figure 4.3B, lanes 5-8) as compared to passage 10 (Figure 4.3B, lanes 1-4). AR expression was lost at passage 30 (results not shown). From these experiments, it was concluded that the AR protein in the MFVD A6 cell line is stabilized in the presence of ligand and that AR protein expression is lost after 30 passages.

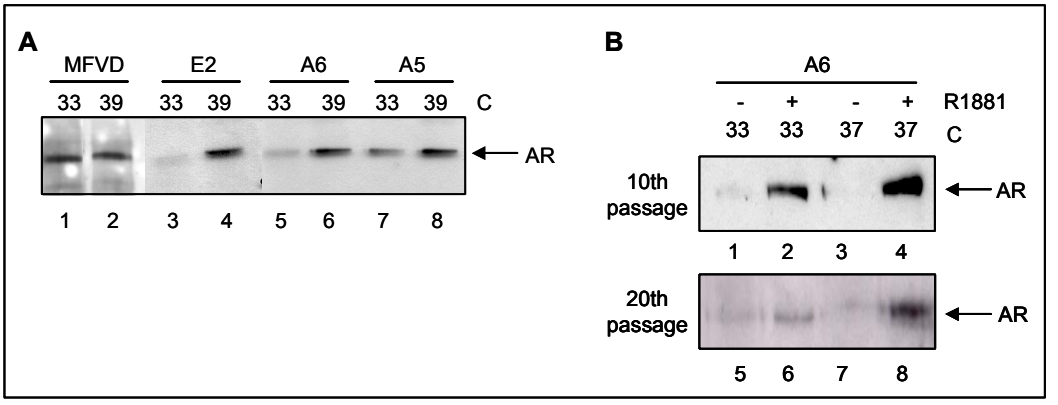

Figure 4.3 Androgen receptor (AR) protein expression
A) AR immunoprecipitation in MFVD parental and clonal cell lines cultured at 33 C and 39 C.
B) AR protein expression in A6 cell line at passages 10 and 20. A6 cells were cultured at 33 C or 37 C in the presence of vehicle (0.1% ethanol) or 1 nM R1881, as indicated in the figure.

CBF-A and mElfin are differentially expressed in MFVD A6 cells

In order to identify proteins that are regulated by androgens, the MFVD A6 cell line was used for proteomic analysis. MFVD A6 protein lysates from 4 separate experiments were used to generate analytical 2DE gels. Typically, silver-stained 2DE gels showed approximately 1500 protein spots. Comparison of protein expression patterns of control and androgen-treated cells revealed only minor qualitative changes, except for one part of the gels which showed, repeatedly (n=4), a differential protein expression in a region focussed between pH 6 and 7 and between a molecular mass of 30-35 kDa. In order to identify these differentially expressed proteins by MALDI-TOF-MS, preparative 2DE gels were run. MFVD A6 cells were either treated for 24 hours with vehicle, 1 nM R1881, or 10 nM testosterone. A colloidal Coomassie stained gel from cells treated with vehicle is shown in Figure 4A. Approximately 400 protein spots could be visualized on these gels. Protein expression patterns of the cells cultured under different hormonal conditions were much alike, except for the region that is boxed in Figure 4A. A more detailed representation of this part of the gel is shown in Figure 4B, for all three culture conditions. Protein spots a, b, c, and d represent non-changing anchors which were identified by MALDI-TOF-MS (Table 4.I). Protein spots 1 and 2 present in the vehicle situation are subject to changes upon androgen treatment. While spot 1 is present in the vehicle situation, this protein disappears and three other proteins appear at positions 1a, 1b and 1c, upon treatment with either R1881 or testosterone. The exposure to R1881 or testosterone resulted in migration of protein spot 2 to position 2a and position 2b, respectively. Protein spots 1, 1a, 1b, and 1c could all be identified with MALDI-TOF-MS as CArG-binding factor A (CBF-A) (Table 4.I).

Protein spots 2, 2a, and 2b were all found to represent mElfin (Table 4.I). From the gels it is apparent that addition of either 10 nM of the natural ligand testosterone or 1 nM of the synthetic androgen R1881 has the same effect on the position of the protein spots representing CBF-A. However, 1 nM R1881 appears to have a different effect on the 2DE migration of mElfin as compared to the effect of 10 nM testosterone. No change in position of CBF-A and mElfin was observed when cells were treated with 1 nM testosterone (preliminary results, data not shown), which may be the consequence of the lower affinity of testosterone for the AR as compared to dihydrotestosterone or R1881. Furthermore, addition of 100 nM of an AR antogonist, hydroxyflutamide, together with 1 nM R1881 blocks the effect of R1881 (preliminary results, data not shown).

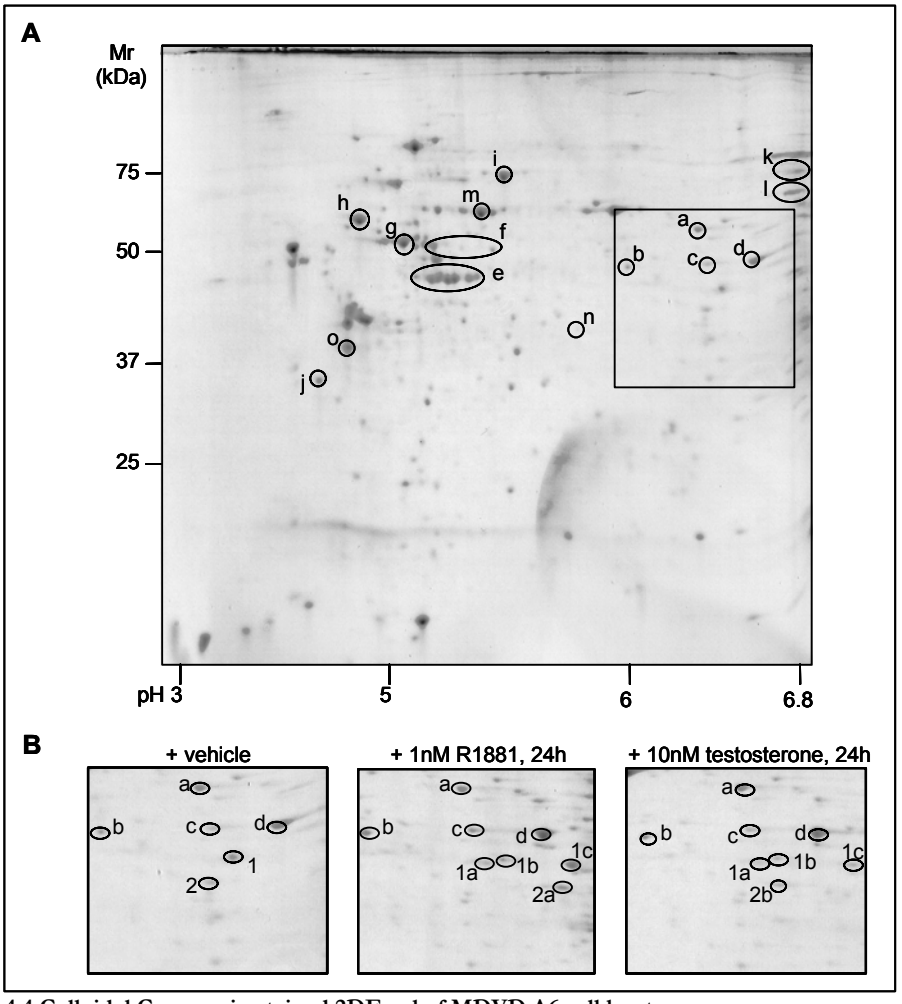

Figure 4.4 Colloidal Coomassie-stained 2DE gel of MDVD A6 cell lysate
A) Part of a 2DE gel from cells cultured in the presence of vehicle (0.1% ethanol) for 24h. Proteins identified by MALDI-TOF-MS are encircled and lettered a-o. Boxed is a region with differential protein expression.
B) The boxed region from A) is shown in more detail. The same region is shown in detail for 2DE gels from cells cultured in the presence of 1 nM R1881 or 10 nM testosterone. Differentially expressed proteins are numbered as 1 and 2, for cell incubations without ligand (vehicle). In the gels representing the cells incubated with either R1881 or testosterone, the differentially expressed proteins are numbered as 1a, 1b, 1c, and 2a or 2b, respectively.

Proteins a-d in Figure 4B and e-o encircled in Figure 4A were also identified to provide a further characterization of MFVD A6 cells and are all listed in Table 4.I, together with their molecular mass, theoretical and experimental pI, and Accession number. In some cases, proteins did not run in the gel according to their theoretical mass or pI, and these proteins may have undergone modifications that change their position in the gel. Using this proteomic approach, we have performed a small-scale analysis to further characterize the MFVD A6 cell line. Several identified proteins (protein disulfide isomerase ER-60, laminin A, laminin C, ATP-synthase β-chain, and mitochondrial stress protein-70) have been reported to be expressed in mouse fibroblasts [582]. The presence of these proteins in the MFVD A6 cell line emphasizes the common mesenchymal characteristics.

Table 4.I. Peptide mass fingerprinting by MALDI-TOF- MS

Spot*	Protein#	MM (kDa)	tpI	epI	Accession #
a	Chaperonin containing TCP-1β–chain	57	6.0	6.3	BAA81874
b	Ornithine amino transferase precursor	47	6.1	6.1	AAH08118
c,d	α–Enolase	47	6.4	6.4-6.6	P17182
e	β-Actin	41	5.3	5.1-5.4	P02570
f	Vimentin	54	5.1	5.2-5.5	P20152
g	ATP synthase β–chain	56	5.0	5.1	P56480
h	Protein disulfide isomerase precursor	57	4.8	4.8	P09103
i	Mitochondrial stress-70 protein precursor	73	5.5	5.5	P38647
j	Tropomyosin fibroblast isoform 1	33	4.6	4.6	P46901
k	Laminin A	74	6.5	6.7-6.8	P48678
l	Laminin C	64	6.4	6.7-6.8	P11516
m	Mitochondrial matrix precursor protein P1	60	5.4	5.4	P19226
n	γ-Aminobutyric acid A receptor, α-6	32.9	5.7	5.7	AJ222970
o	Proliferating cell nuclear antigen	28.8	4.7	4.7	P17918
1,1a,1b,1c	CARG-binding factor-A	30.8	7.7	6.4-6.8	JQ0448
2,2a,2b	mElfin	35.7	6.4	6.4-6.6	AAH04809

* Index according to Figure 4.4. # Proteins identified with a significance of $p<0.05$ (Mascot software, Matrix Science). Accession # according to NCBI Blast. SWISS-PROT ExPASy data base. MM, molecular mass; tpI, theoretical pI as calculated from the; epI, experimental pI measured from the gels.

The presence of both CBF-A and mElfin under different culture conditions but at changing positions in the gels strongly suggests that these proteins undergo post-translational modification upon androgen treatment. Comparison of the peptide mass fingerprint spectra of CBF-A isoforms 1, 1a, 1b, and 1c revealed some differential peptide peaks. In isoform 1, 1a, and 1b, a peptide with a mass to charge ratio (m/z) of 1939.71 was observed that was not present in isoform 1c. On the other hand, isoform 1c showed a peptide with a m/z of 1539.79 that could not be observed in isoform 1, 1a, or 1b. According to the MSDB database search, which is based on the peptide masses only and not on the precise sequence, both these peptide masses match with CBF-A amino acid residues 259-274 assuming that the 1939.71 peptide has an extra mass that corresponds with 5 phosphate residues (Table 4.II). Similarly, a putative phosphopeptide was found with a m/z of 1189.58 corresponding to amino acid residues 90-97 with two phosphorylated residues. For mElfin, a putative phosphopeptide with m/z 1159.75 was found only in isoform 2. Other predicted phosphopeptides that were found in mElfin are listed in Table II, together with the putative phosphorylation sites.

Table 4.II. Proposed phosphopeptides according to differential MALDI-TOF-MS

Protein	Modified peptide m/z	Unmodified peptide m/z	Sequence	Residues	Candidate phosphorylation sites
CBF-A	1939.71	1539.70	GSGGGQGSTNYGKSQR	259-274	S260,S266,T267,Y269,S272
	1189.58	1029.52	DLKDYFTK	90-97	Y94,T96
mElfin	1159.75	1079.73	CGTGIVGVFVK	261-271	T263
	1805.94	1565.74	IKGCADNMTLTVSR	70-83	T78,T80,S82
	2116.89	17.96.82	GDADNMTLTVSRSEQK	72-87	T87,T80,S82,S84

m/z= mass to charge ratio

Discussion

The present study addresses the development and characterization of conditionally immortalized clonal cell lines from mouse fetal vas deferens. Immortalized cell lines from mouse urogenital ridge [583] and prepubertal mouse vas deferens epithelium [584,585] have been reported previously. Other studies were performed using primary epithelial cells from adult [586] and primary mesenchymal cells from fetal reproductive tract [143]. However, so far no clonal cell line was described originating from fetal vas deferens mesenchymal cells. The MFVD A6 cell line is homogeneous because of its clonal background, it is of stromal origin, and expresses a functional androgen receptor. Therefore, these cells provide a cellular background that is suitable to study androgen regulation of gene and protein expression in relation to fetal genital tract development.

In addition to cDNA microarrays, proteomics is becoming a widely used approach to study cellular mechanisms. The study of proteins and post-translational modifications adds to what can be learned from genomic studies. Androgenic effects on gene and protein expression have been studied for prostate cancer [336,337,517]. However, the work presented herein is the first proteomics study on the effects of androgens in relation to genital tract development. In the MFVD A6 cell line, we have identified CBF-A and mElfin as two proteins that are regulated by androgens at the post-translational level. It is evident that a genomics approach would not have provided information about such a functional change in protein expression.

In the differential 2DE analysis of androgen-treated MFVD A6 cells versus untreated cells, we primarily focussed on qualitative differences. It was found that both CBF-A and mElfin were present in the stimulated and non-stimulated cells, but as different protein isoforms. Comparing the peptide mass fingerprint spectra of the different protein isoforms resulted in identification of peptide masses that were present in either one or the other protein isoform. Peptide phosphorylation could account for these differences in mass, and possible phosphopeptides were identified using the MSDB database. In the case of the m/z 1939.71 peptide, the predicted presence of 5 phosphorylated amino acid residues is remarkable. It can, however, not be excluded that some other kind of modification, e.g. glycosylation, could result in a peptide of the same mass as the putative phosphopeptide.

The MALDI-TOF-MS analysis of 2DE gels shows that post-translational modification of CBF-A and mElfin occurs upon androgen treatment and that peptide mass fingerprints can provide clues as to what kind of modification is taking place. Thus far, no androgenic regulation of CBF-A and mElfin at the post-translational level has been described.

CBF-A belongs to a subfamily of highly homologous A/B-type heterogeneous nuclear ribonucleoproteins and functions in both transcriptional and post-transcriptional processes of gene regulation [587]. CBF-A was originally described as a ubiquitously expressed protein, which binds to CArG-box motifs and to single-stranded DNA, and functions as a

transcriptional repressor [588]. In a different context, the protein was found to interact with the SP6κ promoter as a co-activator of transcription [589]. In addition, CBF-A activates transcription of the Ha-ras promoter in mammary cells, and appeares to bind the Ha-ras element 1 with higher affinity than the CArG-box [590]. Furthermore, it was postulated that CBF-A is subject to post-translational modification [589], and in a recent study it was found that multiple modified isoforms of CBF-A associate with actin and are involved in nuclear-cytoplasmic shuttling of mRNA [591]. In MFVD A6 cells, post-translational modification of CBF-A might influence the expression of specific androgen target genes.

mElfin is a family member of the Enigma proteins that possess a PDZ domain at the amino terminal and a LIM domain at the carboxyl terminal, which is expressed in a variety of tissues [592]. Expression of mElfin is found as early as gestational day E8 in the developing heart of mouse embryos [593]. LIM domains are defined by cysteine-rich sequences that form two zinc fingers, and are involved in protein-protein interactions (reviewed in [594]). LIM domains are found in a variety of proteins with different cellular functions, especially in many key regulators of developmental pathways [594]. mElfin was found to associate to the F-actin-rich cytoskeleton by interacting with α-actinin via its PDZ domain, which in turn binds to F-actin [592,595,596]. It was suggested that mElfin functions as an adapter protein by recruiting signaling kinases to the cytoskeleton via its protein-protein interaction motifs [593,595]. A functional relationship between mElfin and the AR pathway can be similar to what has been observed for FHL2, a four-and-a-half LIM domain protein which was found to be expressed preferentially in the heart [597]. FHL2 is also expressed in epithelial cells of the prostate, where it overlaps with nuclear AR expression and functions as an AR-specific, ligand-dependent co-activator of transcription [238].

Another functional link to AR signaling may involve the binding of CBF-A and mElfin to F-actin. It was described that several proteins initially characterized as actin-binding proteins or complexes were found to co-activate transcriptional regulators, including AR. The F-actin binding protein filamin has been found to interact with the AR and is involved in nuclear translocation of the AR [275]. Likewise, β-catenin, which plays a pivotal role in cell-cell adhesion by linking cadherins to α-catenin and the actin cytoskeleton, and which is a downstream effector of the Wnt signaling pathway, was found to enhance androgen dependent transcriptional activity of the AR [598]. It is tempting to postulate that post-translational modification of mElfin and CBF-A in the presence of androgens may link a change in cytoskeletal architecture to androgen-regulated gene transcription.

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Chapter 5

Proteomic profiling of epididymis and vas deferens: identification of proteins regulated during rat genital tract development

Arzu Umar*, Marja P. Ooms*, Theo M. Luider#, J. Anton Grootegoed* and Albert O. Brinkmann*

* Department of Reproduction and Development, and
# Department of Neurology and Erasmus Center for Biomics,
Erasmus MC, Rotterdam, The Netherlands

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Summary

Epididymis and vas deferens form part of the male internal genital tract, and are dependent on androgens for their growth and development. To better understand the molecular action of androgens during male genital tract development, protein expression profiles were generated using 2-dimensional (2D) gels, for rat epididymides and vasa deferentia isolated on embryonic days (E) 17-21. Proteins that were differentially expressed between E17 and E21 were cut from the gels, digested into tryptic peptides and analyzed on a MALDI-TOF mass spectrometer. Using this approach, 20 proteins could be identified that were regulated in time and were categorized into cytoskeletal proteins, nuclear proteins, transport proteins, chaperones, and enzymes (mainly glycolytic). Furthermore, epididymides and vasa deferentia isolated on E19 were cultured *in vitro* in the absence or presence of 10nM of the synthetic androgen R1881, for 9, 24, and 48 hours. Under these conditions, regulation and post-translational modification were observed for glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, hnRNP A2/B1 and hnRNP A3, similar to the observed changes *in vivo*. In addition, post-translational modification of RhoGDI1 (also named RhoGDIα) was found in response to androgen. Androgen-induced post-translational modification of RhoGDI1 and glycolytic enzymes may be an important functional link between signaling pathways and cytoskeletal rearrangements in control of growth and development of the male internal genital tract.

Introduction

Androgens play an important role in regulation of male development and physiology, ranging from male sex differentiation during embryogenesis, several aspects of puberty and spermatogenesis, to adult male function [179,568,599]. The principal androgen testosterone (T) is essential for the development of the internal sex organs derived from the Wolffian duct system: epididymis, vas deferens, and seminal vesicle. Dihydrotestosterone (DHT), the 5α reduced form of T, is involved in development of the prostate and external genitalia from the urogenital sinus and the tubercle [2,600]. Both T and DHT perform their actions via one and the same intracellular receptor, the androgen receptor (AR), but DHT is a more potent androgen. Binding of either T or DHT to the AR might be a mechanism to regulate distinct androgenic effects in target tissues [600]. In humans, the differential action of T and DHT is illustrated in clinical syndromes such as androgen insensitivity (AIS) and 5α-reductase type2 deficiency. Complete AIS patients have a non-functional AR, which results in the absence of Wolffian duct and urogenital sinus derived structures, and feminized external genitalia (1). In contrast, patients with a 5α-reductase type2 deficiency have normally developed epididymides and vasa deferentia but have feminized external genitalia [107]. Surprisingly, studies in mice deficient for steroid 5α-reductase 1 and 2, have shown that treatment with non-reducible T analogues was sufficient for secondary sex organ formation and growth [110]. Therefore, it seems that in rodents, DHT acts largely as a signal amplifier (5). In experimental setups, the synthetic androgen R1881 (methyltrienolone) is often used because it is not further metabolized and exerts both T and DHT effects.

The AR is a member of the steroid/nuclear receptor superfamily, and functions as a transcription factor upon androgen binding [226]. Structurally, the AR protein can be divided into separate domains, e.g. the N-terminal transcription activation domain, the DNA binding domain, and the C-terminal ligand binding domain. The ligand binding domain contains a strictly ligand-dependent transcription activation function and interacts with

nuclear cofactors [205,601]. Upon ligand binding, the AR binds to specific genomic androgen response elements, thereby regulating transcription of specific genes.

AR expression in the developing male genital tract occurs in a strict temporal, and cranial to caudal fashion, which is first detected in mesenchymal cells and later in epithelial cells. Mesenchymal AR is expressed along the rat genital tract as early as embryonic day 14 (E14), whereas epithelial expression in the epididymis starts at E18 in the caput epididymis and after birth in the cauda epididymis and vas deferens [570]. Thus, initiation of androgen-dependent differentiation of the Wolffian duct system into epididymis and vas deferens occurs before epithelial cells express a detectable level of AR protein. During development, mesenchymal cells are important androgen targets that elicit androgenic effects in epithelial cells via paracrine factors and mesenchymal-epithelial interactions [122,602].

Morphologically, differentiation of the epididymis is characterized by growth and heavy coiling of the epithelial duct. In molecular and mechanistic terms, however, it is not completely understood which factors are involved in growth and differentiation of the Wolffian duct. We have recently described the effect of androgen on protein expression in a mouse fetal vas deferens (MFVD) cell line using a proteomics approach. Stimulation of MFVD cells with androgen resulted in post- translational modification of two actin binding proteins, mElfin and CBF-A [514]. In the present investigation, we have applied proteomics to study and identify proteins that are involved in the androgen-dependent development of fetal rat epididymis and vas deferens, using freshly isolated or cultured tissues. Proteins regulated during fetal development were categorized as cytoskeletal proteins, nuclear proteins, transport proteins, chaperones, and glycolytic enzymes. Androgen-regulation *in vitro* involved post-translational modification of nuclear proteins and glycolytic enzymes, as well as RhoGDI1, a signaling molecule. Post-translational modification of proteins by androgen stimulation may be a key regulatory event in genital tract development, suggesting a functional link between signaling pathways and cytoskeletal proteins.

Materials and methods

Animals

Pregnant female Wistar rats (16 to 20 weeks old) were purchased from Harlan Winkelmann GmbH (Bohren, Germany); the company determined the day of gestation by checking the presence of a vaginal plug in the morning after mating, which is referred to as E0. The rats were housed for 1 to 3 days in our in house facilities under standard animal housing conditions in accordance with the NIH Guide for the Care and Use of Laboratory Animals. This study was approved by the Dutch Experimental Animal Committee, Protocol Number 124-01-04.

Tissue preparation

Rats were sacrificed by CO_2 gas and cervical dislocation. Fetuses were removed from the uteri and placed in PBS on ice. Epididymides and vasa deferentia were isolated as a whole from male fetal rats on embryonic days (E) 17, 18, 19, 19.5, 20, and 21. Dissection was performed in PBS in a sterile hood. All tissues were either immediately frozen on dry ice or fixed in Bouin's solution. In addition, tissues from fetuses of E19 were used for organ culture studies. Epididymides together with vasa deferentia were placed in a drop of medium on a Millicell-CM 0.4 μm culture plate insert (Millipore Corp., Bedford, USA) that was floating on top of 0.5 ml medium (DMEM/F12 + 2% v/v charcoal-stripped fetal calf serum, 10 μg/ml insulin, 10 μg/ml transferrin, and a mixture of penicillin (100 IU/ml),

streptomycin (100 μg/ml), and fungizone (0.6 μg/ml), all from Sigma, St. Louis, USA. in a 4-well plate (Nunc, Roskilde, Denmark) [64]. The two epididymides and vasa deferentia from one male were always cultured in either the absence of hormone (0.1% v/v ethanol vehicle) or in the presence of 10nM of the synthetic androgen R1881, methyltrienolone (New England Nuclear, Boston, MA, USA), for 9, 24, or 48 hours. For each culture condition, 25 tissues were collected. For the frozen material, 40 tissues of E17 and E18 each and 25 tissues of E19-E21 were collected.

2-Dimensional gel electrophoresis (2DE)

All collected tissues from one time point were pooled and lysed in 500 μl 2D lysis buffer (7M urea, 2M thiourea, 4% w/v CHAPS, 40mM Tris-HCl, pH8.8, 40mM DTT and 0.5% v/v Immobilized pH gradient (IPG) buffer pH3-10) using the Sample Grinding kit (Amersham Biosciences, Piscataway, NJ, USA). High purity PlusOne chemicals were from Amersham Biosciences. Insoluble proteins and cell debris were pelleted at 100 000 rpm for 10 min at 4 C, using the Optima TLX Tabletop Ultracentrifuge (Beckman). Protein concentration was determined using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) and either 75 or 100 μg protein from total tissue lysate was used for isoelectric focussing (IEF). IEF was performed in an IPGphor apparatus according to the instructions of the manufacturer (Amersham Biosciences) using 24 cm non-linear IPG strips, pH3-10 (Amersham Biosciences). The strips were actively rehydrated overnight in rehydration buffer (8M urea, 2% w/v CHAPS, 40mM DTT and 0.5% v/v IPG buffer) containing the sample applying a voltage of 30V per hour (Vh). After rehydration, the strips were run until 100kVh was reached. Following IEF, the strips were equilibrated in SDS-buffer (6M urea, 2% w/v SDS, 30% v/v glycerol, 50mM Tris-HCl, pH8.8) containing 65mM DTT for 15 min and thereafter in SDS-buffer containing 135mM iodoacetamide, for 2 min. 2nd dimension gel electrophoresis was performed on 10% v/v Duracryl gels (Genomic Solutions, Perkin Elmer, Boston, MA, USA) using the Ettan Dalt gel caster and electrophoresis device (Amersham Biosciences) at a constant power of 3W for 30 min and 180W for 3-4 hours, depending on the amount of gels run simultaneously, at 25 C. Gels were fixed overnight in 40% v/v methanol, 5% v/v ortho-phosphoric acid, and stained with colloidal Coomassie using a Colloidal Blue staining kit (Invitrogen, Paisley, UK). Images were scanned with the Bio-Rad GS800 densitometer and analysed using the PDQuest software package (Bio-Rad).

Mass spectrometry

Peptide samples were prepared as described previously [514]. Briefly, proteins of interest were manually excised from the gel, destained twice with 30% v/v acetonitrile (ACN) in 50mM ammoniumhydrogen carbonate for 15 min, dried in a Speed Vac Plus (Savant, NY, USA) for 30 min and enzymatically digested overnight using sequencing grade trypsin (Promega, Madison, WI, USA). Peptides were eluted from the gel with 30% v/v ACN/0.1% v/v trifluoric acid, and 0.5 μl peptide sample was mixed with 4 volumes matrix solution (2 mg alpha-cyano hydroxycinnamic acid in 100% ACN), which was spotted on an AnchorChip™ target plate (Bruker Daltonik, Bremen, Germany). Mass spectra were generated on a Biflex III MALDI-TOF-MS (Bruker Daltonik) and peptide fingerprints were analysed using BioTools (Bruker Daltonik) and Mascot software (Matrix Science) and the NCBI database.

Immunoprecipitation, SDS-PAGE, and Western immunoblotting

Immunoprecipitation was performed on 25 μg protein from total tissue lysate from rat E19 tissues cultured in the absence or presence of 10nM R1881. Goat anti- rabbit-agarose beads were coupled to RhoGDIα antibodies for 1 hour at room temperature, cell lysate in PBS was added and the mixture rotated for 2 hours at 4 C. The agarose-antibody precipitate was washed with PBS and the pellet was resolved in 25 μl Laemmli sample buffer for SDS-PAGE. Total tissue lysate (12.5 μg protein) or immunoprecipitate were loaded on 10% v/v and 15% v/v acrylamide gels. Gels were run on the mini PROTEAN gel system (Bio-Rad) and proteins were blotted to a nitrocellulose membrane (Schleicher & Schuell Keene, NH, USA). Western immunobloting was performed using polyclonal antibodies against α-fetoprotein (clone E19, 1:1000) and RhoGDIα (clone A20, 1:1000) both from Santa Cruz (CA, USA). Proteins were visualized by chemiluminescence detection (Western Lightning, Perkin Elmer, Boston, MA, USA).

Histology and immunohistochemistry

Tissues were fixed in Bouin's solution for 24 hours and were then washed in 70% v/v ethanol for 24 hours. The fixed tissues were first embedded in 2% w/v agar before they were embedded in paraffin. Paraffin sections were cut at 7 μm and stained with hematoxylin/eosin. For immunohistochemistry, paraffin sections from E17 and E21 were incubated with an α-fetoprotein antibody at a dilution of 1:500. Paraffin sections of E19 tissues cultured either in the absence or presence of R1881 for 48 hours were used for immunohistochemistry with the RhoGDIα antibody (1:500). Antibody incubations were performed overnight at 4 C. Secondary antibodies were coupled to peroxidase using the Strept ABComplex/HRP (DAKO A/S Denmark), visualized with DAP stain (Pierce, Rockford, Ill, USA) and counterstained with hematoxylin.

Results

Analysis of growth-related proteins in developing epididymis and vas deferens

Epididymides and vasa deferentia from developing rats were isolated as a whole on E17, E18, E19, E19.5, E20 and E21. The developmental changes in these tissues are illustrated in Figure 1. On E17, the epididymis is relatively small and underdeveloped. The epithelial duct consists of single layer of cuboidal cells with a small lumen, and no coiling of the duct is apparent (Figure 5.1A). On E21, the epididymis has grown much larger and the epithelial duct shows multiple coiling, which is apparent in the figure as multiple small cross-sections through the duct (Figure 5.1B). It is obvious that marked tissue remodeling is needed to achieve such large changes in tissue structure, and this may require, and result in, differential expression and modification of a variety of proteins. To get more information about the spectrum and identity of proteins involved in growth-related tissue remodeling, we used a 2DE-driven proteomic approach, to identify proteins that are differentially regulated in time during this process of growth. Tissue lysates from the combined epididymides and vasa deferentia isolated on E17-E21 were used to separate proteins on 2D gels. All samples were run either in duplicate or triplicate. Protein expression profiles from E17-E21 were compared using the PDQuest software package.

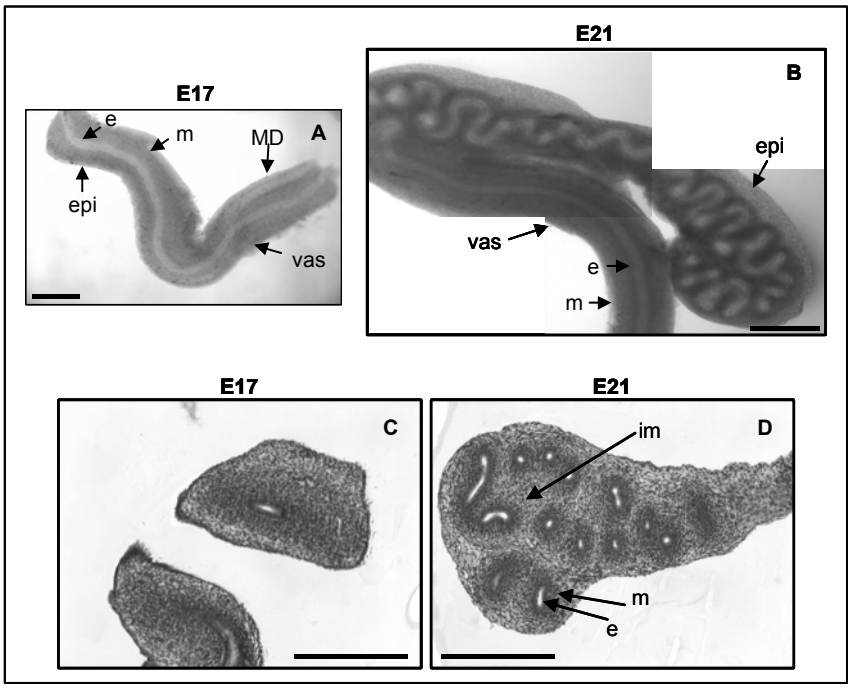

Figure 5.1. Rat epididymis and vas deferens on E17 and E21
Whole mount epididimis and vas deferens photographed on E17 (**A**) and E21 (**B**). On E17, the Wolffian duct organs have not fully developed yet, which is clearly visible by the presence of Müllerian duct remnants next to the vas deferens. On E21, the development of the Wolffian duct tissues is obvious by the gain of size and heavily coiling of the epithelial duct of the epididymis. **C** and **D** are histological sections of the tissues from A (E17) and B (E21). Hematoxylin/eosin staining. MD, Müllerian duct; e, epithelium, m, mesenchyme; im, interstitial mesenchyme. Scale bar in all pictures = 500 μm.

Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), we have identified 20 distinct proteins (numbered in Figure 5.2) representing at least 30 protein isoforms (Table 5.I). Analysis of the Coomassie-stained 2D gels, showed up-regulation of 37 protein isoforms (Figure 5.2, red circles) and down-regulation of 18 protein isoforms (Figure 5.2, green circles), which were consistently regulated throughout all duplicate gels and statistically significant. Inconsistently regulated proteins spots (e.g. doublet of spots 10 o'clock of spots 9) were excluded from the analysis. Furthermore, for three proteins we observed a shift in pI or other changes in one or more of the protein isoforms during development (Figure 5.2, blue circles). These changes most probably represent post-translational protein modifications.

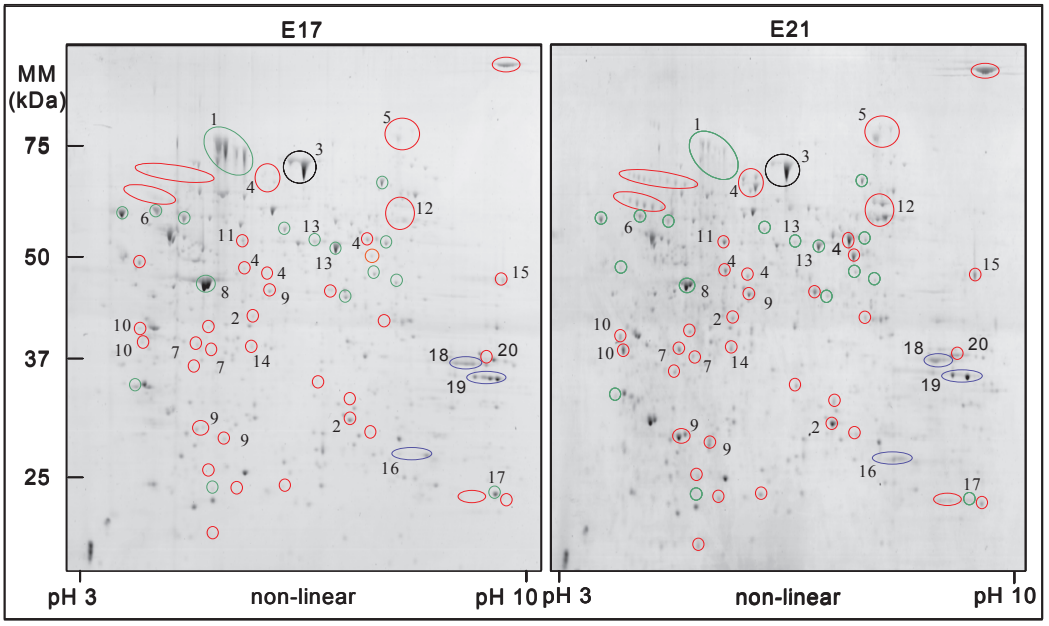

Figure 5.2. 2D gel analysis of total tissue lysates (100 µg protein) from epididymis/rat vas deferens at E17 and rat E21

Proteins were separated by isoelectric focussing on pH3-10 gel strips, and according to molecular mass (MM) on SDS-PAGE. Proteins that were down-regulated during development are encircled in green, up-regulated proteins are encircled in red, and the proteins that shifted in position are encircled in blue. The protein encircled in black did not change in expression. All proteins identified by MALDI-TOF-MS are numbered. Proteins that were identified to represent isoforms of one and the same protein, have the same number (2,4,7,9,10,13).

Table 5.I. Identification of proteins by peptide mass fingerprinting*

Spot	Protein	MM (kDa)	pI	Regulation (fold)	Accession #
1	α-Fetoprotein precursor (AFP)	68	5.8	6-10 down	A93561
2	AFP fragments	26, 38	6.0-6.7	2-3 up	A93561
3	Serum albumin precursor (SA)	68	6.1	no	A93872
4	SA fragments	45, 50, 60	6.0-6.8	4-6 up	A93872
5	Transferrin	76	6.9	3 up	BAA07458
6	Tubulin α-chain	50	4.9	3x down	A92869
7	Tubulin α-chain, fragments	30	5.3	3-4x up	A92869
8	β-Actin	42	5.3	3x down	A38571
9	β-Actin, fragments	25	5.3	5x up	A38571
10	Tropomyosin 5	29	4.7	4-6x up	P97726
11	Fibrinogen γ-chain precursor	50	5.4	2-3x up	P02680
12	Fibrinogen β-chain	54	7.9	11x up	AAA64866
13	α-Enolase	47	6.2	2-3x down	P04764
14	F-Actin capping protein α-2	33	5.6	2-3x up	AAC00567
15	HSP-47 precursor	46	8.9	2x up	A40968
16	Triosephosphate isomerase (TPI)	27	6.5	Acidic ptm	P48500
17	Probable thioredoxin peroxidase 2	22	8.3	3-4x down	I52425
18	Glyceraldehyde 3-phosphate dehydrogenase	36	8.4	Acidic ptm	P04797
19	Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1	36	8.7	Acidic ptm	AF073993
20	hnRNP A3	39	8.7	5-7x up	P51991
a	RhoGDI1	23	4.9-5.1	Acidic ptm	Q99PT1
b	TPI	27	6.5	Acidic ptm	P48500
c	Glyceraldehyde 3-phosphate dehydrogenase	37	8.4	Acidic ptm	P04797
d	hnRNP A3	39	8.7	3x up	P51991
e	hnRNP A2/B1	36	8.7	Acidic/ Basic ptm	AF073993

* Identification with p<0.05 (Mascot, Matrix Science, UK). *Numbering* according to Figure 5.2; *lettering* according to Figure 5.6A. Accession # according to the NCBI database. PI, isoelectric point; ptm, post-translational modification.

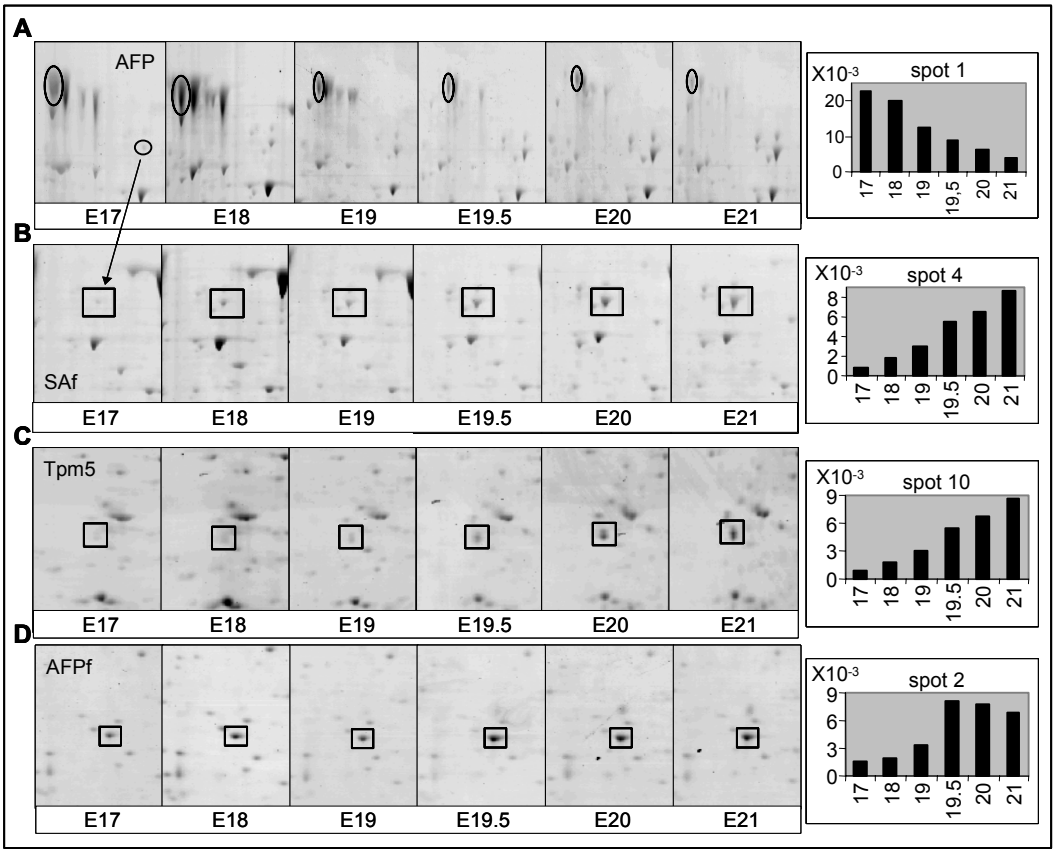

Figure 5.3. Zoom-in regions of E17-E21 2DE gels

A) Down-regulation of one of the full-length α-fetoprotein (AFP) precursor isoforms (spot 1 according to Figure 5.2).

B) Up-regulation of one of the serum albumin precursor fragments (spot 4 according to Figure 2).

C) Up-regulation of tropomyosin 5 (spot 10 according to Figure 5.2).

D) Up-regulation of an AFP fragmented isoform (spot 2 according to Figure 5.2), with peak intensity at E19.5.

All tissue lysates were run on duplicate gels and all gels were analyzed using the PDQuest software package. Corresponding arbitrary spot intensities of encircled (A) and boxed (B, C, D) proteins are represented as bars in the adjacent histograms. Proteins were selected to be up- or down-regulated at least 2-fold with a significance of p<0.05, as calculated by PDQuest software.

Analyzing the gels in more detail revealed that temporal up- or down-regulation of the proteins occurred gradually. One of the proteins that was regulated in time was α-fetoprotein precursor, AFP (spot 1, Figure 5.2). AFP occurred in the 2D gel as a 68 kDa multi-isoform protein and was down-regulated in time (Figure 5.3A). However, 2 separate and fragmented isoforms of AFP were up-regulated in time. The up-regulation of the smaller AFP fragments was gradual but peaked at E19.5, after which the expression was going down again. The expression pattern of one of the AFP fragments is shown in Figure

5.3D. Tropomyosin 5 was also up-regulated with a peak intensity at E19.5 (Figure 5.3C). MALDI-TOF-MS revealed that fragmentation not only occurred for AFP but also for serum albumin precursor (SA), β-actin and α-tubulin. Fragmentation of these proteins has been reported in relation to particular physiological roles [603-605]. All the SA fragments were up-regulated gradually in time, of which one is illustrated in Figure 5.3B.

The presence of transferrin (spot 5, Figure 5.2), and the regulation of AFP (spots 1 and 2, Figure 5.2) and SA fragments (spots 4, Figure 5.2), raised the question whether the tissue lysates contained serum proteins from infiltrating capillaries. To address this question, immunohistochemistry for AFP was performed on E17 and E21 tissues (Figure 5.4A and B). The staining shows that there is no detectable AFP in the epithelial duct, nor in the mesenchymal cells directly surrounding the duct, but there is positive staining in the cytoplasm of interstitial mesenchymal cells on E17. On E21, AFP staining in the interstitial mesenchyme was more abundant and some staining was also observed in the mesenchyme directly surrounding the epithelial duct. The staining was uniform and not restricted to capillaries, indicating that the tissue lysates contain serum proteins originating from the (interstitial) mesenchymal cells. Furthermore, no clear-cut difference in subcellular localization of the AFP staining between E17 and E21 tissue sections was visible. Western blot analysis of the tissue lysates showed a slight decrease of the two main bands of AFP (68 kDa) and an increase of some of the fragments, in agreement with the data of the 2D gels (Fig. 5.4C).

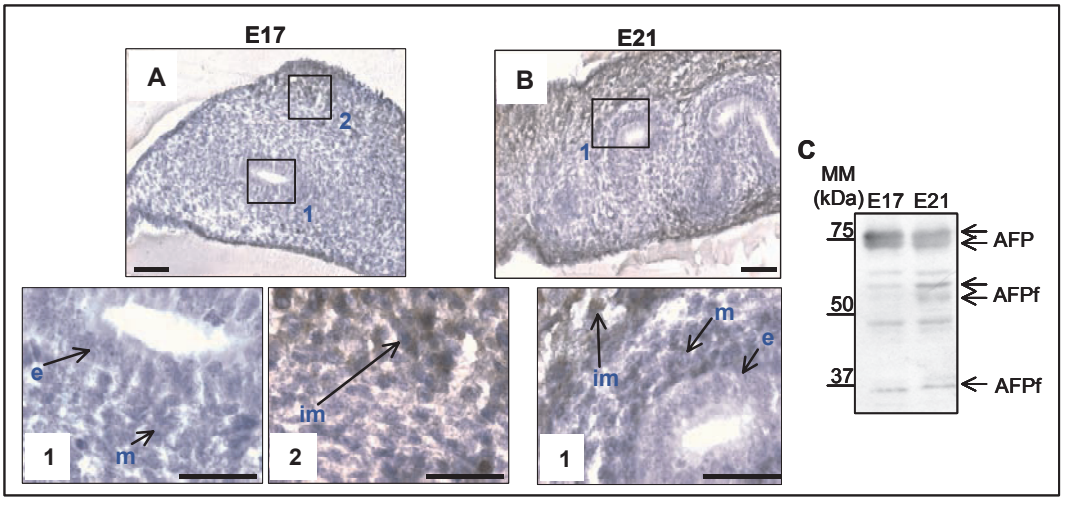

Figure 5.4. Immunohistochemistry for alpha-fetoprotein (AFP) of rat E17 (A) and E21 (B) epididymis sections

On E17, AFP stained positive in the interstitial mesenchyme. On E21, some staining was also visible in the mesenchyme directly surrounding the epithelial duct.

Scale bar = 100 μm (**A** and **B**), and 50 μm (A-1, A-2, and B-1); e, epithelium; m, mesenchyme; im, interstitial mesenchyme.

C) Western immunoblot for AFP. Total tissue lysate (12.5 μg protein) from rat E17 and E21 epididymis/vas deferens was separated on SDS-PAGE and immunoblotted. Note the down-regulation of the two 68 kDa AFP form and up-regulation of some of the fragments (AFPf).

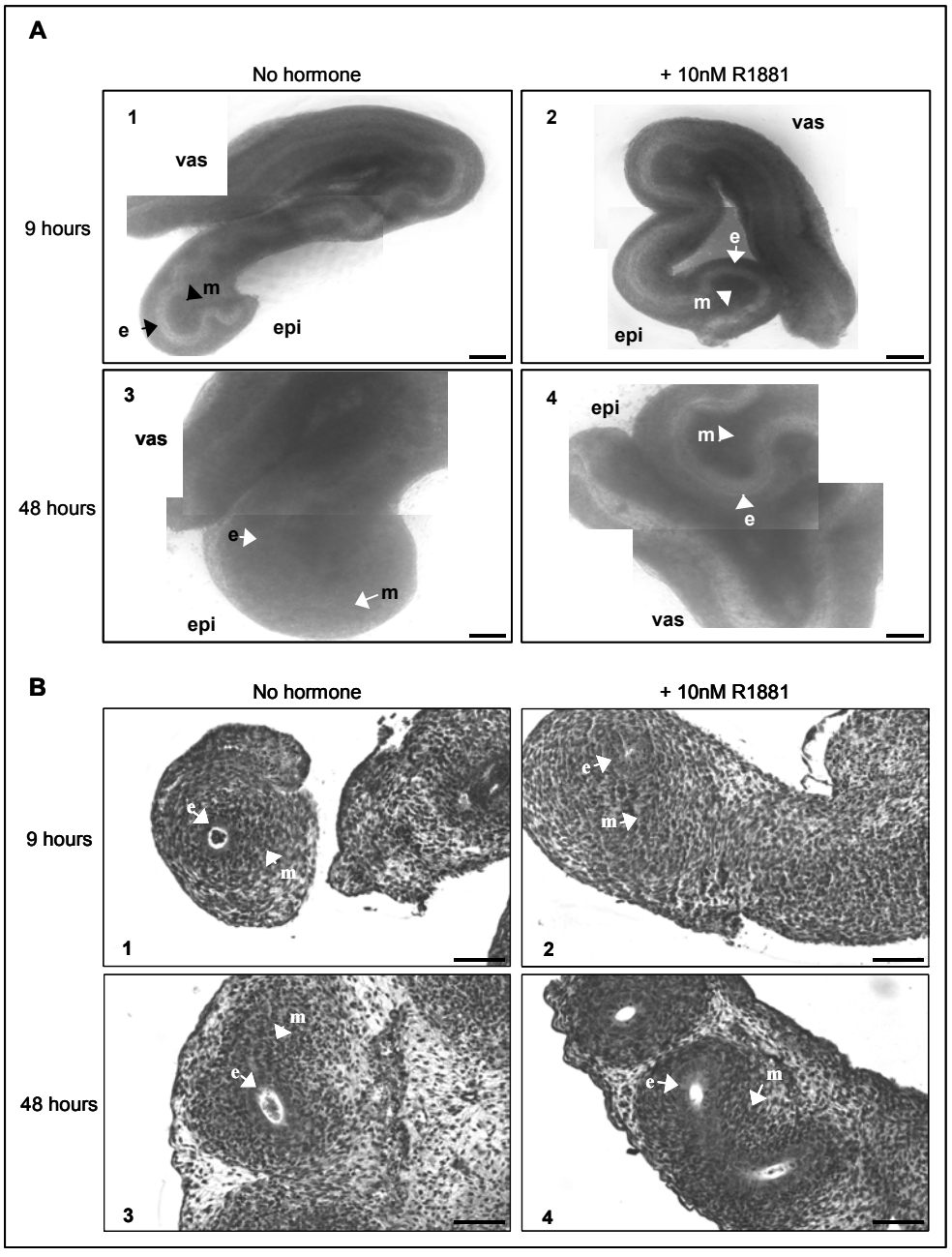

Figure 5.5. Rat E19 vas deferens and epididymis tissues

A) Photographs of E19 tissues cultured in the absence (panels 1 and 3) or presence (panels 2 and 4) of 10nM synthetic androgen R1881 for 9 hours (panels 1 and 2) and 48 hours (panels 2 and 4). Scale bar = 100 μm.

B) Histological sections of E19 epididymis cultured in the absence (panels 1 and 3) or presence (panels 2 and 4) of 10nM R1881 for 9 hours (panels 1 and 2) and 48 hours (panels 2 and 4). Scale bar = 100 μm. Hematoxylin/eosin staining. Epi , epididymis; vas, vas deferens; e, epithelium; m, mesenchyme.

Note that the epithelial duct regressed in the absence of androgen, whereas it differentiated in the presence of R1881.

Analysis of androgen-regulated proteins in cultured epididymis and vas deferens

During isolation of the epididymides and vasa deferentia from the different developmental stages, it became apparent that the largest morphological changes occurred between E19 and E20. Therefore, epididymides and vasa deferentia were isolated on E19 and cultured in either the absence or presence of synthetic androgen R1881, for 9, 24, and 48 hours, to analyze regulation of protein expression induced by androgen. At the time points mentioned, photographs were taken and some tissues were used for histology. Culturing the tissues for 9 hours in the absence or presence of R1881 did not result in macro- (Figure 5.5A) and microscopical (Figure 5.5B) changes. After 9 hours of culture, the epithelial duct is clearly visible as a lighter part of the tissues, and shows some minor coiling starting in the epididymis (Figure 5.5A-1, 2). However, 48 hours of culture in the absence of androgen resulted in regression of the epithelial duct, as demonstrated by the absence of the lighter duct in the tissue (Figure 5.5A-3). In histological sections, some remnants of the epithelial duct are still present (Figure 5.5B-3). Culturing the tissues in the presence of androgen for 48 hours resulted in stabilization of the epithelial duct, and in most cases in increased coiling of the duct as a sign of differentiation (Figure 5.5A-4). In histological sections, this coiling and stabilization is visible as cross-sections at three different positions (Figure 5B-4). Comparing the sections of Figure 1B and Figure 5.5B-4 illustrates that development of the epididymis *in vitro* during a culture period of 48 hours, although much slower, occurs in a similar pattern as *in vivo*. Furthermore, we also noticed some swelling of the tissues in culture, irrespective of the presence or absence of androgen.

Although morphological differences in the cultured tissues occurred visibly within 48 hours (and to a lesser extent already visible after 24 hours), we expected that most molecular changes, which eventually lead to morphological differences, will precede these changes. Therefore, 2DE was performed on whole tissue lysates from organs cultured in the absence or presence of androgen and the gels were analyzed using PDQuest. At first instance, no major regulation in protein expression was observed between the different culture conditions, nor as compared to the *in vivo* situation. However, a closer examination of the Coomassie-stained gels revealed that 5 proteins spots were regulated upon androgen treatment. Four of these proteins showed regulation of one or more isoforms indicating post-translational modification (RhoGDI, TPI, G3PD, and hnRNP A2/B1; Figure 5.6A spots a-c and e, and Table I), and one protein was visibly up-regulated (hnRNPA3; Figure 5.6A, spot d). Comparison of these results with Figure 5.2 and Table I showed that TPI, G3PD, hnRNP A2/B1, and hnRNP A3 were also regulated in the *in vivo* situation.

RhoGDI1 was regulated by R1881 at the post-translational level *in vitro* (Figure 5.6B). One protein isoform of RhoGDI1 was identified in E19 tissues that were either directly frozen or cultured. However, culturing the tissues in the presence of androgen for 48 hours resulted in the appearance of a second, more acidic, protein isoform that was also identified by MALDI-TOF-MS as RhoGDI1. The glycolytic enzyme triosephosphate isomerase (TPI) was regulated by R1881 *in vitro* as well as *in vivo* at the level of post-translational modification (Figure 6C). In the presence of androgen (E19, t=0 and +10nM R1881 for 9 hours and 48 hours), expression of a more basic isoform was relatively high, although the intensity decreased with increasing culture time. Androgen withdrawal resulted in a further decrease in the expression of the basic isoform, whereas a more acidic isoform appeared (Figure 5.6C and 5.6D). Apparently, androgens play a role in maintaining TPI in a more basic isoform.

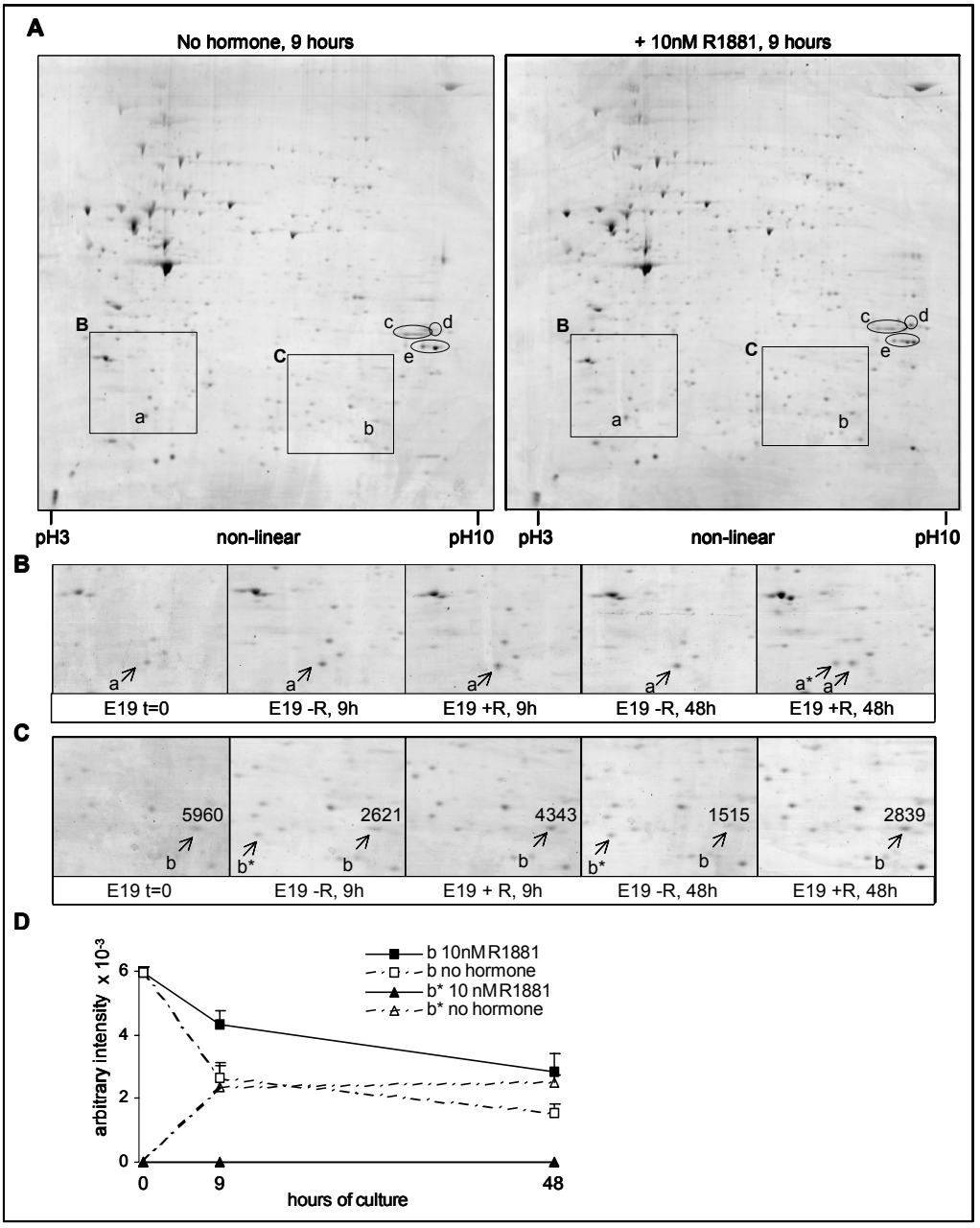

Figure 5.6. 2DE gels of tissue lysates from rat E19 epididymis/vas deferens

A) 2DE gels of tissues cultured for 9 hours in the absence (-R) or presence (+R) of 10nM synthetic androgen, R1881.

B) Zoom-in region B of gels from tissues cultured for 0, 9, or 48 hours in the absence or presence of 10nM R1881. Regulation of a second RhoGDI1 isoform, spot a*.

C) Zoom-in region C of gels from tissues cultures for 0, 9, or 48 hours in the absence or presence of 10nM R1881. Regulation of two triosephosphate isomerase (TPI) isoforms, spots b and b*.

D) Arbitrary spot intensities of TPI isoforms b and b* during 48 hours of tissue culture in the absence or presence of 10nM R1881. In the absence of R1881, spot b is down-regulated and spot b* is up-regulated. Each point in the graph represents the mean (SD) intensity of spots from two separate gels.

RhoGDI1 is an interesting candidate protein to analyze in more depth, since it was reported that this protein can increase transcriptional activity of the AR [606]. We questioned whether addition of androgen resulted in a change in subcellular localization of RhoGDI1 and whether the second isoform represented a differential phosphorylated isoform, since phosphorylation of RhoGDI1 has been reported [607]. Immunohistochemical analysis of epididymis cultured in the absence of androgen for 48 hours, confirmed the cytoplasmic localization that was already reported [608]. As expected, no nuclear localization of RhoGDI1 was observed after androgen treatment (Figure 5.7A/C and B/D). Although the epithelial duct regresses in the absence of R1881, RhoGDI1 is still expressed in the cytoplasm of the remnants of the duct, and also in the surrounding mesenchyme. Western immunoblot analysis of tissue lysates showed an equal expression of RhoGDI under all culture conditions (Figure 7E). Immunoprecipitation of RhoGDI1 from the lysates with a RhoGDI1 antibody and subsequent immunoblot analysis with a phospho-serine antibody confirmed that RhoGDI1 is a phosphoprotein (Figure 5.7F).

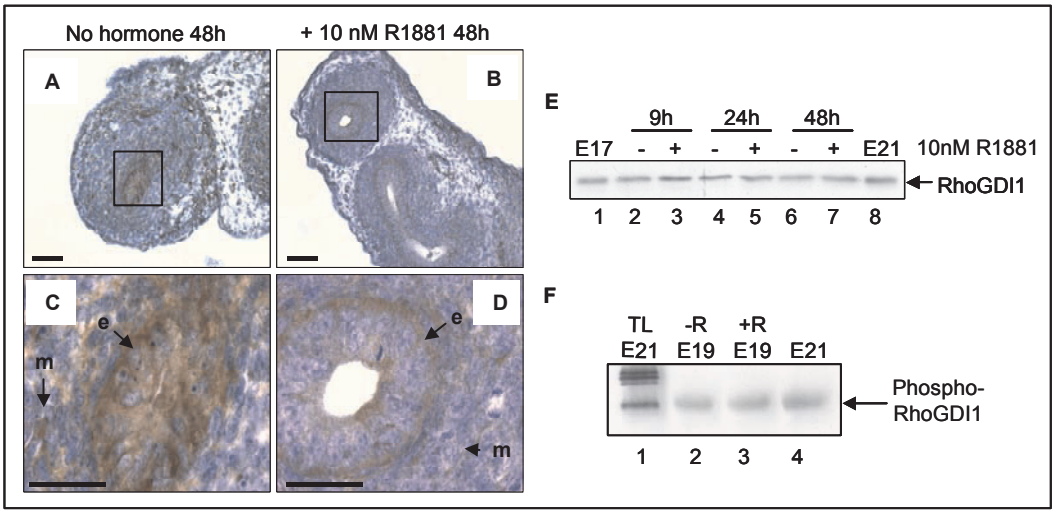

Figure 5.7. RhoGDI1 expression
Immunohistochemistry for RhoGDI1 in rat E19 epididymis cultured in the absence (**A**, **C**) or presence (**B**, **D**) of 10nM R1881 for 48h. Scale bar = 100 μm (**A**, **B**), and 50 μm (**C**, **D**).
E) RhoGDI1 Western immunoblot of E17 (lane 1), E19 (lanes 2-7), and E21 (lane 8) epididymis/vas deferens tissue lysates. RhoGDI1 protein expression was equal under all culture conditions.
F) RhoGDI1 immunoprecipitation and phospho-serine Western immunoblot. Lane 1= E21 total lysate input; lane 2= E19 no hormone, 48h; lane 3= E19 + 10nM R1881, 48h; lane 4= E21. Phosphorylated RhoGDI1 was detected after immunoprecipitation in lanes 2-4. In lane 1 several other phosphorylated proteins were detected on the total tissue lysate.

Discussion

Androgen-controlled growth and differentiation of the epididymis and vas deferens involves regulation of specific proteins. It was our aim to identify proteins that are involved in growth and differentiation, and more specifically proteins that are regulated by androgen during this process. The dramatic change in morphology of the epididymis, and to a lesser extent of the vas deferens, during the last phase of the fetal period, was the starting point for the present study.

Identification of growth-related proteins

Using 2DE driven proteomics, 20 proteins were identified that are regulated during fetal growth and development of the rat epididymis and vas deferens. The proteins identified can be categorized into enzymes (α-enolase, TPI, glyceraldehyde 3-phosphate dehydrogenase (G3PD), probable thioredoxin peroxidase 2), cytoskeletal proteins (α–tubulin, β-actin, tropomyosin 5, F-actin capping protein α-2), transport proteins (AFP, SA, transferrin, fibrinogen β/γ), RNA binding proteins (hnRNP A2/B1/A3), and chaperones (Hsp-47 precursor). Furthermore, fragmented isoforms of SA, AFP, α-tubulin, and β-actin were identified, which were up-regulated in time during genital tract development.

AFP is a specific fetal glycoprotein that is secreted by embryonic tissues and is involved in up- and down-regulation of cell growth [609], in immunosuppression, and in apoptosis [610]. AFP belongs to the proteins encoded by the serum albumin multigene family, which present three structurally homologous domains I, II, and III [603]. Fragmentation of AFP has been reported, with cleavage products of 32 and 38 kDa resulting from domains I and III. These products can be further fragmented into proteolytically stable isoforms of 23 and 26 kDa [604]. A similar kind of fragmentation was described for SA, but with many more proteolytic cleavage products possible [611]. These reported fragments of AFP correspond in size to the fragments that were identified in the present study. Furthermore, others have described that distinct AFP isoforms accumulate at different stages of fetal development [612,613] and that AFP can interact with nuclear receptors [614]. Thus, the up-regulation of AFP fragmented isoforms may be physiologically relevant for growth and differentiation of epididymis and vas deferens.

Glycolytic enzymes interact directly (α-enolase, G3PD), or indirectly (TPI), with structural proteins and complexes like actin and microtubules [615,616]. It was recently described for TPI that it binds to the actin-binding protein cofilin, which in turn binds to Na,K-ATPase upon phosphorylation by the Rho-signaling pathway [617]. TPI takes part in production of energy, which is transduced to the cytoskeleton that modulates cell function, proliferation, and differentiation [617]. The cytoskeletal proteins actin and tubulin, and in particular specific cleavage of these proteins, have been implicated in several regulatory processes, including apoptosis [605] and other processes such as meiosis resumption in starfish oocytes (30) Our finding that fragments of β-actin and α-tubulin accumulate taken together with the observation of post-translational regulation of glycolytic enzymes could reflect the processes of vigorous tissue remodeling during the process of genital tract development.

Identification of androgen-regulated proteins during growth

Since it is known that androgen action is essential for growth and development of the male genital tract, it was our aim to identify androgen-regulated proteins that are involved in this process. Epididymides and vasa deferentia cultured *in vitro* in the absence and presence

of androgen were subjected to 2DE, and the differentially expressed proteins identified were compared to the proteins identified *in vivo*. Glycolytic enzymes G3PD and TPI, and nuclear proteins hnRNP A2/B1/A3 were identified to be regulated by androgen, as was also observed *in vivo*. In addition, RhoGDI1 was identified as a protein that was regulated by androgen at the level of post-translational modification. Although the regulation of these proteins occurs under the action of androgen, we cannot conclude that this is the result of a direct and/or indirect androgen action. Furthermore, we did not observe any obvious regulation of the AR protein in the 2D gels. However, the AR protein is of lower abundance and therefore not easily detectable in such a broad range 2D gel.

Rho guanine nucleotide dissociation inhibitor 1(RhoGDI1 or RhoGDIα), is a cytoplasmic protein originally identified as a negative regulator of RhoGTPases [608]. RhoGTPases are molecular switches that cycle between an active membrane-associated GTP-bound state and an inactive cytoplasmic GDP-bound state. This switch is carefully controlled by exchange factors (GEFs), activating proteins (GAFs), and dissociation inhibitors (GDIs) [618]. RhoA, Rac and Cdc42 belong to the family of RhoGTPases, which regulate many signal transduction pathways, including those linked to the actin cytoskeleton, microtubule dynamics, vesicular transport dynamics, regulation of cell polarity, gene transcription, G1 cell cycle progression, and a variety of enzymatic activities [618,619]. Members of the RhoGDI family block GDP dissociation from RhoGTPases and control cytoplasmic localization of RhoGTPases. In addition, it was described that RhoGDI1 specifically increases the transcriptional activity of estrogen receptors (ERα, ERβ), glucocorticoid receptor (GR) and AR [606]. This activation is mediated via repression of RhoGTPases, which demonstrates that the Rho-mediated signaling pathway is an important regulator of ERs, GR, and AR transcriptional activity [606]. Although RhoGDI1 is ubiquitously expressed in all tissues, *RhoGDI1* knockout mice show specific and progressive impairment of kidneys and reproductive organs probably as a result of the destruction of the actin cytoskeleton [620], meaning that the function of RhoGDI1 in the kidney and reproductive organs is non-redundant.

The testis of *RhoGDI1* -/- mice reveals structural abnormalities, the number of germ cells is dramatically decreased and mature sperm cells are only rarely detected in seminiferous tubules and epididymides [620]. *RhoGDI1* -/- female mice have an intrinsic defect in their reproductive system, which is most evident in the postimplantation development of *RhoGDI1* -/- embryos. For the epididymis, no functional developmental defect in the knockout mice was reported [620].

Bourmeyster et al., (1996) have detected RhoGDI1 on a 2D gel as a single protein spot with a pI of 5.1, which represents the free, unbound form of RhoGDI. In the case were RhoGDI was associated with RhoA GTPase, they detected two RhoGDI protein spots with pI 4.6-5.1, which could be reduced to one protein spot after phosphatase treatment [607]. Furthermore, they have shown that RhoA-RhoGDI1 association depends on RhoGDI1 phosphorylation. Thus, phosphorylation and dephosphorylation of RhoGDI1 determines the activation state of RhoA GTPase [607]. In the present tissue culture experiments, 48 hours of androgen treatment resulted in the appearance of a second, more acidic, RhoGDI1 isoform that could represent a phosphorylated isoform. If RhoGDI1 is indeed phosphorylated by androgens, this could lead to complex formation with Rho GPTase and repression of the Rho GTPase activity. Taken the fact that RhoGDI1 enhancement of AR transcriptional activity occurrs via RhoGTPase repression [606], one could postulate that

RhoGDI1 phosphorylation leads to enhanced AR transcription activation, and as a result regulation of RhoGDI1 activity by phosphorylation and dephosphorylation might function as a feed-back loop in androgen signaling.

Heterogeneous nuclear ribonucleoproteins (hnRNP) form a large family of proteins that are categorized on the basis of structural/functional motifs, and of which the A/B type are the most abundant hnRNP proteins [591]. Besides a large number of post-transcriptional isoforms, the hnRNP A/B proteins also show extensive post-translational modifications. For a specific subset of hnRNPs, hnRNP A2, hnRNP A3 and DBP40/CBF-A, an interaction with nuclear actin was described, and an interaction was suggested for cytoplasmic actin [591]. In a previous study, we have identified CBF-A, which is a minor variant of hnRNP A2, to be regulated by androgen at the level of post-translational modification in the mouse fetal vas deferens cell line MFVD [514]. This coincides with the present finding that androgens regulate hnRNPs post-translational modification in genital tract tissues.

Glycolytic enzymes, like TPI and G3PD, are dependent on androgen action for their activity, as was demonstrated by castration experiments. Enzymatic activity was found to be decreased in rat epididymis and monkey seminal vesicle after castration, which was restored after androgen replacement [621-623]. Our present results suggest that androgenic regulation of glycolytic enzyme activity is controlled at the level of post-translational modification.

In summary, our study shows that changes in protein expression profiles can be detected on 2D gels, when developing genital tract tissues are followed in time *in vivo*. Analyses of androgen-regulated proteins in epididymis and vas deferens tissues exposed to androgen, also revealed several changes in protein expression profiles when grown *in vitro*. In the present system, androgen action occurs mainly at the level of post-translational modification. We hypothesize that during the process of androgen stimulation, which will lead to growth and differentiation of the male genital tract, post-translational modification of glycolytic enzymes regulates their activity and their association with cytoskeletal proteins. Furthermore, androgen-induced post-translational modification of RhoGDI1 and glycolytic enzymes may be an important functional link between signaling pathways and cytoskeletal rearrangements in control of growth and development of the male internal genital tract.

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Chapter 6

General Discussion

Introduction

Actions of androgens are essential for male sexual development [2,624]. The circulating androgen testosterone and its locally 5α-reduced metabolite DHT stimulate the Wolffian duct, urogenital sinus, genital tubercle, genital fold and swelling, to develop into the internal sex organs (epididymis, vas deferens, seminal vesicle, and prostate gland), and the external sex organs (penis, with urethra and scrotum). Testosterone and DHT can bind to the AR, which is present in mesenchymal cells of reproductive tract tissues during the critical time of development. The AR is a transcription factor that recognizes specific androgen response element sequences in promoter regions or in far upstream enhancer regions of androgen-target genes, thereby facilitating regulation of gene expression, and ultimately of protein expression (described in **Chapter 1**). Paracrine factors play an important role in transmitting the androgenic signal from AR positive mesenchymal cells to AR negative epithelial cells, but the underlying mechanism, how androgens drive the mesenchymal cells to produce and secrete these factors, is largely unknown. In the neonatal and adult epididymis and vas deferens, numerous androgen-regulated proteins have been identified, of which many play a role in sperm cell maturation or maintenance of organ physiology (**Chapter 1.3**, Table 1.II). However, only a few studies describe androgenic regulation of proteins that are involved in differentiation and development of other male internal sex organs, or the external sex organs.

For the research described in this thesis, a proteomic approach was chosen, to try to identify androgen-regulated proteins in an androgen target cell line, MFVD cells, and in target tissue, epididymis and vas deferens (**Chapters 4 and 5**). The results of these studies are discussed below in a broader perspective.

Proteomics and androgen-regulated male development

Proteomics research involves a technology-driven approach to systematically determine diverse properties of proteins, such as identity, quantity, subcellular distribution, and structure. The first applications were in the field of "discovery science", meaning that identification and cataloguing of all proteins in a system were the goal, irrespective of any hypothesis in relation to a function of that system. The completion of the draft human genome sequence, together with the continuous development of technology, has resulted in incorporation of proteomics research into studies on understanding biological systems [625,626].

As described in Chapter 3, proteomics research can be divided into a "2DE-driven", classical approach or a "non-2DE-driven" approach, which is becoming more easily accessible and therefore gaining popularity. Both approaches have their advantages and disadvantages, but starters in the field will most probably begin their proteomics research with 2DE for two reasons. First of all, 2D gels can be easily run on the bench, although preparation of the 2D gels remains a tedious task, and secondly, 2DE-driven proteomics does not have to be overwhelmingly expensive. In contrast, multi-dimensional protein separation and identification strategies omitting the 2D gel often require the use of highly specialized and expensive equipment, which is usually not readily accessible for many researchers. Whatever protein separation technique is used, ultimately proteins need to be identified, and the mass spectrometer is the apparatus of choice. Many different types of mass spectrometers are available, which all have certain specific properties, but accuracy and sensitivity are issues that apply to all. However, one of the most easily accessible mass spectrometers is the MALDI-TOF, which has the capacity to relatively easily and quickly create peptide finger prints of, and screen, many samples.

For these reasons, we have chosen to use 2D gels as a protein separation step, and MALDI-TOF mass spectrometry for protein identification. Two approaches were taken in an attempt to study androgen-regulated protein expression. In **Chapter 4**, the generation of an androgen-target cell line, MFVD, is described. Using this cell line for proteomics studies has the advantage of yielding relatively large quantities of sample, while the experiments are easily repeated, and the experimental conditions are reproducible. Furthermore, the effects of androgens on one particular cell type, mesenchymal vas deferens cells in this case, can be studied, without being influenced by surrounding factors. In addition, in **Chapter 5** the effect of androgens has been studied in cultured tissues. In this organ culture system, both epithelial and mesenchymal cells are present, but also infiltrating blood cells (in capillaries) and connective tissue. As a consequence, the androgenic effects found in an organ culture system cannot be directly linked to a certain cell type. However, there is the advantage that such an *ex-vivo* system provides a more physiological environment in which the androgens act. A limitation of using fetal tissue for 2D analysis, is that many samples need to be collected before a small but workable amount of sample can be extracted.

While in the experiments described herein, the organ-culture system was performed using fetal rat epididymis and vas deferens, the cell line was derived from fetal mouse vas deferens. Therefore, it was questioned whether the androgenic effects observed in 2D gels of the two systems would be similar and comparable. We did not identify identical proteins in the two systems studied that were regulated upon androgen treatment, but common observations include that:

1. *Androgens influence post-translational modification of several proteins.*
2. *Signaling molecules that appear to be regulated by androgens are associated with the actin cytoskeleton.*

The actin cytoskeleton mediates a variety of essential biological functions in all eukaryotic cells, by providing a structural framework, and the driving force for cells to move and divide. The Rho family of signaling molecules are key regulators of the actin cytoskeleton that link surface receptors to the organization of the actin cytoskeleton through interaction with multiple target proteins [619]. In Figure 6.1, the effect of Rho activation on the actin cytoskeleton is illustrated.

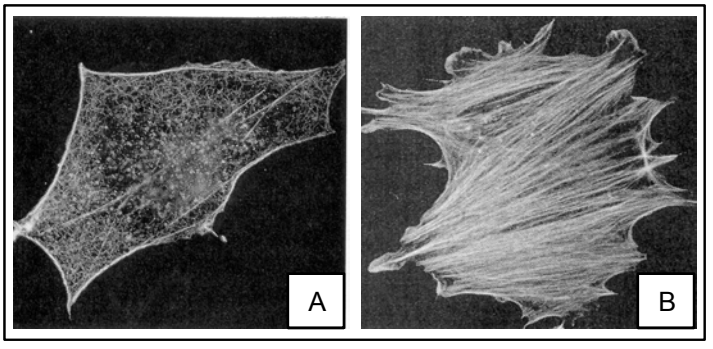

Figure 6.1. The actin cytoskeleton
A) Quiescent, serum-starved Swiss 3T3 fibroblasts contain very few actin filaments.
B) Activation of Rho results in formation of actin stess fibers.
Adapted from Hall, 1998 [619].

Androgens, the actin cytoskeleton, and signaling molecules

Androgenic treatment of MFVD cells resulted in post-translational modification of mElfin and CBF-A (**Chapter 4**). In fetal rat epididymis and vas deferens, post-translational modification upon androgen treatment occurred for hnRNP A2/B1, glycolytic enzyme TPI, and signaling protein RhoGDI1 (**Chapter 5**). These proteins can be categorized into certain functional protein families. CBF-A and hnRNP A2/B1 are members of the family of A/B type hnRNP proteins, which are present in pre-mRNP particles. CBF-A has been reported to have a dual function: transcriptional regulation, and post-transcriptional regulation at the RNA level [588,590]. CBF-A and hnRNP A2/B1 shuttle between nucleus and cytoplasm [591,627], they are associated with either globular (G)- or filamentous (F)-actin, and are involved in transport of mRNA into the cytoplasm [591,628]. Since a large fraction of mRNA is associated with cytoskeletal structures [629], it was proposed that mRNA undergoes a nuclear preparatory event by a subset of hnRNP proteins complexed to actin, in order to function properly in the cytoplasm [591,628]. What role androgen-regulated post-translational modification plays in this process has, not yet, been described. One could, however, speculate that post-translational modification of hnRNPs is necessary to become fully functional in regulation transcription and translation of androgen-target genes and mRNAs. Perhaps, it is also possible that post-translationally modified hnRNPs, bound to actin in the cytoplasm, regulate translation of mRNAs that are already present in the cytoplasm, thereby bypassing an AR mediated genomic event (this will be discussed in the next paragraph).

What groups mElfin, RhoGDI1 and TPI together is their, direct or indirect, association with the cytoskeleton and their connection to signaling pathways. mElfin contains two protein-protein interaction domains, a PDZ and a LIM domain, with which it associates to F-actin through binding to α-Actinin, and through which it functions to recruit kinases to the cytoskeleton [595,596,630]. RhoGDI1 is one of the players in the Rho-signaling pathway, and functions as an inhibitor of Rho GTPase activity. RhoGDI1 blocks GDP dissociation from Rho GTPase and keeps it in the cytoplasm in an inactive state, thereby preventing interaction with Rho GTPase and its effectors [608]. Rho GTPases are key regulatory molecules that, in addition to many other pathways, link surface receptors to the organization of the actin cytoskeleton [618,619]. RhoGDI1 plays a central role in regulation of signal transduction cascades mediated by Rho GTPases [631]. The glycolytic enzyme TPI has also been found to be associated with the actin cytoskeleton, where it interacts with the F-actin binding protein Cofilin [617]. Cofilin can be phosphorylated by LIM-kinase, a member of the Rho signaling cascade, after which the Cofilin-TPI complex is directed to the cell membrane to provide glycolytic energy to Na^+,K^+-ATPase [617]. What role post-translational modification of mElfin, RhoGDI, and TPI plays in androgenic regulation, and whether these modifications are a direct or indirect effect of androgen treatment is not known. Studies on related proteins, however, may provide clues about the regulatory mechanisms involved.

FHL2, a LIM-only protein, was initially reported to function as an adaptor protein involved in integrin signaling pathways [632]. Later on it was described as a tissue-specific co-activator of transcription factors like CREM (cAMP response element modulator) and CREB (cAMP response element binding protein) [633]. Furthermore, FHL2 appears to translocate from the cytoplasm to the nucleus upon activation of the Rho signaling pathway [634], and it functions as a tissue-specific co-activator of the AR, thereby linking Rho signaling to the activation of transcription factors [238]. Interestingly, FHL2 has also recently been identified as a co-activator for the Wnt signaling mediator β-Catenin, potentiating the

transcription of Wnt-response genes [635]. Although β-Catenin itself has been identified as an AR co-activator as well [598], β-Catenin and FHL2 have no synergistic effect on AR-mediated transcription [635]. Thus, it appears that the regulatory function of proteins like β-Catenin and FHL2 depends on the activating signal and the promoter context. Detailed analysis of the subcellular localization of mElfin upon androgen treatment and co-transfection studies with the AR, would provide more information about the role and function of mElfin in the androgen signaling pathway, since mElfin, like FHL2 and β-Catenin, may be involved in several different pathways, exerting different functions.

Recently, more and more actin-binding cytoskeletal proteins and proteins of the Rho signaling pathway have been associated with AR translocation and transactivation, such as Supervillin [277], Filamin-A [275,276], and PAK6 [636]. Supervillin is an actin binding protein, and interacts specifically with the AR NH2-terminal and ligand binding domains in a ligand-dependent manner. It enhances transactivation of AR, also in cooperation with other co-activators, such as ARA55 or ARA70 [277]. Filamin-A is a component of the cytoskeleton and is involved in translocation of the AR to the nucleus [275]. In the nucleus a fragmented form of Filamin-A binds to the hinge region of the AR and inhibits transcription activation by interfering with TIF2 binding [276]. PAK6 (p21-activated kinase 6), which is primarily a cytoplasmic protein containing a Cdc42 Rho GTPase interacting domain and a kinase domain, inhibits AR transactivation by interacting with the hinge region [636]. The inhibiting effect of PAK6 was not due to the sequestration of Cdc42, although Cdc42 itself can also inhibit AR (as well as ER and GR) transcription activation activity [606]. The inhibiting activity of Cdc42 can be reversed by the Rho GTPase inhibitor RhoGDI1, which in turn activates transcription of AR, ER and GR [606]. Thus, binding of Rho signaling proteins such as PAK6 and RhoGDI1 to the AR, and other steroid hormone receptors, provides and regulates a mechanism of cross-talk between diverse signal transduction pathways and steroid hormone receptors.

More evidence for cross-talk between the Rho signaling pathway and steroid hormone receptors comes from a study on the RhoA effector protein PRK1 (protein kinase C-related kinase1) [637]. PRK1 is a serine/threonine kinase that stimulates the transcriptional activity of AR (and also PR, MR, and p160 co-activators) in the presence of testicular and adrenal androgens but also in the presence of antagonists, through interaction with the AR AF-5 transactivation domain. This novel signaling pathway links RhoA effectors to AR transactivation. Since PRK1 is up-regulated in prostate tumors and activates the AR in the absence of testicular androgens, as well as in the presence of antagonists [637], it may be of importance in prostate cancer progression,.

Non-genomic effects of androgens

Apart from cross-talk with signaling pathways that regulate AR transcriptional activation activity, cross-talk between steroid hormone receptors and the MAPK (mitogen-activated protein kinase) signaling pathway has been described, that is not directly associated with genomic actions. Rapid responses to steroid hormones (androgens, progestins and estrogens) can be effectuated by non-genomic mechanisms, still mediated by steroid receptors [341,342]. In addition, some reports indicate that non-genomic effects may be mediated by plasma membrane-bound receptors [352-354]. In LNCaP cells, a plasma membrane testosterone receptor was described, which is distinct from the classical AR. Androgen binding to this membrane receptor resulted in a rapid redistribution of the actin cytoskeleton, which may regulate trafficking and secretion of molecules [638].

Whether or not androgens stimulate AR to trigger gene transcription or not, might be dependent on the amount of receptor present in the cell, as was recently described for mouse embryo fibroblast NIH3T3 cells. The NIH3T3 cells express a very low level of AR, compared to LNCaP cells, and stimulation with androgen does not result in nuclear translocation and transcription activation of the AR [639]. However, a very low androgen concentration (1 pM) stimulated AR association with c-Src, which induced S fase entry of quiescent NIH3T3 cells, and a much higher androgen concentration (10 nM) stimulated Src-mediated activation of the Rho GTPase Rac, which rapidly caused changes in actin assembly resulting in membrane ruffling [639]. Thus, a hormone-regulated correlation between cytoskeletal changes and the activation of Rho GTPase Rac would represent a new signaling pathway. Similar to NIH3T3 cells, other mesenchymal cells, such as MFVD cells, also contain low amounts of steroid hormone receptors [640,641]. It might be possible that MFVD cells respond to androgen stimulation with cytoskeletal changes and activation of signaling pathways, not necessarily directed by transcriptional activity of the AR. In conclusion, increasing evidence shows that steroid hormones play an important role in controlling cytoskeleton function. This novel aspect of steroid hormone action opens an interesting area of research, where many questions are open to new research efforts.

From the data presented in this thesis, it is not clear whether androgen-regulated post-translational modification of the proteins mentioned is part of a transcription-mediated genomic effect, or rather represents a non-genomic mechanism. Arguments can be raised in favour of both possibilities. Post-translational modification of hnRNPs and RhoGDI1 were found to occur after 24 and 48 hours, respectively, and not at shorter time intervals after initiation of the exposure to androgen, excluding a rapid (within seconds to minutes) non-genomic effect. Regulation of hnRNP proteins suggests that a transcription process has taken place, although hnRNPs also act in the cytoplasm. On the other hand, recruitment of signaling proteins in the cytoplasm, associating with the cytoskeleton, may indicate that signaling pathways are activated, exerting a non-genomic effect. Whether the effects described in this thesis are part of a pathway acting upstream of the AR, in parallel with the AR, or perhaps even completely bypassing the AR, will need to be established. Incorporating the data presented in this thesis into the present literature, a model of androgen-induced mechanism of action of post-translational modification is proposed in Figure 6.2.

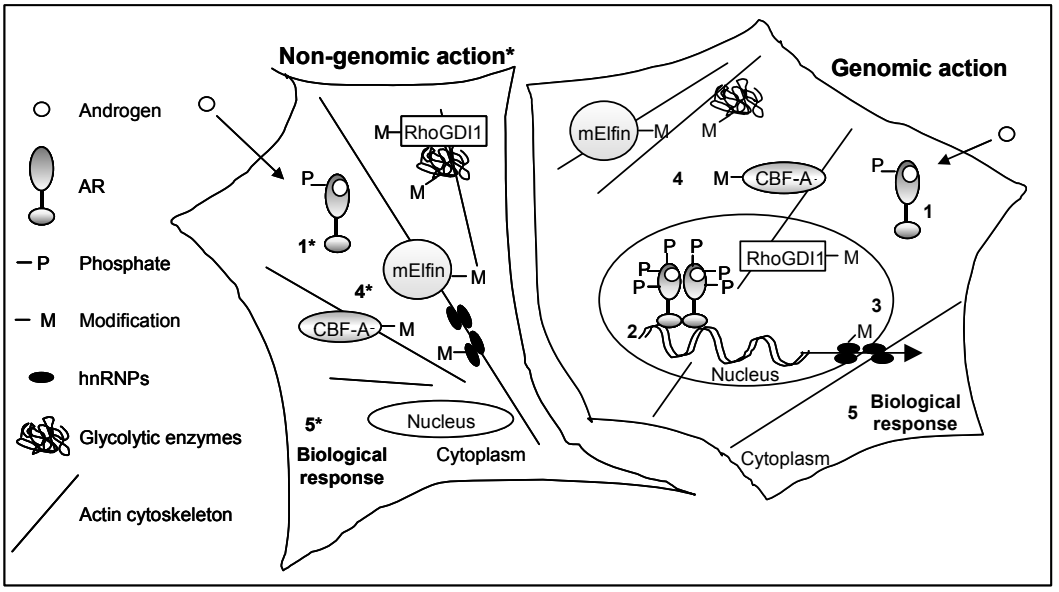

Figure 6.2. Model of androgen induced post-translational modification and mechanism of action
Genomic action: Androgens enter their target cell and bind to the androgen receptor (1), which becomes phosphorylated, translocates to the nucleus, binds to DNA response elements, and activates transcription. This process may be enhanced by phosphorylated RhoGDI1 (2). Post-translationally modified hnRNP proteins bind mRNA , which is transported into the cytoplasm and translated into protein (3). Subsequently, actin-associating proteins (mElfin, CBF-A, glycolytic enzymes) become post-translationally modified (4), which leads to a biological response (5).
Non-genomic action*: Androgens enter their target cell and bind to the androgen receptor (1*), after which signaling molecules (RhoGDI1) and actin-associated proteins (mElfin, CBF-A, glycolytic enzymes) become post-translationally modified by androgens, either directly or indirectly (4*). The activated signaling cascades result in a non-genomic biological action (5*).

Defective androgen action

While this thesis is mainly focused on the effect of androgens on protein expression in a target cell during normal male development, the syndrome of androgen insensitivity is a very appropriate example of defective androgen action *in vivo*. In order to understand the phenotypes observed in individuals with AIS, much attention has been paid to the effect of mutations on AR function [179,357] (Chapter 2). However, identification of molecular pathways that are disturbed by defective AR function is an area of research that is still at its infancy. Recently, Holterhus *et al.*, 2003, have performed a genome wide screen in genital skin fibroblasts (GSFs) from normal males and compared it with gene expression patterns of GSF of 46,XY individuals with AIS [642]. They have used cDNA microarrays representing 33,000 genes, and identified 404 transcripts that were expressed at significantly different levels in GSFs from normal males as compared with those from AIS-affected individuals. Some of the differentially expressed genes were involved in sex steroid hormone metabolism, such as reductases, dehydrogenases, and in intracellular signal transduction, such as MAPK, and their expression was reduced in GSFs from AIS individuals. The different gene expression profiles observed between GSFs from 46,XY males and 46,XY AIS individuals, suggest that the AR is involved in setting long-lasting gene expression patterns in GSFs. In addition, the study indicated that GSFs from both normal and AIS

individuals did not show a detectable response to androgen treatment at the transcriptional level [642]. It would be of interest to perform a proteome screen on these GSFs to see whether androgen treatment induces post-translational modification of signaling proteins, cytoskeleton-associated proteins, or glycolytic enzymes. This would yield an indication for a non-genomic effect of androgens in GSFs that express relatively low levels of androgen receptors.

Conclusions

This thesis describes the role of androgens, AR, and androgen-regulated proteins in the process of male genital tract development. To investigate these issues, several questions were raised in Chapter 1. Based on the results presented in the different chapters of this thesis, together with the discussion in Chapter 6, the questions can be addressed and answered as follows:

1. *Is it possible to develop an androgen-responsive cell line derived from Wolffian duct mesenchymal cells and use it as a model system for studying androgen-induced regulation of gene expression?*

We have developed a mouse fetal vas deferens cell line, MFVD, which was derived from WD mesenchymal cells, and which shows features of WD stromal cells. The MFVD cells express a relatively low amount of AR protein, similar to the expression level in human genital skin fibroblasts, but lower as compared to AR protein expression in the prostate cancer cell line LNCaP. The endogenous transcriptional activity of AR in MFVD cells is rather low, and therefore we performed transfection of AR cDNA into the MFVD cells, which resulted in a much higher transcriptional activity. We conclude that the MFVD cells are androgen-responsive and can function as a good model system for studying androgen-regulated protein expression.

2. *Can detailed functional analysis provide insight into the effect of a novel mutation in the AR gene in an individual with PAIS, and do the results of this analysis correlate with the observed phenotype?*

Numerous mutations in the *AR* gene of AIS patients have been described, and most of them reside in the LBD. When a novel mutation is detected, the position of the mutation can be indicative for the effect of the mutation on AR functioning, but this is not always predictive, due to the lack of sufficient knowledge about AR structure-function relationship. The detailed functional analysis performed for ARQ902K, such as N/C interaction and TIF2 co-activation, appeared to result in information about the role of residue Q902 in AR functioning. Furthermore, several recent reports and our own results emphasize the importance of these intra- and intermolecular interactions in determination of the AIS phenotype.

3. *What are the changes in protein expression patterns of androgen target cells upon treatment with androgen as detected by means of 2DE analysis, and can differentially expressed androgen-regulated proteins be identified by mass spectrometry?*

Both MFVD cells as well as cultured epididymides and vasa deferentia show changes in the protein expression pattern on 2D gels upon androgen treatment. The differentially expressed proteins could be identified by peptide mass fingerprinting using a MALDI-TOF mass spectrometry approach. Upon protein identification, it appeared that the differentially expressed proteins were post-translationally modified isoforms of proteins also present in the untreated target cells. Further to these qualitative changes, no obvious quantitative

changes (up- or down- regulation) in protein expression were observed upon androgen treament, except for hnRNP A3 identified in cultured tissues.

4. *Do the androgen-regulated proteins identified in a cell line provide relevant information about the androgenic signaling pathway of a whole organ?*

In MFVD cells, androgen treatment resulted in post-translational modification of mElfin and CBF-A, whereas in cultured epididymides and vasa deferentia, post-translational modification was observed for RhoGDI1, hnRNP A2/B1, and TPI. Although not identical proteins appeared to be regulated by androgens in the two model systems used, the pathways involved are rather similar. In both systems the common protein scaffold interacting with proteins that undergo androgen-induced post-translational modification, seems to be the actin cytoskeleton. It is either regulated by the signaling pathway involved or functions as a site for interaction to which signaling molecules are recruited.

Future directions

Proteome analysis using 2DE and MALDI-TOF mass spectrometry is developing into a powerful technology, but it still has limitations when looking at the protein side of life. It is obvious that the 2D gels used for the studies described in **Chapters 4 and 5** can resolve and visualize only a limited number of protein isoforms and are highly biased for high abundant proteins. Looking at the "tip of the iceberg", we have been able to detect and identify androgen-regulated protein isoforms. However, the question is how many more did we miss, and is there a way to detect these missing pieces of the puzzle? Advances in proteomics technology have been described in **Chapter 3,** to indicate the possibilities of quantitation techniques and new types of mass spectrometers in combination with multidimensional sample separation methods in solution. Until current issues of sensitivity, accuracy, dynamic range, and data analysis have been solved in proteomics research, we will be unable to identify all components of a particular proteome. However, it can be predicted for the near future that proteomics will mature into a technology suitable for identifying more and more regulatory proteins in different biological systems [626]. Therefore, to investigate the role of androgens in either normal or defective male genital tract development, application of a combination of genome and proteome screening and eventually high-throughput functional protein analyses will allow identification of multiple interconnected networks.

Summary

Development of sex organs in both male and female embryos first requires formation of the so-called indifferent gonads and the indifferent genital tract. Once the indifferent stage has been established, the developmental program continues in either a male or a female direction, starting with differentiation of the gonads into testes or ovaries. Testicular androgens are essential for differentiation of the male genital tract into epididymis, vas deferens, and seminal vesicle, and absence of androgenic signals results in feminization of the genital tract. The action of androgens is exerted via the androgen receptor (AR), which is a transcription factor that belongs to the family of steroid hormone receptors. Upon ligand binding, the AR translocates to the nucleus, where it binds specific DNA sequences, so-called androgen-response elements, which are present in promoter and enhancer regions of androgen-regulated genes. Regulation of gene expression by androgens involves chromatin remodeling, interaction with co-factors, and assembly of the transcription initiation complex. During androgen-dependent male genital tract development in the fetus, AR is present in mesenchymal cells surrounding the epithelial ducts. Therefore, androgenic signals in mesenchymal cells must be transduced to epithelial cells via secreted paracrine factors. Some growth factors, such as KGF and FGF10, play an important role as paracrine factors, but do not appear to be regulated by androgens *in vivo*. Furthermore, it is still largely unknown which proteins are regulated by androgens in mesenchymal cells during fetal development. In **Chapter 1**, we have postutaled 4 questions that are being addressed in this thesis:

1. *Is it possible to develop a mouse fetal vas deferens cell line that can be used as a model system for studying androgen-regulated gene expression?*
2. *Can detailed functional analyses of a novel AR mutation provide information that can be correlated to an AIS phenotype?*
3. *Can changes in androgen-induced expression patterns of mouse fetal vas deferens cells (MFVD) be detected by 2-dimensional gel electrophoresis (2DE), and can androgen-regulated proteins be identified by mass spectrometry?*
4. *Do androgen-regulated proteins as detected in cultured MFVD cells play a part in the androgenic signaling pathway of a whole organ?*

Many different mutations, deletions, or insertions in the AR gene result in defective androgen binding or a defective action of the AR, which forms the basis of the androgen insensitivity syndrome. Individuals with AIS show a wide spectrum of phenotypes, depending on the severity of the AR defect. In **Chapter 2**, the effect of the Q902K mutation on AR transcription activation, co-activator association, and intramolecular interaction potential was studied. It could be demonstrated that residue Q902 is only partially involved in AR transcription activation, and plays a more pronounced role in co-activator TIF2 association and in intramolecular interaction of the AR NH2-and COOH-termini. From this study it is concluded that the severity of disturbance of intermolecular interactions of AR with co-activator TIF2 and also AR intramolecular N/C interaction is indicative for the severity of AIS phenotypes.

Gene regulation by transcription factors, such as the AR, precedes regulation of protein expression. Proteins are the effector molecules of gene expression in the cell, and can be further regulated at the post-translational level. **Chapter 3** describes in which way great efforts in genomic research have resulted in the emergence of the field of proteomics.

The proteome is defined as the total protein complement of the genome, and it is estimated that a cellular proteome contains tens- to hundreds- of thousands of proteins. Proteomics is a field that combines classical protein separation techniques, such as 2-dimensional gel electrophoresis and liquid chromatography, with state-of-the art mass spectrometry and bioinformatics for protein identification. Proteomics as a technology is now becoming widely used for applications in a large variety of fields ranging from the biology of embryonic development to studies on human disease. In **Chapter 3**, some examples are given of applications in endocrinological research, and future developments are discussed.

To identify proteins that are regulated by androgens during genital tract development, a proteomic approach of 2DE and MALDI-TOF mass spectrometry was chosen. In **Chapter 4,** the development of a mouse fetal vas deferens cell line, MFVD, is described, which is an androgen-responsive target cell line of mesenchymal origin. In this cell line, two proteins, mElfin and CBF-A were found to be regulated by androgens at the post-translational level. It is suggested that mElfin and CBF-A are phosphorylated upon androgen treatment of MFVD cells. Since MFVD cells were derived from an isolated part of the genital tract, androgen-induced proteomic changes in whole organs were studied and described in **Chapter 5**. The differentiating epididymis in the fetus responds to androgens by heavily coiling of the epithelial duct and by overall organ growth. This response to androgens can be reproduced *in vitro* by culturing early stage epididymides in the presence of androgens. In this defined organ culture system, changes in proteome profiles can be ascribed to the direct and indirect effects of androgens. Androgen-induced regulation and post-translational modifications were observed for the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase, for heterogeneous nuclear ribonucleoproteins A3 and A2/B1, and for the signaling protein RhoGDI1. It is hypothesized that post-translational modification of RhoGDI1 and glycolytic enzymes in genital tract tissues may be important functional links between signaling pathways and cytoskeleton rearrangements, in control of growth and development.

The studies descibed in **Chapters 4** and **5** have two overlapping features. First, androgen-induced regulation involves post-translational modification of proteins. Second, androgen-regulated proteins in both MFVD cells and whole organs interact with the actin cytoskeleton or cytoskeleton-binding proteins. In **Chapter 6,** these findings are incorporated into a broader context. It is discussed that androgens exert an effect on cytoskeleton function in various androgen target tissues by regulating post-translational modifications of key proteins. Furthermore, it is suggested that these effects can be achieved by a transcription-mediated genomic effect, but may also involve a non-genomic mechanism.

Samenvatting

De ontwikkeling van geslachtsorganen in zowel mannelijke als vrouwelijke embryo's begint met de vorming van zogenoemde indifferente gonaden en geslachtsgangen. Nadat de indifferente weefsels zijn gevormd vervolgt de ontwikkeling zich in ofwel een mannelijke ofwel een vrouwelijke richting, beginnend met de differentiatie van de gonaden tot ovaria of testikels. Testiculaire androgenen zijn essentieel voor de differentiatie van de mannelijke geslachtsgang tot de bijbal (epididymis), de zaadleider (vas deferens) en de zaadblaas (seminal vesicle), en afwezigheid van een androgeensignaal zorgt voor het vervrouwelijken van de geslachtsgangen. Het effect van androgenen wordt bewerkstelligd door de androgeen receptor (AR). De AR is een transcriptiefactor die tot de familie van steroidhormoonreceptoren behoort. Nadat de AR het androgeen heeft gebonden, verplaatst het zich naar de celkern waar het aan specifieke DNA sequenties bindt. Dit zijn zogenoemde androgeen responsieve elementen die zich in promotor en enhancer regionen van androgeen-gereguleerde genen bevinden. Bij de regulatie van gen expressie door androgenen wordt het chromatine gemodificeerd, vinden er interacties met co-factoren plaats en wordt het transcriptie initiatie complex geassambleerd. Tijdens de androgeen-afhankelijke ontwikkeling van de mannelijke geslachtsgangen in de foetus is het AR eiwit aanwezig in mesenchymale cellen die de epitheliale gangen omringen. Epitheliale AR expressie is echter pas detecteerbaar nadat het androgeen doelwitweefsel is begonnen zich te ontwikkelen. Daarom zullen androgene signalen vanuit het mesenchym moeten worden overgedragen naar epitheliale cellen via zogenoemde paracriene factoren. Sommige groeifactoren, zoals KGF en FGF10, zijn belangrijke paracriene factoren, maar blijken *in vivo* niet te worden gereguleerd door androgenen. Verder is het nog grotendeels onbekend welke eiwitten worden gereguleerd door androgenen in mesenchymale cellen tijdens de foetale ontwikkeling. In **Hoofdstuk 1** hebben we een viertal vragen gepostuleerd die in dit proefschrift worden behandeld.

1. *Is het mogelijk om een muize vas deferens cellijn te ontwikkelen en deze te gebruiken als modelsysteem om androgeen-gereguleerde genexpressie te bestuderen?*
2. *Kunnen gedetailleerde functionele analyses aan een nieuwe AR mutatie informatie verschaffen die gecorreleerd kunnen worden aan een AIS fenotype?*
3. *Kunnen androgeen-geïnduceerde veranderingen in expressiepatronen van vas deferens cellen van de foetale muis (MFVD) gedetecteerd worden met behulp van 2-dimensionale gel electroforese, en kunnen androgeen-gereguleerde eiwitten geïdentificeerd worden met behulp van massa spectrometrie?*
4. *Spelen androgeen-gereguleerde eiwitten die zijn gedetecteerd in gekweekte MFVD cellen een rol in androgeen signaleringsmechanismen van een orgaan?*

Vele verschillende mutaties, deleties en inserties in het AR gen resulteren in defecte androgeen binding of een defect androgeen werkingsmechanisme, hetgeen de basis vormt van het androgeen ongevoeligheidssyndroom (AIS). In **Hoofdstuk 2** is het effect bestudeerd van de Q902K mutatie op AR transcriptie activatie, co-activator associatie, en intramoleculaire interactie potentieel. Er is aangetoond dat aminozuur Q902 slechts gedeeltelijk betrokken is bij AR transcriptie activatie. Echter, het speelt een prominentere rol bij co-activator TIF2 associatie en intramoleculaire interacties tussen de NH2- en COOH uiteinden van de AR. Er kan geconcludeerd worden dat de mate van verstoring van inter-moleculaire interacties van de AR met co-activator TIF2 en intra-moleculaire interacties van de NH2- en COOH uiteinden van de AR een indicatie is voor de ernst van het fenotype in AIS patienten.

Genregulatie door transcriptiefactoren, zoals de AR, gaat vooraf aan regulatie van eiwit expressie. Eiwitten zijn de uitvoerende moleculen van een cel, en kunnen verder gereguleerd worden op post-translatie niveau. In **Hoofdstuk 3** wordt beschreven hoe grootschalig genoomonderzoek ertoe heeft geleid dat het proteomicsonderzoek van de grond is gekomen. De definitie van het woord proteoom wordt gegeven als het totale eiwit complement van het genoom, en een cellulair proteoom bestaat uit naar schatting 10 tot 100 duizenden eiwitten. Proteomics is een onderzoeksgebied dat klassieke eiwit scheidingstechnieken, zoals 2DE en vloeistof chromatografie, combineert met moderne massa-spectrometrie en bioinformatica om eiwitten te identificeren. Als techniek wordt proteomics steeds vaker toegepast in zeer uiteenlopende onderzoeksgebieden, van ontwikkelingsbiologie tot studies aan humane ziektes. In **Hoofdstuk 3** worden enkele voorbeelden gegeven van toepassingen in endocrinologisch onderzoek en worden toekomstperspectieven bediscussiëerd.

Om eiwitten te identificeren die worden gereguleerd door androgenen tijdens de ontwikkeling van de geslachtsgangen, werd gekozen voor een proteomics toepassing van 2DE en MALDI-TOF massa spectrometrie. In **Hoofdstuk 4** wordt de ontwikkeling van een cellijn van MFVD beschreven. Deze cellijn is een androgeen responsieve doelwitcellijn van mesenchymale afkomst. Er werd gevonden dat mElfin en CBF-A worden gereguleerd door androgenen op post-translationeel niveau. Er wordt gesuggereerd dat mElfin en CBF-A worden gefosforyleerd wanneer MFVD cellen worden gestimuleerd met androgenen. Aangezien MFVD cellen afkomstig zijn van een geïsoleerd stukje van de geslachtsgang, hebben we ook hele organen in kweek gebracht om androgeen-geïnduceerde veranderingen in expressiepatronen te bestuderen. Deze resultaten zijn beschreven in **Hoofdstuk 5**. Het effect van androgenen op de zich ontwikkelende epididymis van de foetus uit zich in de groei van het orgaan maar ook in het ontstaan van een kronkelende structuur van de epitheliale gang. Dit effect van androgenen kan *in vitro* nagebootst worden door middel van orgaankweek van de epididymis in aanwezigheid van androgenen. In dit gedefiniëerd orgaankweek systeem zijn veranderingen in eiwitexpressiepatronen geheel toe te schrijven aan directe of indirecte effecten van androgenen. In dit systeem worden de glycolytische enzymen glyceraldehyde 3-fosfaat dehydrogenase en trifosfaat isomerase, de heterogene nucleaire ribonucleo-eiwitten A3 en A2/B1, en het signaaleiwit RhoGDI1 gereguleerd door androgenen op post-translatieoneel nievau. Er wordt gehypothetiseerd dat post-translatie modificatie van RhoGDI1 en glycolytische enzymen een belangrijke functionele link is tussen signaleringspaden en veranderende activiteit van het cytoskelet, die betrokken zijn bij regulatie van groei en ontwikkeling.

De studies die zijn beschreven in **Hoofdstuk 4** en **5** hebben twee overlappende kenmerken. Allereerst worden in MFVD cellen en epididymis weefsel eiwitten op post-translatie niveau gereguleerd door androgenen. Ten tweede hebben de androgeen-gereguleerde eiwiten in MFVD cellen en gekweekte epididymides gemeen dat ze interacteren met het actine cytoskelet of met cytoskelet bindende eiwitten. In **Hoofdstuk 6** worden deze bevindingen in een bredere context geplaatst. Er wordt bediscussiëerd dat androgenen een effect uitoefenen op het cytoskelet door post-translationele modificaties van belangrijke eiwitten te reguleren in verschillende androgeen doelwitweefsels. Verder wordt er gesuggereerd dat deze effecten bereikt kunnen worden door transcriptie-gemediëerde genomische effecten van androgenen, maar ook niet-genomische mechanismen kunnen omvatten.

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Sevgili anneciğim ve babaciğim, sizlerin sayesinde bu basamağa da ulaşabildim. Anneciğim, herzaman okuyup adam olmamı istedin ve bunu başarabilmek için senin örneğin benim için çok önemli. Babacığım, bu kitabı yazana kadar makalelerimi okuyup bana akıl fikir verdin. Yardımınız, anlayışınız ve sevginiz için çok teşekkürler. Sizleri çok seviyorum.

Lieve Robin, zonder jou was dit boekje er waarschijnlijk niet gekomen. Jouw advies en kritiek, als wetenschapper en vriend, hebben me net op tijd weer op de rails gezet. Bedankt voor je (letterlijk) grenzeloze steun en begrip en vooral vertrouwen in mij, die vaak doorgaat als het bij mij al is gestopt.

List of Publications

This thesis

Arzu Umar, Theo M. Luider, Cor A. Berrevoets, J. Anton Grootegoed, and Albert O. Brinkmann. Proteomic analysis of androgen-regulated protein expression in mouse fetal vas deferens cells.
Endocrinology 2003; **144**(4)**:**1147-1154.

Arzu Umar, Marja P. Ooms, Theo M. Luider, J. Anton Grootegoed and Albert O. Brinkmann. Proteomic profiling of epididymis and vas deferens: identification of proteins regulated during rat genital tract development.
Endocrinology 2003; **144**(10)**:**4637-4647.

Arzu Umar, Cor A. Berrevoets, N. Mai Van, Marije van Leeuwen, Micheal Verbiest, Wim J. Kleijer, Dennis Dooijes, J. Anton Grootegoed, Stenvert L.S. Drop, and Albert O. Brinkmann. Functional analysis of a novel androgen receptor mutation, Q902K, in a patient with partial androgen insensitivity.
Submitted for publication.

Arzu Umar, Theo M. Luider, J. Anton Grootegoed, Albert O. Brinkmann. Proteomics and applications in endocrinology.
Manuscript in preparation.

Other

Pettersson, A., T. Prinz, A. Umar, J. van der Biezen, and J. Tommassen. Molecular characterization of LbpB, the second lactoferrin receptor protein of *Neisseria meningitidis*. *Mol. Microbiol.* 1998; **27:**599-610.

Cor A. Berrevoets, Arzu Umar and Albert O. Brinkmann. Antiandrogens: selective androgen receptor modulators.
Mol.Cell Endocrinol. 2002; **198**(1-2)**:**97-103.

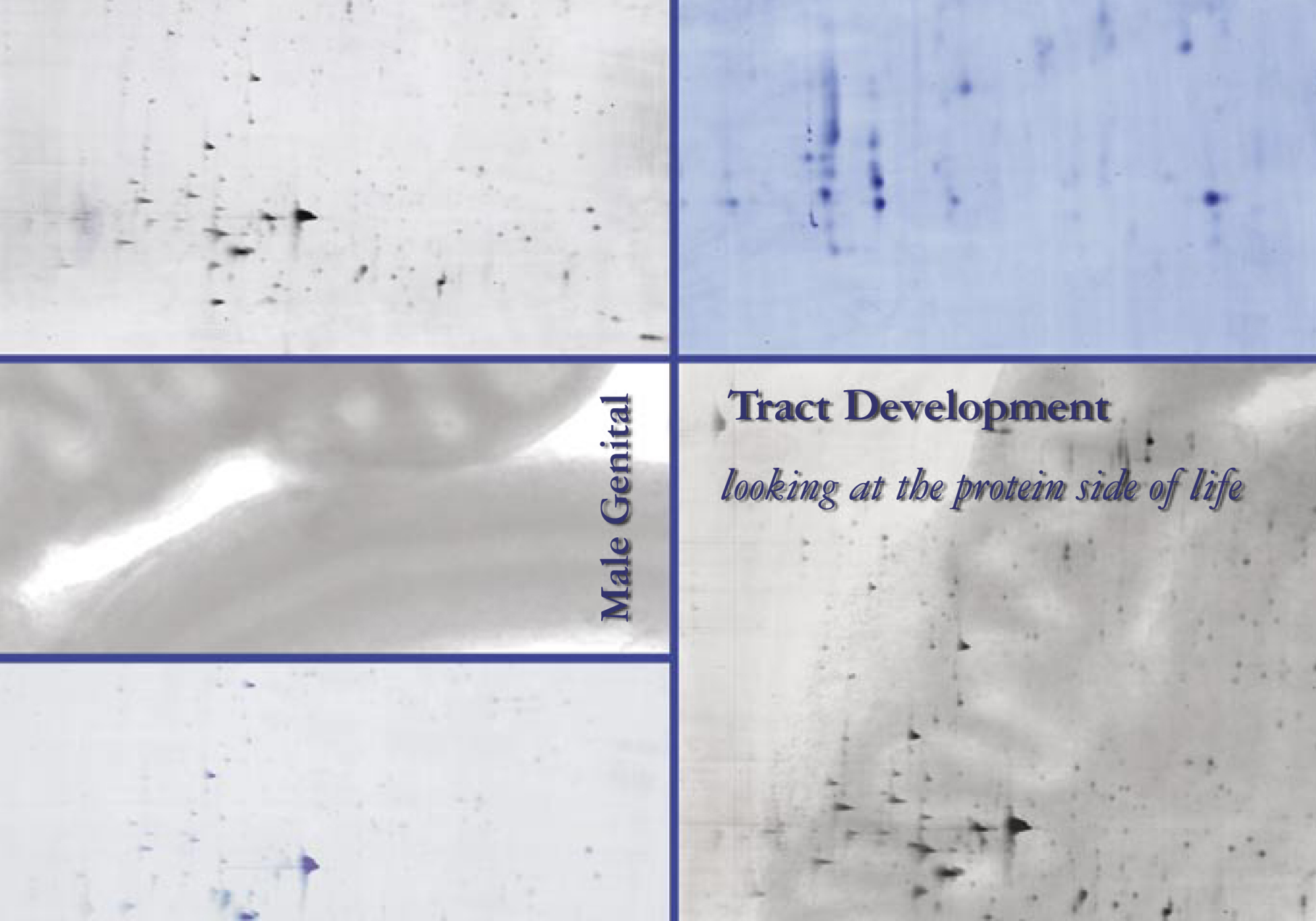
C. A. Berrevoets, A. Umar, J. Trapman, and A. O. Brinkmann. Differential modulation of androgen receptor transcriptional activity by the nuclear receptor corepressor (N-CoR).
Conditionally accepted for publication in J. Biochem.

Curriculum Vitae

Arzu Umar was born on September 2nd 1975 in Rotterdam. Her secondary education started in September 1987 at the Erasmiaans Gymnasium, from which she graduated in July 1993. In September of the same year she started her study Biology at the University of Utrecht. From January 1996 to April 1997, she followed her first doctoral stage at the department of Molecular Cell Biology at the University of Utrecht and the RIVM in Bilthoven, entitled "Molecular Characterisation of the *LbpB* gene of *Neisseria meningitidis*, strain BNCV" under the supervision of Dr. A. M. Pettersson and Prof. Dr. W. P. M. Hoestra. During that first stage she followed a three month's course in Immunology at the University of Lund as an Erasmus exchange student. Her second doctoral stage was from September 1997 to April 1998 at the department of Immunology of the Pharmaceutical company GlaxoWellcome (nowadays known as GlaxoSmithKline), Stevenage, UK, under the supervision of Dr. J. Turner. The Biology study was succesfully completed in June 1998.

From September 1998 to August 2003, she worked as a PhD student on the research project presented in this thesis at the department of Reproduction and Development (formerly known as Endocrinology and Reproduction), at the Erasmus MC Rotterdam, under the supervision of Dr. A. O. Brinkmann and Prof. Dr. J. A. Grootegoed.

The author is currently writing an application for a grant to start a collaboration with Prof. Dr. L. Pasa-Tolic at the PNNL in Richland, Washington, USA, Dr. D. J. Cahill at the University of Dublin, Dr. T. M. Luider at the Erasmus Center for Biomics and department of Neurology, and Dr. J. A. Foekens at department of Medical Oncology, Josephine Nefkens Institute Rotterdam, to work as a post-doctoral researcher. On August 22nd this year she married Robin Peeters.


Male Genital
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