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Androgen Signaling in Sertoli Cells

Lavinia Vija

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UNIVERSITE PARIS-SUD

ÉCOLE DOCTORALE :
Signalisation et Réseaux Intégratifs en
Biologie

Laboratoire Récepteurs Stéroïdiens, Physiopathologie Endocrinienne et Métabolique

Reproduction et Développement

THÈSE DE DOCTORAT

Soutenue le 09/07/2014
par

Lavinia Magdalena VIJA

SIGNALISATION ANDROGÉNIQUE DANS LES CELLULES DE SERTOLI

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Motto:

*“I am not searching for the answers,
I am just trying to understand the questions”*

Confucius

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Lavinia Vija, Kahina Boukari, Hugues Loosfelt, Geri Meduri, Say Viengchareun, Nadine Binart, Marc Lombès and Jacques Young*Mol Cell Endocrinol. 2014 Mar 25;384(1-2):32-42*

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Second part: Androgen receptor coregulators (SRC-2 and HBO1):
expression and characterization during testicular development

Paper2. Expression and characterization of androgen receptor coregulators, SRC-2 and HBO1, during human testis ontogenesis and in androgen signaling deficient patients.

Lavinia Vija, Geri Meduri, Eva Comperat, Viorel Vasiliu, Vincent Izard, Sophie Ferlicot, Kahina Boukari, Philippe Camparo, Say Viengchareun, Elisabeth Constancis, Constantin Dumitrache, Marc Lombès, Jacques Young. *Mol Cell Endocrinol.* 2013 Aug 15;375(1-2):140-8.

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Lavinia Vija, Sophie Ferlicot, Diana Paun, H  l  ne Bry-Gauillard, Gabriela Berdan, Issam Abd-Alsamad, Marc Lomb  s and Jacques Young *accepted in BioMedCentral Endocrine Disorders May 2014*

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List of aminoacids

A	Alanin	I	Isoleucine	R	Arginine
C	Cystein	K	Lysine	S	Serine
D	Aspartic acid	L	Leucine	T	Threonine
E	Glutamic acid	M	Methionine	V	Valine
F	Phenylalanine	N	Asparagine	W	Tryptophan
G	Glycin	P	Proline	Y	Tyrosine
H	Histidin	Q	Glutamine		

ABBREVIATIONS

ABP	Androgen binding protein
AMH	Anti Mullerian hormone
AR	Androgen receptor
5ARD	5 α -reductase type 2 deficiency
ARE	Androgen responsive elements
DBD	DNA-binding domain
DHH	Desert hedgehog gene
ER	Estrogen receptor
FSH	Follicle stimulating hormone
FSHR	FSH receptor
GnRH	Gonadotropin releasing hormone
HAT	Histone acetyl transferase
HBO1	Histone acetyltransferase binding to origin recognition complex
hCG	Human chorionic gonadotropin
INSL3	Insulin-like 3
LBD	Ligand binding domain
LH	Luteinizing hormone
LHR	LH receptor
LNCaP	Lymph node carcinoma of the prostate
NCoR	Nuclear hormone receptor corepressor
N-ter	Amino terminal
Pem	Placental embryonic oncofetal gene
PTM	Peritubular myoid cells
SF1	Steroidogenic factor 1
SGP-2	Sulphated-glycoprotein -2
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SRC-2	Steroid receptor coactivator-2
SRY	Sex determining region of Y
SOX9	Sry-like HMG box9
TAU-1,5	Transcription activation unit 1,5
TDF	Testis determining factor
mRNA	Messenger RNA
RNApolII	RNA polymerase II
3 β HSD	3 β -hydroxysteroid deshydrogenase
WAGR	Wilms' tumor, Aniridia, Genitourinary abnormalities and mental retardation
WT1	Wilms' tumor

Foreword

The androgen receptor (AR) is essential for the development and maintenance of the masculine phenotype and for spermatogenesis. Whether androgens are produced as early as around 7 gestational weeks, in accordance with the expansion of Sertoli cells number during the latter half of the first trimester and the beginning of the second trimester of gestation, Sertoli cells do not express AR during fetal development in both rodent and human. Moreover, AR expression in Sertoli cells exhibits a specific pattern during postnatal testicular development. During fetal life and in the neonate, during the “mini-puberty“period, the transient coexistence of high concentrations of androgens and AMH are related to physiological androgen insensitivity, explained by the absence of functional AR expression in SC during these developmental periods. At puberty, androgenic stimulation of a functional AR in Sertoli cells is associated with the entry in meiosis and spermatogenesis. Moreover, AMH expression significantly decreases in adulthood, in association with the androgenic stimulation of a functional AR present on Sertoli cells, as well as with the meiotic entry of spermatocytes.

The main objective of the thesis was the better comprehension of the physiological molecular mechanisms of AR regulation in Sertoli cells during murine and human ontogenesis.

The first part of this manuscript, represented by the Introduction, presents the scientific background, related to the expression and regulation of the androgen receptor (AR) and its molecular partners, during testicular developmental stages. The introduction is organized in five chapters. The first chapter presents the main factors contributing to prenatal testicular differentiation and development. The second chapter is meant to be a short overview about testicular development from birth to adulthood, presenting data related to the contribution of testicular cellular components to the main testicular functions: steroidogenesis and spermatogenesis. In the last part of this chapter I insisted on the hormonal regulation of spermatogenesis, in order to introduce the main contributions of the androgenic regulation. The third chapter shortly introduces general data related to nuclear receptors structure and classification, whereas the fourth chapter is related to the molecular aspects of androgens and the androgen receptor. The last chapter is dedicated to AR molecular partners, notably to the roles and specific expression of the AR coregulators in physiology, in specific animal models, as well as their known implications in human pathologies related to androgen regulation.

Foreword

The introductory part is followed by a general presentation of the objectives of the thesis project. The objectives are organized as a list of questions.

The results are organized in three parts and focus several aspects related to the androgenic regulation of Sertoli cells. Each part includes either the published manuscript related to the question announced in the Objectives area, or the manuscript submitted. Each paper is preceded by an introductory part including the objectives of the work and followed by additional data covering discussions about the limits and the potential perspectives related to the presented work.

The first paper describes the characteristics of a novel, immortalized murine Sertoli cell line, which was generated in the INSERM U693, the research unit where I developed my thesis project. This mature Sertoli cell line exhibits important endogenous AR expression, as well as androgenic transcriptional regulation and posttranslational stabilization. Moreover, we used this novel cellular model to test the hypothesis that in mature, postpubertal Sertoli cells, AMH extinction is directly regulated by the AR.

The second paper describes the expression and characterization of two AR coregulators (steroid receptor coactivator-2, SRC-2 and histone acetyltransferase binding to origin recognition complex, HBO1) during human testicular developmental stages and in patients with impaired androgen signaling, due to mutations in the AR (androgen insensitivity syndromes). We show for the first time the cellular distribution of these coregulators in human testis and we provide arguments related to their roles in the regulation of the AR transcriptional activity. In addition to the publication, the expression of these coregulators studied during murine ontogenesis and also in several other human pathologies related to impaired androgen production or infertility are also presented in the last part of this chapter.

The third part presents two additional papers that I sign as the first or the third author, in which different aspects of androgen regulation in Sertoli related to the contribution of testosterone or its reduced metabolite, dihydrotestosterone, in the regulation of spermatogenesis, or the contribution of both androgens and spermatogenesis to AMH extinction in mature seminiferous tubules, are presented, starting each one, from a case report. The first paper describes the histological and immunohistochemical characteristics of a patient

Foreword

with 5- α reductase type 2 deficiency, while the second paper describes the first healthy birth after testicular extraction of sperm and intracytoplasmic sperm injection from an azoospermic man with mild androgen insensitivity syndrome.

The last chapter, named General conclusions, summarizes the most important and original contributions of this work for the better understanding of human testicular physiology. The manuscript is finalized with a presentation of the future perspectives and research themes that might contribute to better understanding, diagnosis and treatment of male infertility.

Foreword

Chapter 1

Testicular differentiation and development

The process of human sex differentiation is complex and comports three sequential stages, as well as the combined action of hormones and cell signaling, allowing the formation of a male or female urogenital apparatus.

Distinct from the fact that genetic sex is determined during the fecundation of an X-chromosome bearing oocyte by an X or Y chromosome bearing spermatozoid, the gonads remain undifferentiated until the 7th week of gestation. It has been generally accepted that the presence of the Sry gene on the Y chromosome determines the gonads to become testis, while testicular hormones are important for the differentiation of internal and external male genital organs and for the development of the secondary sexual characteristics.

Factors involved in male sex determination

A.Undifferentiated state (bipotential gonad)

The bipotential gonadal ridge, develops at 4 gestational weeks in the human embryo as a thickening of the mesodermic mesonephros covered by the coelomic epithelium, after the division of the urogenital ridges (precursors of the urinary and adreno-gonadal ridge), followed by the division of the adreno gonadal ridges (common precursor of the adrenal and gonadal glands). Gonads are subsequently colonized by the primordial germ cells (of ectodermal origin) that have migrated from the dorsal part of the yolk sac wall near the base of the allantois, after having crossed the dorsal mesentery of the hindgut (Fig.1.). The mesonephros and the coelomic epithelium also give rise to Wolffian ducts (primordial for epididymes, vasa deferentia and seminal vesicles in the male fetus) and to Mullerian ducts (that give rise to Fallopian tubes, uterus and upper vagina in the female).

Biochemical, genetic and molecular experimental studies in mice and clinical observations in patients with disorders of sexual differentiation (DSD), allowed the identification in the XY embryo, of several factors that play essential roles in the differentiation of the gonads into a testis.

The bipotential gonad expresses genes such as WT1, SF1, DMRT1. Subsequently, GATA4/Fog2, WT1 and SF1 promote the expression of SRY in male.

Several factors such as PAX2, EMX2, LHX9 have been identified in the urogenital system of mouse embryos; however, no genitourinary defects or sex development defects have been identified in humans (Biaison-Lauber 2010).

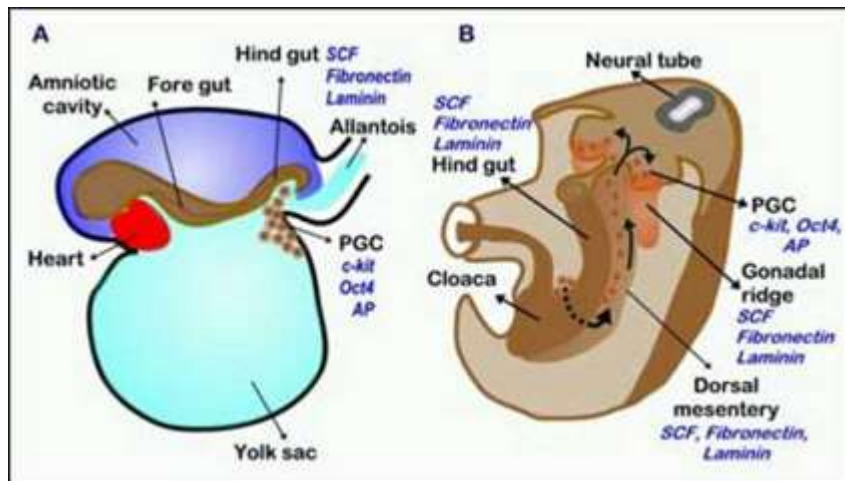


Fig.1. Migration of primordial germ cells (Rey, R, Josso N, 2009; Endotext)

WT1

The WT1 (Wilms tumour 1) gene encodes a zinc finger DNA-binding protein that plays important roles in development as well as tumour suppression. WT1 functions upstream of two orphan nuclear receptors: SF1 (steroidogenic factor 1) and DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, X chromosome). WT1 and SF1 have a simultaneous expression in the undifferentiated gonad and synergize to enhance transcription of AMH (Anti Mullerian Hormone) while DAX 1 represses this interaction (Nachtigal, Hirokawa et al. 1998; Park and Jameson 2005).

WT1 is expressed in the gonadal ridge and its expression is maintained throughout development to adulthood. Once the gonad has differentiated, WT1 is expressed in Sertoli cells.

Its invalidation in mice is lethal leading to absence of the gonads, kidneys and adrenal glands (Kreidberg, Sariola et al. 1993).

Mutations in the WT1 gene have been identified in patients with renal tumours (Wilms tumour), or isolated diffuse mesangial sclerosis, and complex tumours such as Wilms tumour, aniridia, genitor-urinary anomalies and mental retardation syndrome (WAGR syndrome), Frasier syndrome, Denys-Drash syndrome (DDS) or Meacham syndrome (Jeanpierre, Denamur et al. 1998; Royer-Pokora, Beier et al. 2004; Biason-Lauber 2010).

SF1

Human SF1 (*NR5A1*) (steroidogenic factor 1) is a protein belonging to the nuclear receptor superfamily, expressed in the developing adrenal gland and in the bipotential gonad (Ramayya, Zhou et al. 1997; Hanley, Ball et al. 1999). Its expression is maintained in the somatic cells of the differentiated testis where, together with SRY supports SOX9 expression (Sekido and Lovell-Badge 2008). In Sertoli cells, SF1 activates AMH expression at around 7-8 weeks of gestation, leading to the regression of the Mullerian ducts in the male fetus (Fig.1.2. and 1.3.). Starting from 8 weeks of gestation, SF1 activates the steroidogenic cascade in Leydig cells, leading to the androgenization of male external genitalia (Biason-Lauber 2010).

Deletion of *NR5A1* gene, encoding for SF1 in XY mice results in impaired adrenal development, testicular dysgenesis with persistence of Mullerian structures and female external genitalia (Lin and Achermann 2008).

SF1 dysfunctions related to heterozygous loss of function mutations in *NR5A1* or single nucleotide polymorphisms are associated with a wide range of reproductive phenotypes in humans ranging from complete testicular dysgenesis with Mullerian structures, through individuals with mild clitoromegaly/micropenis or genital ambiguity or cryptorchidism, to severe penoscrotal hypospadias or even anorchia (Lin and Achermann 2008).

DMRT1

Another human gene specifically expressed in the testis is DMRT1 (Doublesex and mab-3 related transcription factor 1). DMRT1 presents sequence similarity to genes that regulate the sexual development of nematodes and insects (Raymond, Shamu et al. 1998; Matson, Murphy et al. 2011).

Matson et al (Matson, Murphy et al. 2011) found that combined hemizygoty of DMRT1 and DMRT2, genes mapping on the 9p, is responsible for gonadal dysgenesis, with a pleiotropic spectrum, varying from streak gonad to hypoplastic testes.

DMRT1, expressed in murine Sertoli and germ cells, contributes to male determination by repressing multiple female-promoting genes (such as FOXL2) and activating male-promoting genes. Matson et al. demonstrated that deletion of DMRT1 during fetal development induces postnatal feminization of the testis, causing male-to-female primary sex reversal. Moreover, deletion of DMRT1 in adults can re-program differentiated Sertoli cells into apparent granulosa cells. DMRT1 mutant mice are born male, but present with anomalies in Sertoli cells and lack germ cells, suggesting that this gene is not essential for the gonadal differentiation (Raymond, Murphy et al. 2000). In humans, deletions of the long arm of chromosome 9, removing DMRT1, are associated with XY male-to-female sex reversal.

GATA4

GATA4, a member of the GATA family of transcription factors, is abundantly expressed in Sertoli cells throughout embryonic development (Biason-Lauber 2010).

GATA4 enhances AMH gene transcription through a direct interaction with the nuclear receptor SF-1 (Tremblay and Viger 1999). In 46, XY humans, GATA4 mutations were related to atrial septal heart defects and genital ambiguity (Biason-Lauber 2010).

B. Gonadal differentiation

Starting from the 7th week of gestation, in the XY embryo, a specific panel of factors regulates the differentiation process of the undifferentiated gonad into a testis. Genetic approaches on human sex-reversal and sexual dysgenesis syndromes as well as mouse gene knockout studies allowed the identification of sex-determining and gonad-formation genes, such as those encoding for the transcription factors *SRY*, *SOX9*, *SF1*, *DAX1*, *DMRT1* and cell-signaling molecules *AMH*, *WNT4* and *FGF9*.

SRY

In 1940, Alfred Jost performed elegant surgical experiments on rabbit embryos revealing that the ablation of the undifferentiated gonad leads to female phenotype (Jost 1947). On the other hand, sex determination was shown to be chromosomally controlled. On the basis of these two principles it was hypothesized that the Y chromosome must encode for a so-called testis-determining factor (TDF), comprising one or several genes on the Y chromosome. Moreover, molecular analysis related to the presence of the Y chromosome in several human pathologies, such as Klinefelter syndrome, which is caused by a sexual aneuploidy, demonstrated that Y chromosome was associated exclusively to a male phenotype (Nordqvist 1995). Initially named Testis Determining Factor (TDF), this factor then became SRY (Sex-Determining Region of Y), as Goodfellow's team revealed in 1989 that 46, XX males expressed a fragment of the Y chromosome (Palmer, Sinclair et al. 1989). Furthermore, Jager et al presented a case of 46, XY female with a frameshift mutation of the SRY gene (Jager, Anvret et al. 1990). SRY is the site of the majority mutations responsible for XY gonadal dysgenesis.

However, despite more than 20 years of research, little is known about SRY function and mechanism of action. As it is located on the Y chromosome, SRY is minimally conserved and vulnerable to degradation. It belongs to the HMG (high mobility group)-box containing family of nuclear transcription factors and contains a HMG DNA-binding motif, representing the only region conserved between species (Biaison-Lauber 2010).

Expressed only in the XY gonadal ridge, SRY is enhanced by WT1 and SF1, whereas an overexpression of WNT4 and DAX1 inhibits SRY action (Rey and Grinspon 2011).

Human and mouse SRY are expressed before the differentiation of the bipotential gonad into a testis. Moreover, SRY has been detected in both nucleus and cytoplasm of Sertoli cells in human fetal and neonatal testis as well as in spermatogenic cells from adult human testis (Modi, Shah et al. 2005).

SOX9

SOX9 (Sry-like HMG box9) is another candidate gene and a transcription factor, critical for testicular differentiation and chondrogenesis. Human SOX9 contains a HMG domain, capable of directing nuclear import and DNA bending and shares 70% amino acid homology to the

HMG box of SRY. In addition, the SOX9 protein contains additional protein domains, including two transcriptional activation domains, downstream of the HMG box. Unlike SRY, it is very highly conserved.

SOX9 is up regulated in the male gonad under the influence of SRY, driving Sertoli cell differentiation. If SRY is absent or inactive, SOX9 is silenced, leading to the development of the follicle cells and the ovary.

SOX9 has a cytoplasmic expression in both murine and human genital ridges, while its expression increases soon after SRY expression and becomes exclusively confined to the nucleus of Sertoli cells in the differentiated testis (Morais da Silva, Hacker et al. 1996; de Santa Barbara, Moniot et al. 2000). In female mice, SOX9 expression is absent in the differentiated ovary, while transcripts and weak cytoplasmic expression is still detected in human female after the gonadal differentiation (Morais da Silva, Hacker et al. 1996; de Santa Barbara, Moniot et al. 2000; Harley, Clarkson et al. 2003).

When sexually undifferentiated female gonads were cultured in the presence of Leptomycin B (an inhibitor of nuclear export) a sex reversal phenotype was observed, due to a potential role for NES (a SOX9 nuclear export sequence) driven nuclear export in the regulation of the sex-specific gene expression (Gasca, Canizares et al. 2002).

Ectopic expression of SOX9 is sufficient to induce testis differentiation in XX mice gonads, suggesting that SOX9 is required downstream of SRY to initiate the male pathway (Vidal, Chaboissier et al. 2001).

Moreover, transgenic mice with an insertional mutation, *odsex* (*ods*), lacking a gonad-specific regulatory element upstream to the SOX9 promoter, that mediates repression of SOX9, develop as sterile XX males lacking SRY and showing no skeletal defects (Bishop, Whitworth et al. 2000).

46 XY, SOX9 null mice develop gonads into ovaries (Kobayashi, Chang et al. 2005). Conditional SOX9 null mutants in Sertoli cells show normal embryonic testis development and are initially fertile, but become sterile with age from dysfunctional spermatogenesis. Moreover, SOX9 and SOX8 double nullizygous mice do not differentiate testis cords into seminiferous testis tubules and present upregulation of ovary-specific markers and downregulation of Sertoli intercellular junctions, highlighting that concerted SOX9 and SOX8 function in Sertoli cells is essential for the maintenance of testicular function (Barrionuevo, Georg et al. 2009).

In humans, SOX9 mutations are responsible for campomelic dysplasia characterized by male to female sex reversal in about 75% of cases, and by a skeletal malformation syndrome characterized by shortness and bowing of the long bones and typical facial appearance that includes hypertelorism, depressed nasal bridge, flat face, low-set ears, micrognathia and macrocephaly (Foster, Dominguez-Steglich et al. 1994; Moog, Jansen et al. 2001).

DAX1

DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; also known as NROB1, nuclear receptor subfamily 0, group B, member 1) encodes for a member of the orphan nuclear receptor family of transcriptional regulators that is expressed in steroidogenic tissues (gonads, adrenals), the ventromedial hypothalamus (VMH), and pituitary (Jadhav, Harris et al. 2011). DAX1 interacts with a series of corepressors, of which some are expressed in human testis (Biaison-Lauber 2010). DAX1 represses SF1 transcriptional activation of steroidogenic genes; it interacts with and represses estrogen-receptor target reporter genes. Several DAX1 mutations cause not only adrenal insufficiency but also hypogonadotropic hypogonadism (Jadhav, Harris et al. 2011), consistent with DAX1 expression in both hypothalamus and pituitary, suggesting a common function for DAX1 as a repressor of SF1 in both adrenal and gonadal tissues.

Transgenic XY mice with DAX1 overexpression show delayed testicular development, while duplications of the X chromosome containing DAX1 cause dosage sensitive sex-reversal (Bardoni, Zanaria et al. 1994; Muscatelli, Strom et al. 1994).

AMH

Also known as Müllerian inhibiting substance (MIS), the Anti Müllerian Hormone (AMH) is a member of the transforming growth factor family. AMH, produced by fetal Sertoli cells, is responsible for regression of the Müllerian duct, starting from the 8th week of pregnancy (Fig.2.). SOX9, SF1, WT1 and GATA4 are transcription factors which contribute to the anti-Müllerian hormone production by Sertoli cells during fetal life (Fig.3.). Mutations of the *AMH* and AMH receptor type II (*AMHR-II*) genes determine persistence of the uterus and

Fallopian tubes in male, syndrome called PMDS (persistent Müllerian duct syndrome)(de Santa Barbara, Moniot et al. 2000).

FGF9 and WNT4

FGF9 and WNT4 act in an antagonistic way to regulate sexual determination (Kim, Kobayashi et al. 2006). FGF9 is a fibroblast growth factor, expressed in seminiferous ducts, involved in testicular development, as XY mice lacking FGF9 develop as phenotypic females showing defects in testis cord formation and organization as a consequence of reduced and abnormal Sertoli cells and/or reduced migration and proliferation of interstitial cells (Colvin, Green et al. 2001).

WNT4 is a member of the WNT family of glycoproteins involved in developmental changes (Vainio, Heikkila et al. 1999). WNT4 is expressed in mesenchymal tissue surrounding Müllerian ducts. The bipotential gonad expresses both FGF9/WNT4. In the XY gonad, SRY stimulates SOX9 expression which favors FGF9 expression, while in the XX gonad, in the absence of the SRY, SOX9 is not anymore induced, and WNT4 participates in the ovary formation. WNT4 knock out female mice are masculinised, with few oocytes in their ovaries, suggesting a role of WNT4 in the fate of female germ cells.

Homozygous WNT4 mutations lead to female to male sex reversal in 46, XX fetuses with ambiguous genitalia, gonadal dysgenesis or to a normal testis, adrenal hypoplasia and pulmonary and cardiac anomalies, called SERKAL syndrome (Mandel, Shemer et al. 2008; Biason-Lauber 2010).

Epigenetic mechanisms involved in sexual differentiation

DNA methylation is a crucial element in the epigenetic regulation. Guo et al. (Guo, Zhu et al. 2014) revealed, in a recent publication in Nature, in July 2014, that in human embryos the major wave of genome-wide demethylation is complete at the 2-cell stage, and that, the demethylation of the paternal genome is faster than that of the maternal genome, so that, at the end of the zygotic stage the genome-wide methylation level in male pronuclei is lower than that in female pronuclei. These data suggest that testicular and Sertoli cell development as well as the disorders of sexual differentiation are not only explained by the detection of

genetic alterations on DNA sequencing, but also related to multiple complex alterations in gene methylation or demethylation, that cannot be easily depicted.

Events in male sexual differentiation	Gestational Weeks (GW)	Events in female sexual differentiation
Development of Wolffian ducts	4-5	
Migration of germ cells	5	Migration of germ cells
	6	Development of Mullerian ducts
Differentiation of seminiferous tubules	7	
Regression of Mullerian ducts Appearance of Leydig cells	8	
	9	First meiotic prophase in oogonia
Prostate and external genitalia begin Complete Mullerian ducts regression	10	Wolffian ducts regression
Fetal testes in the internal inguinal rings Seminal vesicles formation Penile urethra completed	12-14	First primordial follicles
	16	First primary ovarian follicles
Maximum of Leydig cells. Peak of testosterone	17	
Leydig cell regression	20	
	22-24	First multilayer (secondary) ovarian follicles Canalisation of the vagina
Inguinoscrotal phase of testicular descent	27-36	Cessation of oogonia multiplication

Fig.2. Main steps in gonadal differentiation during embryonic development. Data derived and adapted from (Sizonenko 1993; Biason-Lauber 2010). Green- phase of undifferentiated gonad; Blue-phases of differentiation of the male gonad; Red- phases of differentiation of the female gonad

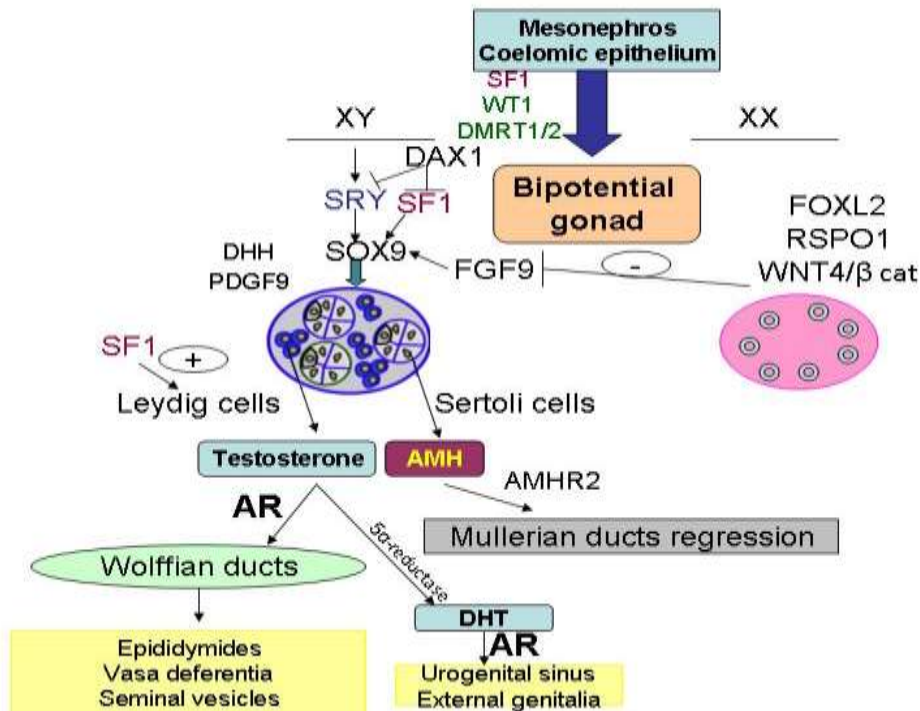


Fig.3. Stages and genetic regulation in normal male sexual differentiation.

Adapted from (Sekido and Lovell-Badge 2008; Biason-Lauber 2010; Rey and Grinspon 2011)

SF1, WT1 and DMRT1/2 are involved in gonadal ridge differentiation from the mesonephric mesoderm and the coelomic epithelium; SRY, positively regulated by WT1 and SF1, and antagonized by DAX1, directs gonadal differentiation to the male pathway, upregulating SOX9. Sertoli cells secrete AMH, responsible for regression of Mullerian ducts. AMH expression is triggered by SOX9 and enhanced by SF1 and WT1. Testosterone binds to the androgen receptor and promotes differentiation of Wolffian ducts into epididymides, vasa deferentia and seminal vesicles, while dihydrotestosterone drives differentiation of the urogenital sinus and external genitalia.

C.Hormonal control of the masculinization process

C.1.Introduction

Masculinization refers to the transformation of the indifferent fetus with a testis into a phenotypic male with internal and external male genitalia. The fetal testes are the most important endocrine regulators for male development and masculinization. Interestingly, in humans and most mammalian species, the endocrine role of the testis in the critical period of fetal masculinization is independent of, and occurs before the regulation by the hypothalamic-pituitary axis(O'Shaughnessy and Fowler 2011).

C.2.Fetal testis development

To summarize, under genetic control, as above mentioned (SRY, SOX9, FGF9), testes differentiate from the genital ridge at about 6 weeks of gestation in human (Fig.2.), and about 12 days post coitum (dpc) in mouse.

Primordial germ cells migrate along the dorsal mesentery and reach the gonadal ridge by 32-35 days, the majority reaching the seminiferous cords. Sertoli cells differentiate from precursors within the coelomic epithelium and migrate at the end of the sixth week, into the developing gonad, where they gather with the primordial germ cells. Sertoli cells begin to express AMH starting from the 8th week, which determines Mullerian duct regression, process completed by the end of the 9th week of gestation (Rey, Lukas-Croisier et al. 2003).

At 7-8 gestational weeks in human (and at 12.5 dpc in mouse), the seminiferous cords arise from the endothelial cells that migrate from the mesonephros towards the developing gonad and enclose Sertoli and germ cells (O'Shaughnessy and Fowler 2011).

At 6 weeks in human and 12.5dpc in mice, fetal Leydig cells start to differentiate from mesenchymal-like stem cells within the interstitial space between the cords (O'Shaughnessy, Baker et al. 2006), under the regulation of factors such as: desert hedgehog gene (DHH) (secreted by the Sertoli cells(Pierucci-Alves, Clark et al. 2001)), the PDGFR- α (Brennan, Tilmann et al. 2003) and the X-linked aristaless-related homeobox gene (Arx)(expressed in the peritubular myoid cells(Kitamura, Yanazawa et al. 2002)).

Peritubular myoid cells (PTM) start to develop at around 8 weeks of gestation in humans and 13 dpc in mice (O'Shaughnessy and Fowler 2011).

Finally, at the end of the 8th week of gestation, the human testis is organized in testicular cords (comprising somatic Sertoli cells and germ cells, surrounded by PTM) and interstitial tissue, containing somatic Leydig cells.

C.3.Fetal testicular hormonal milieu responsible for masculinization

The action of three testicular hormones (AMH, androgens, INSL3) secreted by the fetal testis is determinant for male internal and external genital differentiation.

Sertoli cells secrete AMH since the 8.5th week of gestation, stimulated by direct action of SOX9 and SF1 that acts via type II AMH receptors in the mesenchyme surrounding the Mullerian ducts epithelium, inducing their regression. AMH also might play a role in the maturation of the steroidogenic functions of the Leydig cell population in MIS-KO mice (Wu, Arumugam et al. 2005).

Androgens and INSL3 are predominantly produced by the fetal Leydig cells.

Main testicular androgens are testosterone (T) and dihydrotestosterone (DHT). 5 α -reductase type 2 converts testosterone to dihydrotestosterone in the prostate and external genitalia; moreover, DHT harbors the capacity for activating androgen receptor (AR) regulated genes of almost ten-times of that of T.

Testosterone can be detected in human testis at around 7 gestational weeks, fetal plasma concentration increasing in the early second trimester (11-13 wks), followed by a decline in the late second trimester (17-19 wks). Testosterone expression in fetal testis is in accordance with the expansion of Sertoli cells number during the latter half of the first trimester and the beginning of the second trimester of gestation (O'Shaughnessy, Baker et al. 2007).

Testosterone and dihydrotestosterone are also maintaining the Wolffian ducts and act on the external genitalia to induce formation of a penis and a scrotum.

INSL3 is, along with testosterone, a major product of human prenatal, neonatal and adult testicular Leydig cells.

INSL3 is responsible for the development of the gubernaculum testis, necessary for the testicular descent into the scrotum, process mandatory for normal spermatogenesis and fertility in adulthood. INSL3 knockout mice present with disruption in the abdominal phase of testicular descent (Zimmermann, Steding et al. 1999). In addition to its crucial function in testicular descent, INSL3 is suggested to play a paracrine role in germ cell survival and an endocrine role in bone metabolism. Like testosterone, INSL3 protein and transcript are

present in the human male fetus during the mid and late second trimester (Anand-Ivell, Ivell et al. 2008; Bay, Cohen et al. 2008), followed by an early postnatal peak and increasing secretion during puberty, resulting in high adult serum levels. INSL3 production is entirely dependent on the state of Leydig cell differentiation, and is stimulated by luteinizing hormone (LH). According to animal studies, fetal INSL3 production is, directly or indirectly, sensitive to estrogenic or androgenic compounds, as the gubernaculum testis presents both ER and AR; while androgens regulate growth of the gubernaculum and the inguinal phase of testicular descent, high estrogen exposure in utero can affect the gubernaculum development and suppress INSL3 production by the fetal cells (Emmen, McLuskey et al. 2000; Bay and Andersson 2011)

C.4. Endocrine regulation of the fetal testis major cell types

While in the post pubertal male, normal Sertoli and Leydig cell functions and spermatogenesis are critically dependent on pituitary gonadotropins (FSH and LH), in the fetus, the role of pituitary hormones is less clear and limited to the latter part of gestation (O'Shaughnessy and Fowler 2011). Placental gonadotropin is critical for normal fetal testicular development in both non-human primates and in human (O'Shaughnessy, Baker et al. 2006; O'Shaughnessy, Baker et al. 2007; Scott, Mason et al. 2009).

a) Leydig cells and testosterone production in human fetal testis

Androgens produced by fetal Leydig cells are essential for the fetal masculinization during a period, called “masculinization programming window”, located between 15 to 17 dpc in the rat and between 8 to 12 gestational weeks in the human (Scott, Mason et al. 2009), period when the hypothalamic-pituitary-gonadal axis is not yet functional, suggesting that gonadotropins, such as LH, are not necessary for fetal Leydig cell functions.

This hypothesis was confirmed by mouse models, as the hpg mouse (hypogonadal mouse, which harbors a deletion truncating the GnRH gene (Mason, Hayflick et al. 1986)), but exhibits normal circulating androgen levels at the end of gestation, despite a near-total deficiency of LH values (O'Shaughnessy, Baker et al. 1998), as well as by transgenic mouse models lacking the LH receptor or the LH β subunit, which present with a normal testicular

phenotype at birth despite failure of post-natal Leydig cell development(Zhang, Pakarainen et al. 2004).

The murine testicular steroidogenesis independency from choriogonadotrophins and LH during the period of masculinization of the reproductive tract is contrasted by the crucial dependence on still poorly understood paracrine regulators (such as PDGFA, IGF 1, NAP, and members of the TGF β family (O'Shaughnessy and Fowler 2011)).

In humans, the fetal Leydig cells also go through an early and short phase of independence from hormonal stimulation (6-7 wks of gestation), rapidly followed by the hormonal regulation of chorionic gonadotropin (hCG), which acts through the LH receptor (LHR).

Testosterone production is also independent of GnRH secretion, as this commences in the second trimester of pregnancy, after the “masculinization programming window”, explaining why GnRH mutations do not lead to severe masculinization impairment, but only to hypogonadotrophic hypogonadism with mild signs at birth, such as small penis or cryptorchidism (Young 2012).

The hCG/LH-independent testosterone production at early fetal ages (7 to 10 weeks) is still poorly understood. Rouiller-Fabre's team shown that 7 gestational wk human fetal testis explants do not require hCG/LH stimulation in vitro to maintain testosterone production and that retinoid acid increases testosterone secretion only at this age and not afterwards, suggesting that paracrine factors, such as retinoid acid, might regulate fetal steroidogenesis at this developmental period(Lambrot, Coffigny et al. 2006).

In humans, hCG production peaks between 8 and 12 weeks of gestation paralleling fetal androgen levels, consistent with a primary Leydig cell dependence on hCG. At 12 weeks of gestation, as hCG levels decline, fetal LH begins to rise and subsequently becomes important in maintaining Leydig cell function(Scott, Mason et al. 2009).

Evidence for both the hormone-independent and hCG-dependent phases comes from human individuals carrying inactivating mutations in genes encoding the LH receptor or LH β subunit. In rare inactivating mutations of the *LHR* gene, XY individuals have an external female phenotype, while maintaining epididymis and ductus deferens, confirming a short period of testosterone production independent of LH stimulation, followed by loss of

testicular androgen production during the critical period of sexual differentiation(Wu and Chan 1999). In contrast, in patients with mutations in the *LHB* gene (at present there are 6 *LHB* gene mutations identified in both men and women(Scott, Mason et al. 2009; Basciani, Watanabe et al. 2012)), fetal masculinization appears normal (presumably maintained by hCG) while post-natal masculine development fails in men (Fig.4.).

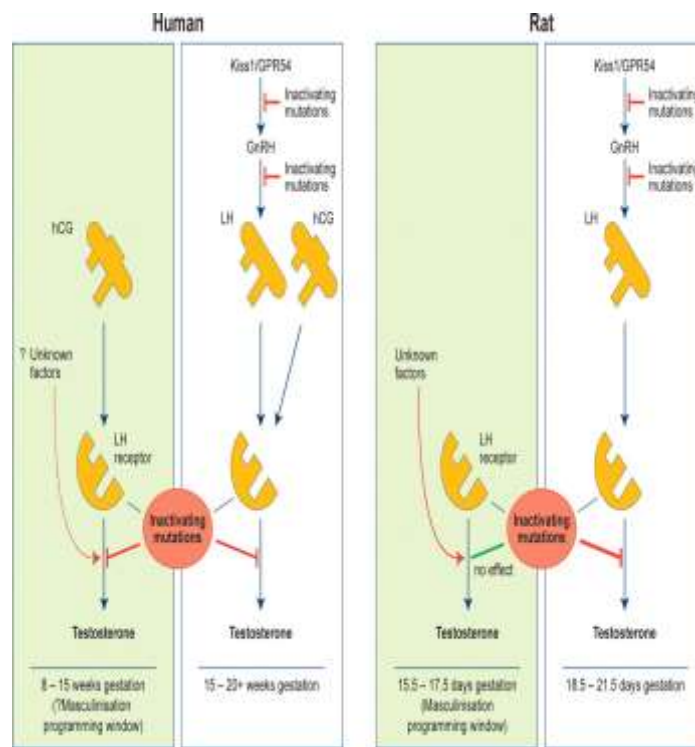


Fig.4. Regulation of testosterone production in the human male fetus. Impact of inactivating mutations in genes encoding main actors involved in the hypothalamic-pituitary-testicular pathway of regulation (reproduced from (Scott, Mason et al. 2009)).

b) Fetal Sertoli cells

Fetal Sertoli cells are essential for the initial differentiation and development of the human testis, inducing differentiation of the fetal Leydig cell population and maintaining the gonocytes and the early spermatogonia populations through expression of different factors, such as Steel factor (kit ligand), glial-derived neurotrophic factor (GDNF), etc(Payne, Gallagher et al. 2010). Sertoli cells also secrete AMH, which acts to prevent Müllerian duct development in the developing male(Rey, Lukas-Croisier et al. 2003).

Sertoli cell proliferation occurs throughout fetal and prepubertal development, and germ cell number and fertility in the adult animal are dependent upon the final numbers of adult Sertoli cells (Sharpe, McKinnell et al. 2003).

While in the adult, Sertoli activity is modulated by FSH and androgens, fetal Sertoli cell development and proliferation are regulated mainly by androgens; altogether, the role of FSH or other hormones such as thyroid hormones are still controversial. FSHR knockout (FSHRKO) mice have normal numbers of Sertoli cells at birth, expressing most markers of Sertoli cell activity (*Dhh*, *Pdgfra*, *Amh*). Androgens appear to play a significant role in the establishment of Sertoli cell number during fetal development, because androgen-insensitive testicular feminized (*Tfm*) mice (Goldstein and Wilson 1972) and androgen receptor-knockout (ARKO) mice (Tan, De Gendt et al. 2005) have a significant reduction in Sertoli cell number at birth (Johnston, Baker et al. 2004).

However, Sertoli cells do not express AR during fetal development in both rodent and human (Shapiro, Huang et al. 2005; Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009), and Sertoli cell numbers are normal at birth in transgenic mice that selectively lack AR in the Sertoli cells (SCARKO mouse (De Gendt, Swinnen et al. 2004)), suggesting that androgens might exert their function on fetal Sertoli cells via another testicular cell type. The intermediate might be the peritubular myoid cells (PTM) as immunohistochemical data suggest that PTM express AR in the fetal testis. This hypothesis was also confirmed by the PTM-specific AR-knockout (PTM-ARKO) mice (Welsh, Saunders et al. 2009), which have impaired Sertoli cell function and develop a 86% reduction in germ cells in adulthood (Welsh, Saunders et al. 2009).

Follicle-stimulating hormone (FSH) is a regulator of Sertoli cell proliferation, as confirmed by FSH-R knockout mice, which have a diminished postnatal Sertoli cell population (Migrenne, Moreau et al. 2012). However, the cessation of neonatal Sertoli cell proliferation occurs despite continual FSH exposure. The role of FSH in fetal Sertoli cell development in human and other mammalian species is not certain. Fetal human anencephaly is associated with reduced testis size, related to reduction in both Sertoli cells but also with severe reduction of Leydig cells (Baker and Scrimgeour 1980).

Thyroid hormones seem to be important in regulating Sertoli cell proliferation, although at present data are not very consistent. Neonatal hypothyroidism in both rodents and human is

associated with increased testicular size and Sertoli cell population, suggesting that hypothyroidism prolongs Sertoli cell proliferation. The precise mechanisms responsible for these effects are still unknown although some mechanistic hypotheses have been proposed (Holsberger and Cooke 2005).

Chapter 2

Human testicular physiology: seminiferous tubules and spermatogenesis

2.1. Testicular development from birth to adulthood

Testicular postnatal structural development is related to its two main functions: steroidogenesis and spermatogenesis. In this respect, testicular structures could be divided in two main compartments: the interstitial tissue, containing Leydig cells, which are responsible for the testicular production of androgens and the seminiferous tubules, containing Sertoli and germ cells, which are peripherally delineated by peritubular myoid cells (Fig.5.A). Spermatogenesis is a complex cellular event consisting of all processes leading from germ cell populations development and proliferation during the pre- and postnatal periods to the formation of spermatids and spermatozooids in the postpubertal male (Fig.5.B).

This chapter aims at presenting the postnatal development of Sertoli, Leydig and germ cells, their involvement in spermatogenesis and steroidogenesis, the hormonal regulation of spermatogenesis, with a focus on Sertoli cells development and their main implications in human spermatogenesis.

2.1.1. Postnatal Sertoli cells

Sertoli cells play major roles in the support, nourishment and organization of germ cells, orchestrating the spermatogenesis.

Sertoli cell proliferation pattern is organized during two periods of life in all species; the first one occurs during fetal and neonatal life and the second during the peripubertal and pubertal period.

In rodents, both number and function of Sertoli cells are essential for the adult testis sperm production; murine Sertoli cells are mitotically active during the first two postnatal weeks with no significant change in Sertoli cell numbers occurring thereafter (Vergouwen, Jacobs et al. 1991).

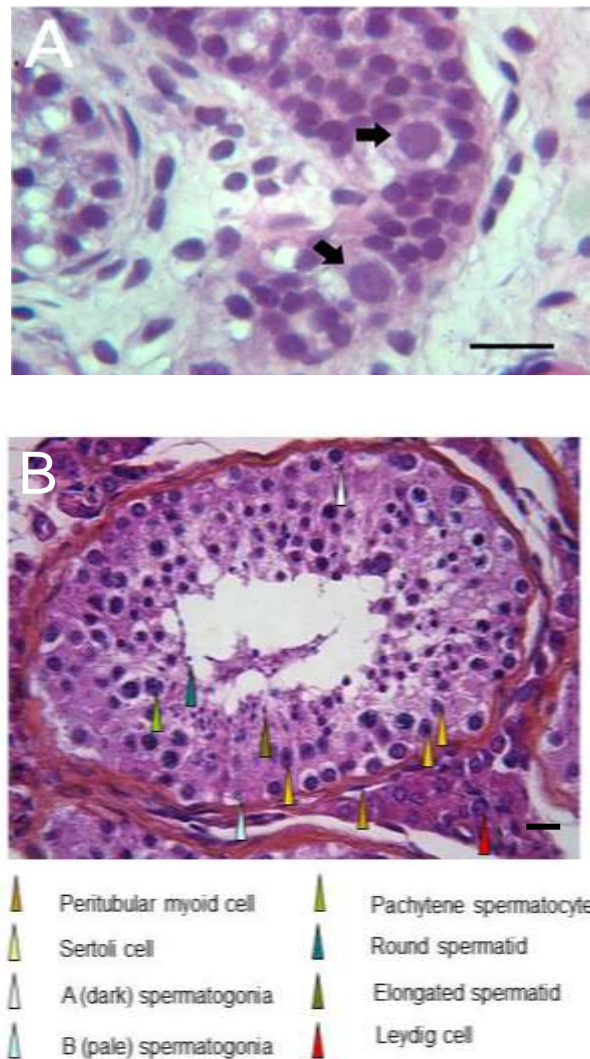


Fig.5. Human testicular sections. A. Newborn testis seminiferous tubules measure 60-65 μ m, have no lumina and contain germ cells (black arrows) and abundant Sertoli cells, with about 26 Sertoli cells per cross-section; B. Adult testis seminiferous tubules are larger (180-200 μ m in diameter) and contain Sertoli cells (yellow markers) (which can be recognized by their large central nucleoli), spermatogonia (basal cells with pale cytoplasm, round nuclei and eccentric nucleoli), spermatocytes and spermatids. Hematoxylin immunostaining. Scale bars- 50 μ m.

In humans, Sertoli cell proliferation was quantified by stereological methods by Cortes *et al*, who established two distinct periods of Sertoli cell proliferation. Sertoli cells proliferate during fetal life, process that continues postnatally and during infancy, while the second proliferation period starts at puberty, in association with the initiation of spermatogenesis(Cortes, Muller et al. 1987) (Fig.6.).

Sertoli cell proliferation is gonadotrophin dependent, being most rapid during the first year of postnatal life, when a sixfold increase in testicular volume is observed. Establishment of an adequate number of Sertoli cells during childhood is crucial, since each Sertoli cell can only sustain a definite number of germ cells, Sertoli cell number determining the efficiency of spermatogenesis.

During neonatal and prepubertal periods, Sertoli cells exhibit immunohistochemical markers of immaturity, such as AMH immunoreactivity(Franke, Pauls et al. 2004); the androgen receptor (AR) is absent at birth, its nuclear expression being evidenced starting from around 4 years of life, increasing throughout adulthood(Chemes, Rey et al. 2008).

At puberty, FSH controls Sertoli cell proliferation, thereby strongly influencing the final number of Sertoli cells in adulthood. Both rodent and human Sertoli cells exhibit a plethora of morphological and functional features as they reach their mature, non-proliferative phenotype. This includes progressive reduction in anti-Müllerian hormone expression, emergence of androgen responsiveness, formation of the blood-testis barrier and polarized secretion, contributing to the formation of a seminiferous tubule with a lumen(Sharpe, McKinnell et al. 2003).

Mature Sertoli cells express AR and have a diminished expression of AMH(Boukari, Meduri et al. 2009); moreover, androgen stimulation of spermatogenesis requires direct androgen action on Sertoli cells(O'Shaughnessy, Verhoeven et al. 2010).

In addition, adult/mature Sertoli cells develop morphological changes (such as distinction of prominent nucleoli) and express adhesion molecules, such as connexin-43, as well as inter-Sertoli tight junction complexes, which represent the morphologic basis for the blood-testis barrier. The blood-testis barrier creates a specific microenvironment necessary for the ongoing spermatogenesis. A recent study, published in Science (Smith and Braun 2012), showed the organization of mature Sertoli cells tight junctions, such as zonula occludens 1, claudin 3 and claudin 11 and their roles in the constitution of the blood-testis-barrier and in spermatogenesis. Mature Sertoli cells synthesize proteins such as androgen-binding protein (ABP), sulfated glycoproteins (SGP), activin and inhibin (inhibin being an

important marker of spermatogenesis), important for the regulation of spermatogenesis, as well as several other proteins involved in the migration of differentiating germ cells (spermatocytes) towards the tubular lumen (such as metalloproteins, α 2-macroglobulin).

The number of Sertoli cells decreases with age, being positively correlated with daily sperm production (Nistal 2008).

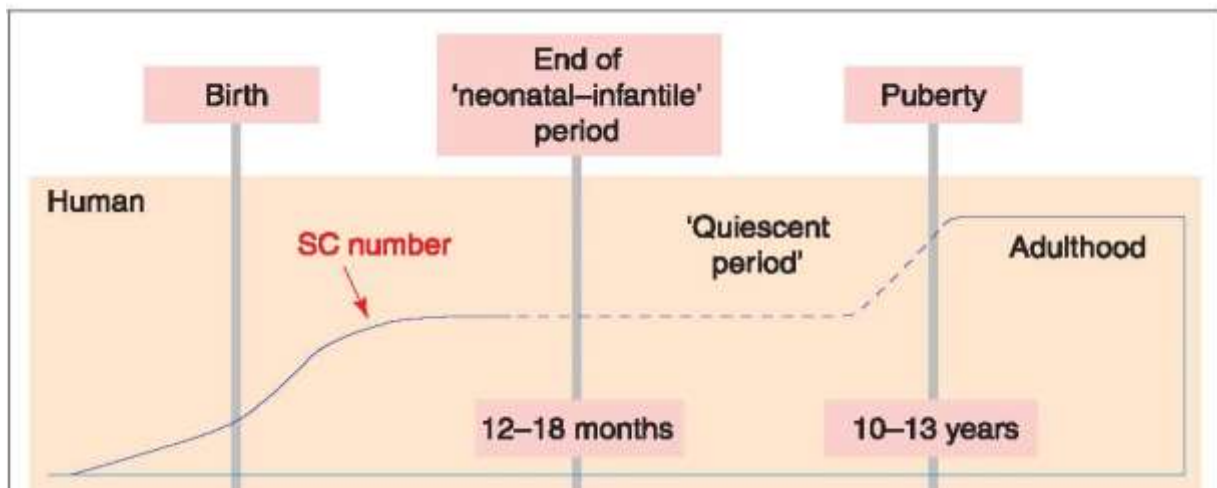


Fig.6. Sertoli cell proliferation in humans follows two periods: the first one starts during fetal life comprising the neonatal and infantile periods, while the second period of proliferation begins at puberty (reproduced from (Sharpe, McKinnell et al. 2003)).

2.1.2. Postnatal Leydig cells

In humans, Leydig cell development is triphasic, as fetal, neonatal and adult Leydig cells are distinguished, all of them representing the main source of testicular testosterone (Prince 2001). Fetal Leydig cells are still present at birth, but disappear by the age of 6 months, explaining the significant levels of serum and testicular testosterone present in neonate boys. A second proliferation period, reaching a peak at 2-3 months postnatally, comprises the neonatal Leydig cells, which contribute to testosterone synthesis under LH stimulation (Codesal, Regadera et al. 1990). At puberty, neonatal population would progressively be replaced by the adult Leydig cell population or the mature Leydig cells. During adulthood (between 20 and 60 years), Leydig cells maintain a steady expression, covering approximately 4% of the mature testicular volume (Shalet 2009).

Postnatal Leydig cell proliferation is regulated by LH/hCG, as suggested by the parallel profiles of Leydig cells proliferation and LH secretion. Conversely, in cases of precocious puberty related to mutations which constitutively activate LHreceptor, Leydig cells become hyperplastic and produce high levels of testosterone in the context of prepubertal levels of luteinizinghormone (LH)(Shenker, Laue et al. 1993).

Leydig cell activity is also regulated by IGF1 as adult mouse models invalidated for IGF1, present diminished numbers of Leydig cells and lower circulating testosterone levels (Wang and Hardy 2004).

AMH modulates the number and steroidogenic function of Leydig cells by regulating the expression of steroidogenic enzymes (Racine, Rey et al. 1998).

In conclusion, Leydig cell development and postnatal activity is strongly regulated by LH, different paracrine factors having also their contribution.

2.1.3. Leydig cells and steroidogenesis

The major circulating androgen is testosterone, which is synthesized from cholesterol in Leydig cells. The conversion of cholesterol to testosterone involves the transfer of cholesterol to the inner mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein and the side chain cleavage of cholesterol by the enzyme P450_{scc}. For final conversion to testosterone, subsequent reactions require enzymes such as 3 β -hydroxysteroid dehydrogenase (HSD17B6), 17 α -hydroxylase and 17 β -hydroxysteroid dehydrogenase type 3 (HSD17B3) (Scott, Mason et al. 2009).

The process of androgen biosynthesis requires the hormonal regulation by LH, which is recognized by its G-protein coupled membrane receptor on Leydig cells. Cholesterol is transferred to the inner layer of the mitochondrial membrane by the Steroidogenic Acute Regulatory Protein (StAR) (CYP11A). Leydig cells are the only cells in the testis that contain cytochrome P450 cholesterol side chain cleavage enzyme (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD), enzymes essential for the first two steps in testicular steroidogenesis, converting cholesterol to pregnenolone and pregnenolone to progesterone. Indeed, pregnenolone is produced in mitochondria, and then transferred to the endoplasmic reticulum, where it can be converted into 17-hydroxy-pregnenolone by the P450 17 α -hydroxylase on the Δ 5 Pathway, or takes the Δ 4 Pathway in which 3 β -hydroxysteroid dehydrogenase converts pregnenolone to progesterone (Fig.7.).

In humans, the $\Delta 5$ Pathway prevails the $\Delta 4$ Pathway; therefore, with the catalytic activities of P450 17α -hydroxylase/17, 20 lyase and 17β HSD type3, steroidogenesis proceeds to 17α -hydroxy-pregnenolone and DHEA (dehydroepiandrosterone) and then to androstendiol and under the enzymatic action of 3β HSD type III, to the ultimate product, testosterone.

Testosterone might be metabolized into 5α -dihydrotestosterone (by the 5α -reductase) (which would be detailed in the forthcoming chapters) or into estradiol (by the P450 aromatase).

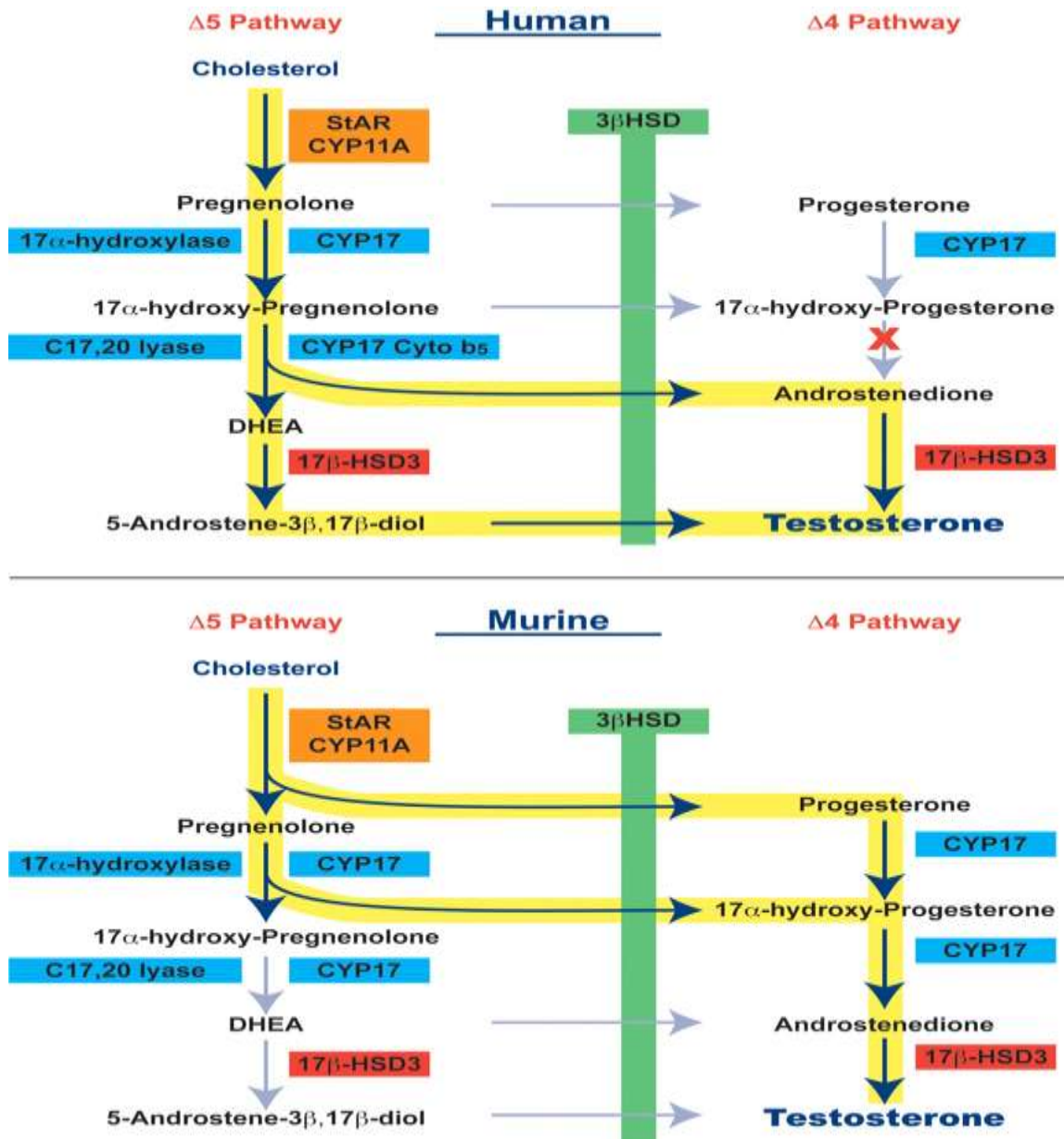


Fig.7. Human testicular steroidogenesis (reproduced from (Scott, Mason et al. 2009))

2.1.4. Postnatal germ cells and spermatogenesis

During fetal life, human germ cells could be divided into three stages of differentiation: gonocytes, intermediate cells, and fetal spermatogonia. At birth, only two types of germ cells are present: gonocytes and spermatogonia (similar to the adult type A spermatogonia)(Fukuda, Hedinger et al. 1975; Nistal 2008).The increased LH secretion and testosterone production during the third to the sixth months of life, stimulate germ cell proliferation and transformation of gonocytes to A dark and some A pale type spermatogonia. By the age of six months gonocytes have disappeared, coinciding with the loss of fetal germ cell markers (c-kit and placental alkaline phosphatase)(Gaskell, Esnal et al. 2004). From the sixth month to the third year of life, testis is in a resting period; however, a second wave of germ cell proliferation occurs before six years of age, despite no elevation of FSH or LH serum concentrations, while a third wave of spermatogenesis begins at nine years coinciding with LH elevation, process that continues during puberty and adulthood(Muller and Skakkebaek 1983).

In human seminiferous tubules, germ cells are surrounded by Sertoli cells and are lining the tunica propria, containing peritubular myoid cells (PTM). Sertoli cells are involved in the maintenance of spermatogenesis as well as in the migration of spermatocytes towards the tubular lumen.

To summarize, at puberty, the human spermatogenesis cycle, consisting of continuous and serial (with a mean duration of 74 days) processes of proliferation, meiosis, and differentiation, generates the transformation of spermatogonium into spermatozoon during an intertwining helical pattern, so that a single tubule cross section may have up to six identified stages represented initially by Clermont (Fig.5.B and 8).

At puberty, spermatogenesis starts with type A spermatogonia differentiation to type B spermatogonia, which in turn proliferate and then transform to preleptotene and then to pachytene spermatocytes in a process known as cell cycle progression(which takes 24 days). Diplotene spermatocytes enter meiosis to form haploid round spermatids. Spermatids undergo spermiogenesis to form elongated spermatids. Elongated spermatids transform into spermatozoa after the shredding of the residual body to be phagocytosed by the Sertoli cells to allow the release of sperm at spermiation.

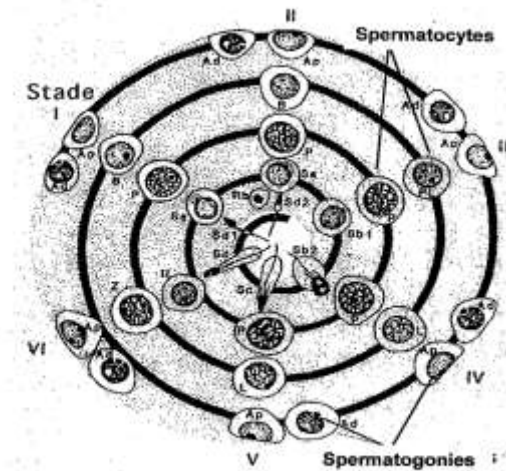


Fig.8. The 6 stages of spermatogenesis defined by Clermont. Germ cell development and spermatogenesis are organized in a centripete helicoidal pattern evolving from the basal peripheral layers of the seminiferous tubules towards the seminiferous tubule lumina (Image reproduced from Young J Régulation hormonale de la spermatogénèse: aspects physiologiques et thérapeutiques. *Méd Clin Endocrin Diab*, 2003, 6:53-61.)

2.2. Hormonal regulation of spermatogenesis

Advances in understanding the hormonal regulation of spermatogenesis (required for the management of male fertility and the potential development of reversible male hormonal contraception) were made possible thanks to several approaches. Studies of rat, monkey and human models of gonadotropin/androgen deprivation and/or replacement associated with the quantitative assessment of germ cell number using stereological techniques, gathering the immunohistochemical exploration of spermatogenesis allowed a better understanding of gonadotropins (FSH, LH) and steroid hormones (mainly androgens-testosterone and dihydrotestosterone, but also estrogens) involvement in human spermatogenesis. However, the majority of studies are based on selective knockout technologies in animal models, including rodents (mouse, rat), or primates (marmoset, etc). Human clinical trials bring up very interesting data, which are limited by the fact that the assessment of spermatogenesis is mainly indirect, by the analysis of the ejaculate samples, and rarely by the analysis of testicular biopsies.

Overall, the hypothalamus secretes gonadotrophin-releasing hormone, which stimulates the pituitary gland in a pulsatile manner to secrete and release luteinising hormone (LH) and follicle-stimulating hormone (FSH). FSH regulates Sertoli cell proliferation and main functions, while LH acts on Leydig cells to stimulate testosterone synthesis. Normal spermatogenesis requires functional somatic cells expressing the androgen receptor (Sertoli, Leydig and peritubular myoid cells), while germ cells express neither FSH receptor (FSHR), nor androgen receptors (AR). Spermatogenesis initiates at puberty, when the adult human testis overcomes the physiological hypogonadotropic hypogonadism (Chemes, Rey et al. 2008) to get to a eugonadal state.

2.2.1. Roles of FSH in spermatogenesis

In primates and humans, FSH acts via specific protein G-coupled receptors that are exclusively expressed in Sertoli cells and controls Sertoli cell proliferation during the perinatal and pubertal period, thus determining the testicular volume and the adult spermatogenic capacity (Sharpe, McKinnell et al. 2003). In prepubertal primates, FSH alone cannot induce spermatogenesis, germ cell differentiation being limited at the B spermatogonia stage (Plant and Marshall 2001).

In rodents, FSH plays a major role in the regulation of the spermatogonial population. Rats treated with FSH following the administration of GnRH, counterbalances the GnRH-induced reduction in the number of pachytene spermatocytes and spermatids and preserve spermatogenesis up to the state of pachytene spermatocytes (Hikim and Swerdloff 1995). Transgenic mouse models show that FSH supports early germ cell development, but is incapable of supporting full spermatogenesis in the absence of androgens or the androgen receptor. In mice lacking testicular AR expression (Tfm or ARKO), spermatogenesis is arrested, as germ cells do not progress from the early stages of meiosis (De Gendt, Swinnen et al. 2004). In contrast, mice lacking FSH or its receptor, although presenting a reduced germ cell number, are still fertile (Abel, Baban et al. 2009; De Gendt, McKinnell et al. 2009). Studies in rats lacking both FSH and androgen receptors in Sertoli cells (FSHRKO/SCARKO rats) reported that FSH and androgens have additional effects in the regulation of spermatogenesis (Abel, Baban et al. 2009; De Gendt, McKinnell et al. 2009). FSHRKO/SCARKO rats presented a marked reduction in the total number of germ cells and an arrest at the pachytene stage, although the total number of Sertoli cells was not

significantly different between FSHRKO versus FSHRKO/SCARKO. These data suggest an additive and synergistic way of both androgens and FSH on the regulation of meiosis and spermiogenesis.

In humans, FSH involvement in spermatogenesis was studied in patients with FSH- β subunit or FSHR inactivating mutations as well as in several clinical studies.

FSH- β subunit or FSHR inactivating mutations associate different phenotypes, as patients with inactivating mutations in the FSH- β present variable degrees of spermatogenic failure, but, surprisingly, do not show azoospermia or absolute infertility (Tapanainen, Aittomaki et al. 1997; Tapanainen, Vaskivuo et al. 1998); this might also be related to a probable, yet not assessed, residual activity of FSH in Sertoli cells (Tapanainen, Aittomaki et al. 1997). The concept that normal FSH action is not an absolute requirement for spermatogenesis is also supported by the FSH β knock-out mice (Kumar, Wang et al. 1997).

Clinical studies revealed that administering hCG after having suppressed gonadotropin secretion and decreased sperm production with androgenic treatment, increases the number of spermatozooids without restoring the normal levels (Bremner, Matsumoto et al. 1981). This process occurs despite FSH suppression. Other studies, such as Matsumoto's, revealed that normal spermatogenesis could have been re-established as early as after 3 months of FSH administration, in subjects with abolished spermatogenesis by an FSH-suppressing hCG treatment (Matsumoto, Karpas et al. 1983).

In patients with hypogonadotropic hypogonadism, FSH and androgen administration, not associated with hCG, fail to initiate or maintain spermatogenesis (Schaison, Young et al. 1993).

In conclusion, FSH exerts an important role in maintaining spermatogenesis; however, FSH alone is not sufficient to completely restore previously interrupted spermatogenesis.

2.2.2. Roles of androgens/LH in spermatogenesis

In order to clarify the contribution of testicular cells expressing AR (Sertoli cells, Leydig cells and peritubular myoid cells) on spermatogenesis, several research groups generated animal models, such as conditional knock-out AR mice in either Sertoli cells (SCARKO) or PTM cells, using the Cre recombinase/loxP system.

Interaction of androgens (testosterone and dihydrotestosterone) with a functional androgen receptor is essential for spermatogenesis, as in GnRH genetically deficient hypogonadal mice (hpg mice) as well as in immature, one year old monkeys (Marshall, Wickings et al. 1984), testosterone alone is sufficient to restore spermatogenesis (O'Shaughnessy, Verhoeven et al. 2010).

Moreover, the global ARKO mice, reproduce the spermatogenic arrest at meiosis of the tfm mice (which harbour a specific phenotype: female somatic morphology, cryptorchidism and spermatogenesis arrested at the level of spermatocytes) suggesting that AR is essential for the development of complete spermatogenesis and for the normal development and maturation of both Sertoli and Leydig cells.

Generation of different models of Sertoli cell AR knockout (SCARKO) mice, developed by several laboratories, brought more evidence that AR expression in Sertoli cells is mandatory for several Sertoli cell maturation features, involving cytoskeletal development and androgenic support of normal spermatogenesis, as well as the progression of germ cells through meiosis and spermatid maturation (Willems, Batlouni et al. 2010; De Gendt, Swinnen et al. 2004; Chang, Chen et al. 2004; Holdcraft and Braun 2004; Lim, Robson et al. 2009).

However, these mouse models prove that androgen action on Sertoli cells is not required for testicular development, Sertoli cell proliferation or initiation of spermatogenesis (Wang, Yeh et al. 2009; Walters, Simanainen et al. 2010; De Gendt and Verhoeven 2012).

Despite important technological and scientific progresses registered during the last 15 years in the field, the mechanisms by which androgen action on Sertoli cell support spermatogenesis are still not understood. For example, it was supposed that AR might regulate the production of androgens as a result of an autoregulatory loop on Leydig cells, but results offered by SCARKO models are divergent (Chang, Chen et al. 2004; De Gendt, Swinnen et al. 2004), suggesting that AR regulation involves various refined mechanisms.

In addition, AR expression in peritubular myoid cells might also be important for normal spermatogenesis, as fertility is impaired in PTM specific AR knockout mouse models. In one of the PTM invalidation mouse model, the mature mice presented oligozoospermia but normal fertility, whereas PTM cells exhibited defects in cells contractility; impaired expression by Sertoli cells of several markers of maturity such as tight junction proteins, connexin-43 was also noticed in this phenotype) (Zhang, Yeh et al. 2006). Another mouse model of AR invalidation in PTM cells associated with azoospermia and infertility, with small testes and

modifications in Sertoli cells abilities to express androgen regulated transcripts, such as (*Rhox5, Eppin, Tubb3*) (Welsh, Saunders et al.2009).

Spermatogenesis induction by testosterone, independently of the FSH action, was also assessed in patients presenting with LH-activating mutations as the cause of familial male limited gonadotropin-independent precocious puberty (FMPP) or testotoxicosis (Soriano-Guillen, Mitchell et al. 2006).

Moreover, male patients with mutations of the luteinizing hormone β -subunit gene (at present there are six identified mutations (Lofrano-Porto, Barra et al. 2007; Basciani, Watanabe et al. 2012), present with hypogonadism and azoospermia associated with an absence of circulating LH.

It is interesting to note that the concentration of testosterone in the rat testis is normally as much as fifty times as high as that in the serum (McLachlan, O'Donnell et al. 2002), while in humans, assessment of intratesticular testosterone concentration revealed by percutaneous aspiration, was found to be more than 100-fold higher than serum testosterone levels(Jarow and Zirkin 2005). Jarow and Zirkin also showed that low doses of hCG (125 units every other day) may maintain baseline levels of intratesticular testosterone concentration in men with gonadotropin withdrawal from exogenous testosterone administration (Coviello, Matsumoto et al. 2005). However, it is still not known how much testosterone is required within the human testis to maintain or to restore normal spermatogenesis.

Another important aspect related to androgenic regulation in spermatogenesis is related to the potentially differential contributions of testosterone (T) versus its metabolite, 5 α -dihydrotestosterone (DHT) to the maintenance of normal spermatogenesis. Performing elegant studies and measures of intratesticular androgens (T and DHT) in human adult fertile subjects, Jarow et al. (Jarow and Zirkin, 2005) revealed that intratesticular DHT represents 2% of intratesticular T. As testosterone bioactivity is 70% that of intratesticular concentration, would argument why, despite 10 fold more important bioactivity, intratesticular DHT concentrations is not significant. These results suggest that in normal fertile male adults, testosterone is the most important androgen, responsible for main testicular functions, such as maintenance of spermatogenesis. Administration of hCG in fertile adult subjects, after suppression of endogenous androgens by administration of testosterone enanthate, restored

intratesticular testosterone levels for doses of hCG of even 125 units every other day (Coviello et al., 2005).

2.2.3. Roles of estrogens in spermatogenesis

There are two forms of the estrogen receptors (ER): ER α and ER β . In the rodent testes, ER α is expressed in Leydig cells when the androgen receptor is not yet expressed, while ER β expression prevails during fetal life, suggesting a major role for ER β in the development and function of male reproductive structures until birth (O'Donnell, Robertson et al. 2001).

Invalidated mouse models present various fertility phenotypes: ER β KO mouse males are fully fertile; ER α KO males present spermatogenic failure associated with impaired efferent ductule function, while aromatase KO present an age-related disruption to spermiogenesis and variable degrees of infertility (O'Donnell, Robertson et al. 2001; Carreau, Bouraima-Lelong et al. 2012). In rodents, estrogens induce proliferation of rat testis gonocytes (Li, Papadopoulos et al. 1997). Recently, Allan et al (Allan, Couse et al. 2010) concluded that estradiol induces spermatogenesis by increasing serum FSH (but not LH) in hpg/WT and hpg/ α ERKO but not in hpg/ β ERKO mice, by an ER α -mediated action of Sertoli cell activation.

Estrogen receptor expression sites in the human male testis are still controversial. In the fetal testis there is no ER α expression, while ER β is expressed in Sertoli, germ and Leydig cells (Boukari, Ciampi et al. 2007). In the adult, information is rather controversial. Some studies locate ER α in the Sertoli and Leydig cells (Cavaco, Laurentino et al. 2009), others only in the Leydig cells (Pelletier and El-Alfy 2000), while others do not identify any ER α expression in the human testis (Makinen, Makela et al. 2001). ER β locates mainly in Leydig cells (Pelletier and El-Alfy 2000; Cavaco, Laurentino et al. 2009), but also in germ cells-mainly spermatocytes and spermatids (Makinen, Makela et al. 2001; Cavaco, Laurentino et al. 2009); few studies identify ER β in Sertoli cells (Pelletier and El-Alfy 2000).

In human males, estrogens seem to modulate sperm maturation, since spermatozoa also express ER α and ER β (Carreau, Bouraima-Lelong et al. 2012). Moreover, estrogens control the quality and the sperm production as recently confirmed by the association of polymorphisms of estrogen-related genes with both sperm concentration and motility (Lee, Kuo et al. 2011).

Congenital estrogen deficiency in men resulting from inactivating mutations of either the aromatase gene (aromatase deficiency) or the ER α gene brought up rather confusing and conflicting results, as these subjects present variable degrees of impaired spermatogenesis and

different responses to estrogen treatment, so that a direct causal relation between infertility and aromatase deficiency could not be demonstrated in these patients (Rochira, Granata et al. 2005; Carreau, Bouraima-Lelong et al. 2012). In adult patients with aromatase deficiency, impaired spermatogenesis is irreversible even after estradiol treatment (Rochira and Carani 2009).

2.2.4. Roles of thyroid hormones in spermatogenesis

Several experimental and clinical studies reported that thyroid hormones are involved in sperm motility and spermatogenesis, as thyroid hormone receptors are expressed in Sertoli cells (Palmero, Prati et al. 1993). Moreover, transient juvenile hyperthyroidism in rats is associated with an early cessation of Sertoli cell proliferation, decreased testis size and altered sperm production, while induced hypothyroidism in prepubertal rats induces a delay in pubertal spermatogenesis, possibly due to retarded differentiation of the Sertoli cells (Francavilla, Cordeschi et al. 1991). TR α KO mice showed testicular phenotypic changes comparable to those observed in the wild type mice following neonatal hypothyroidism, suggesting that T3 actions on neonatal Sertoli cell development are mediated through TR α (Holsberger, Kieseewetter et al. 2005).

In humans, proliferating Sertoli cells express high levels of thyroid receptor α isoforms (Jannini, Crescenzi et al. 2000), as TR α is highly expressed in both fetal and prepubertal testis, while its expression decreases in adulthood.

The precise mechanisms of hormonal regulation mediated by thyroid receptors are still under debate; however, it seems that they might play a role in regulating Sertoli cell proliferation.

Thyroid hormone T3 was shown to modulate estrogen receptor expression and to decrease aromatase activity in Sertoli cells by direct inhibition of the aromatase gene transcription. Moreover, T3 might be involved in the regulation of the postnatal increase in AR expression in Sertoli cells, as studies have shown that T3 increases androgen binding and AR mRNA levels in both immature rat Sertoli and peritubular myoid cells (Horlein, Naar et al. 1995; Wagner, Wajner et al. 2008).

In adults, hyperthyroidism determines oligospermia, while males with thyroid disturbances have an increased risk to develop erectile dysfunction (Clyde, Walsh et al. 1976; Krassas, Tziomalos et al. 2008).

2.2.5. Roles of insulin in spermatogenesis

It has been reported that insulin is involved in the metabolic control of human and mammalian Sertoli cells, as insulin-deprived cells develop alterations in glucose consumption and lactate secretion (Alves, Rato et al. 2013). Recently, studies on transgenic mice with specific Sertoli or germ cell inactivation of either insulin receptor (Insr), IGF1receptor (Igf1r) or both receptors, revealed that both insulin receptor and IGF1 receptor are required for FSH-mediated proliferation of immature Sertoli cells and that insulin signaling pathway is essential in regulating the final pool of Sertoli cells. Despite important consequences on Sertoli cell maturation and proliferation, mice with ablation of Insr/Igf1r did not present alterations in spermatogenesis and maintained normal androgen production as well as normal levels of androgen regulated genes in Sertoli cells (Pitetti, Calvel et al. 2013). There are no data in human suggesting that insulin might play a direct role in the regulation of spermatogenesis.

Chapter 3

Nuclear receptors: classification and general structure

Nuclear receptors (NRs) are members of a large superfamily of DNA-binding transcription factors regulating genes involved in a variety of physiological, developmental and metabolic processes.

They are involved in the regulation of reproductive systems by steroid hormones (ER, PR, AR), the control of growth and development by thyroid and retinoid hormones (TR, RXR) and regulation of bile acid and cholesterol biosynthesis (LXR). Therefore, disturbances in NR signaling contribute to the development of several endocrine-related diseases, such as breast, ovarian or prostate cancer, diabetes and obesity.

Nuclear receptors can modulate transcription through several genomic or nongenomic, ligand-dependent or –independent mechanisms, acting as mediators of genetic repression or activation. They can also be the targets of other signaling pathways that modify the receptor post-translationally (Germain, Staels et al. 2006).

Nuclear receptors may be classified according to either homology(1999) or mechanism of action (Mangelsdorf, Thummel et al. 1995).

3.1. Nuclear receptors (NRs): classifications

- a) Using homology as a classification criterion, The Nuclear Receptor Nomenclature Committee classified the human NRs family into six evolutionary groups of unequal size (1999), as follows:
1. A large group containing TRs (thyroid receptors), RARs (retinoic acid receptors), VDR (vitamin D receptors), PPARs, as well as orphan receptors such as RORs, LXRs, and others.
 2. A group including RXRs, COUP-TF, and HNF-4.
 3. The subfamily of the steroid receptors comprising ERs (estrogen receptors), and the group GRs (glucocorticoid receptors), PRs (progesterone receptors), MR (mineralocorticoid receptor), AR (androgen receptor) and ERRs (estrogen related receptors).

4. A small group contains groups of orphan receptors [NGFI-B (NR4A1), NURR1 (NR4A2), and NOR1 (NR4A3)].
5. Another small group that includes the steroidogenic factor 1 (NR5A1) and the receptors related to the *Drosophila* FTZ-F1.
6. A subfamily containing *SHP*, NR0B2, which do not fit well into any other subfamilies.

b) Nuclear receptors may also be subdivided into the following four mechanistic classes (Mangelsdorf, Thummel et al. 1995):

- **Type I**

Ligand binding to type I nuclear receptors in the cytosol results in the dissociation of heat shock proteins, homo-dimerization, translocation (*i.e.*, active transport) from the cytoplasm into the cell nucleus, and binding to specific sequences of DNA known as hormone response elements (HREs)(Fig.9.).

Type I nuclear receptors include members of subfamily 3 (Mangelsdorf, Thummel et al. 1995), such as the androgen receptor (AR), estrogen receptors (ER), glucocorticoid receptor (GR), and progesterone receptor (PR).

The nuclear receptor/DNA complex recruits other proteins that transcribe DNA downstream from the HRE into messenger RNA and eventually protein, responsible for different cellular functions.

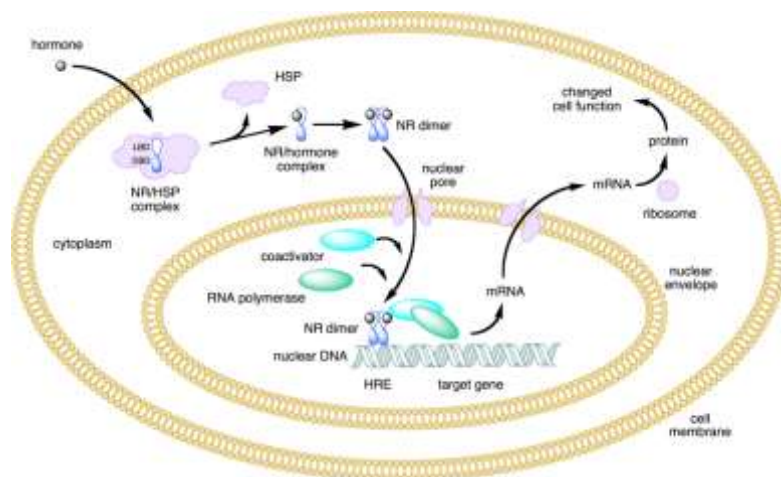


Fig.9. Mechanism for nuclear receptor action of class I nuclear receptors. Class I nuclear receptor (NR) is located in the cytosol in the absence of ligand. Hormone binding to the NR triggers dissociation of heat shock proteins (HSP), dimerization, and translocation to the

nucleus, where the NR binds to a specific sequence of DNA known as a hormone response element (HRE). The nuclear receptor DNA complex then recruits other proteins that are responsible for transcription of downstream DNA into mRNA, which is eventually translated into protein (reproduced from Wikimedia Commons).

- **Type II**

The type II nuclear receptors dimerize with the 9-*cis* retinoic acid receptor (RXR) and include principally subfamily 1 (the receptors for vitamin D3 (VDR), thyroid hormone (TR), all-*trans*retinoic acid (RAR), and the peroxisome proliferator-activated receptors (PPAR) ([, 1999 #626]) (Fig.10). Type II receptors, in contrast to type I, are retained in the nucleus regardless of the ligand binding status. In the absence of ligand, type II nuclear receptors are often complexed with corepressor proteins. Ligand binding to the nuclear receptor causes dissociation of corepressor and recruitment of coactivator proteins. Additional proteins including RNA polymerase are then recruited to the NR/DNA complex that transcribes DNA into messenger RNA.

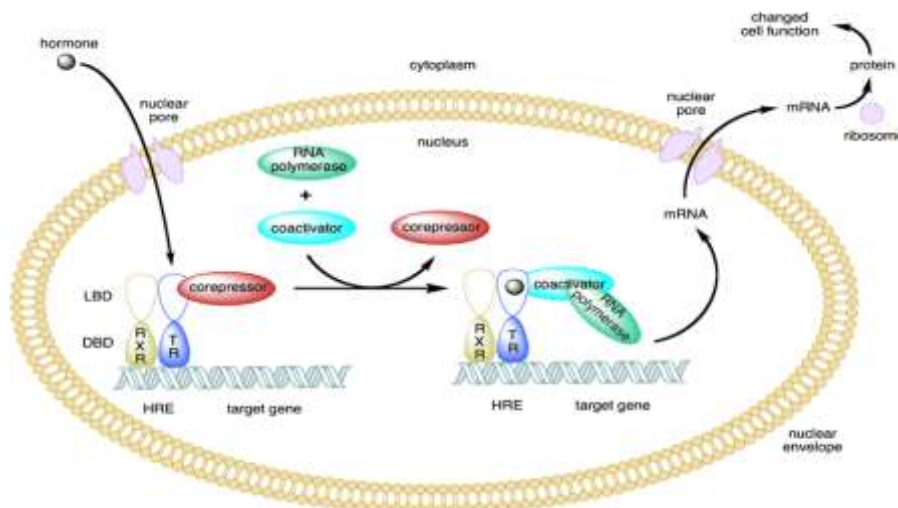


Fig.10. Mechanism for nuclear receptor action of class II nuclear receptors. Class II nuclear receptor (NR), regardless of ligand-binding status, is located in the nucleus bound to DNA. The nuclear receptor in the figure is the thyroid hormone receptor (TR) heterodimerized to the RXR. In the absence of ligand, the TR is bound to corepressor protein. Ligand binding to TR causes a dissociation of corepressor and recruitment of coactivator protein, which, in

turn, recruits additional proteins such as RNA polymerase that are responsible for transcription of downstream DNA into RNA and eventually protein (reproduced from Wikimedia Commons).

- **Type III**

The third type of nuclear receptors is represented by the orphan receptors, such as TR2, TR4, and chicken ovalbumin upstream promoter transcription factor (COUP-TF). They are similar to type I receptors in that both classes bind to DNA as homodimers. However, type III nuclear receptors, in contrast to type I, bind to direct repeat instead of inverted repeat HREs (Heinlein and Chang 2002).

- **Type IV**

Type IV nuclear receptors bind either as monomers or dimers, but only a single DNA binding domain of the receptor binds to a single half site HRE. Examples of type IV receptors are found in most of the NR subfamilies.

Despite a highly evolutionary conserved structural organization, the function and the mode of action of NRs are very diverse. Moreover, among the 48 known NRs of the human genome, only 24 are liganded receptors. These classic receptors are ligand-dependent transcriptional factors that respond directly to a large variety of hormonal and metabolic substances. Ligands trigger changes in the conformational and dynamic behaviour of the receptors that in turn regulate the recruitment of coregulators and chromatin-modifying machineries, a key component of NR signaling. Indeed, the ultimate action of liganded NRs on target genes, after site-specific DNA binding, is to enhance the recruitment and/or function of the general transcription machinery (Roeder 1996).

3.2. Nuclear receptors: general structure

Comparative structural and functional analysis of nuclear hormone receptors revealed a common structural organization in 4 different functional domains: a NH₂-Terminal Domain (NTD), a DNA-Binding Domain (DBD), a Hinge Region and a Ligand Binding Domain (LBD). This structure will be furthermore detailed for the androgen receptor (AR).

Chapter 4

Androgens and the androgen receptor (AR)

Androgens are steroid hormones with important roles in male sexual differentiation, in development and maintenance of secondary male characteristics and in the initiation and maintenance of spermatogenesis (Wilson, Griffin et al. 1993; Brinkmann 2001). The two most important androgens in this respect are testosterone and 5 α -dihydrotestosterone (Wilson, Griffin et al. 1993) (Fig.11.).

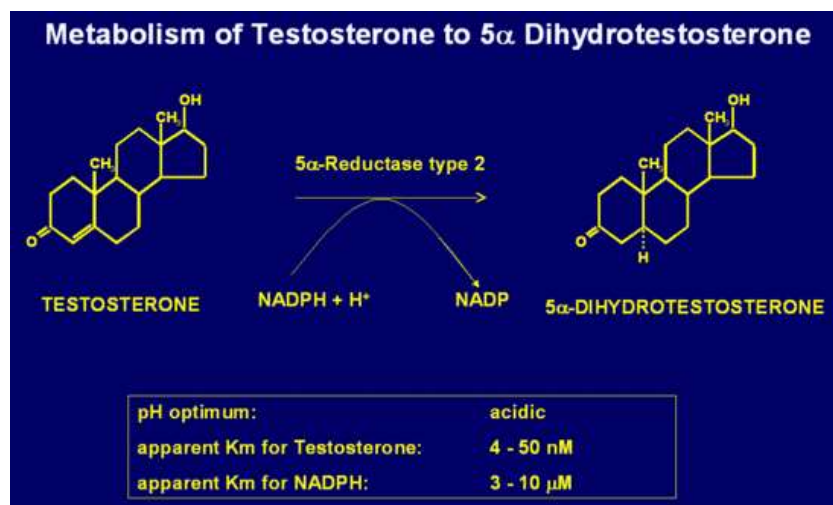


Fig.11. Structure of testosterone and dihydrotestosterone. 5- α reductase mediates conversion of dihydrotestosterone from testosterone (reproduced from (Brinkmann 2009))

4.1. Androgen action

Basic models describe the action of androgens, while androgen signalling pathways are characterized as classical or non-classical.

In the classic model, testosterone or dihydrotestosterone bind to the AR, followed by dissociation of heat-shock proteins in the cytoplasm, accompanied by a conformational change of the receptor protein resulting in a translocation to the nucleus. The receptor then dimerizes with a second molecule, binds to DNA and recruits further additional proteins (such as coactivators, general transcription factors and RNA polymerase II) resulting in specific activation or repression of transcription at discrete sites on the chromatin (Fig.12.a).

In the secondary model, androgen-induced transcription factors (TF) alter the transcription of secondary response genes. These typically lack androgen response elements (AREs) and thus cannot respond directly to androgens.

In non-classical androgen action, rapid signal transduction events (implying Ca^{++} uptake and protein phosphorylation (P)) are induced by androgens either in the presence or in the absence of AR-mediated transcription (Maclean and Wilkinson 2005).

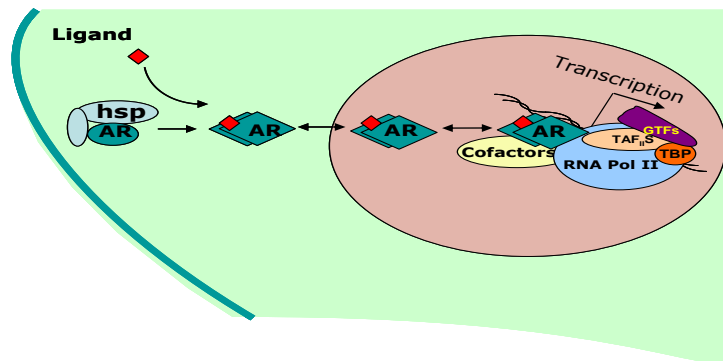
While acting through the same androgen receptor (AR), each androgen has its own specific role during male sexual differentiation: testosterone is directly involved in development and differentiation of wolffian duct derived structures (epididymis, deferential duct, seminal vesicles and ejaculatory ducts), whereas 5α -dihydrotestosterone (DHT), a metabolite of testosterone, is the active ligand in a number of other androgen target tissues, like urogenital sinus and tubercle and their derived structures (prostate gland, scrotum, urethra, penis) (Fig.12.b).

At puberty and in adulthood, androgens (mainly testosterone and dihydrotestosterone) stimulate spermatogenesis through androgen receptors (AR) on Sertoli cells (Chemes, Rey et al. 2008; Rey, Musse et al. 2009) and in lesser extent on peritubular myoid cells (MacLeod, Sharpe et al. 2010). Moreover, in the absence of testosterone or functional androgen receptors (AR), males are infertile because spermatogenesis rarely progresses beyond meiosis as proven in mouse models (Chang, Chen et al. 2004; Wang and Hardy 2004; Tsai, Yeh et al. 2006), then confirmed in human (Hiort and Holterhus 2003).

4.1.1. Testosterone: biosynthesis and metabolism

Testosterone is produced in the fetal human testis starting from the sixth week of pregnancy, when Leydig cell differentiation and the initial early testosterone biosynthesis are independent of luteinizing hormone (LH) (El-Gehani, Zhang et al. 1998). Human chorionic gonadotropin (hCG) promotes the differentiation of Leydig cells and the production of androgens during the 8th week of gestation; the fetal production of the pituitary hormone luteinizing hormone (LH) starts at the 11th-12th gestational weeks.

a)



b)

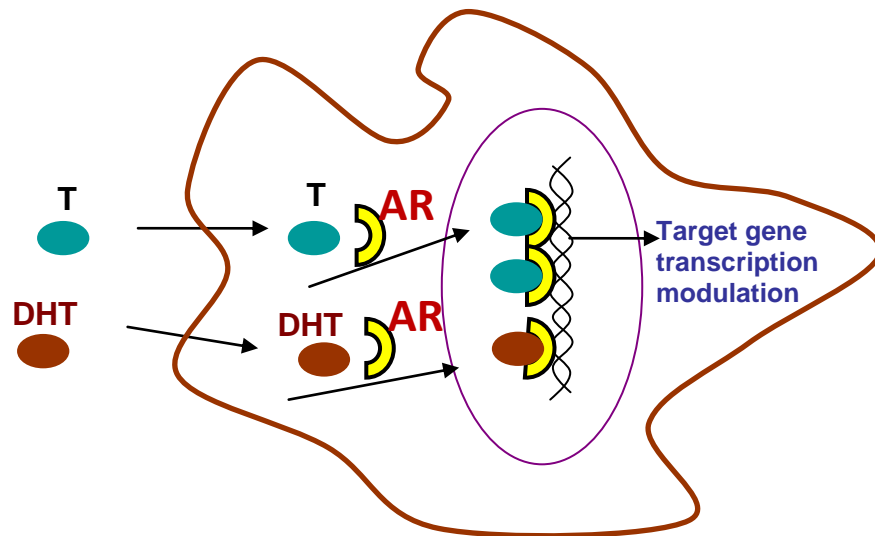


Fig.12.a) Model of androgen action. Both T and DHT (as ligands) bind to the androgen receptor (AR), resulting in functional ligand-receptor complexes interacting with androgen response elements on the DNA. TAFs are TBP-associated factors; TBP: TATA-box-binding protein; GTFs are general transcription factors. **b)** Simplified model for the mechanism of androgen action in Sertoli cells. Testosterone (T) enters the target cell by passive diffusion and is either metabolized to 5 α -dihydrotestosterone (DHT) or interacts directly with the androgen receptor (AR). Both T and DHT can bind to the same androgen receptor resulting in functional ligand-receptor complexes interacting with androgen response elements on the DNA. Each androgen has its own specific role during male sexual differentiation.

It has been established that spermatogenesis does not proceed in the absence of relatively high levels of testosterone (>70 nM in the rat) (Maddocks, Hargreave et al. 1993). Similarly to rodents, testosterone levels in the human testis are 25 to 125-fold greater in the testis (340 to 2,000 nM) as compared to serum (8.7–35 nM) (Jarow, Chen et al. 2001). Thus far, the specific physiological requirements for high levels of testosterone in the testis are not known, although it is hypothesized that this might be related either to the lower affinity of testosterone for the AR or to a state of physiological relative androgen insensitivity. It is known that testosterone (T) has a two to tenfold lower affinity than 5 α -dihydrotestosterone (DHT) for the AR, while the dissociation rate of testosterone from the receptor is five-fold faster than that of 5 α -dihydrotestosterone (Grino, Griffin et al. 1990; Jarow and Zirkin, 2005).

4.1.2. Dihydrotestosterone: biosynthesis and metabolism

Testosterone is metabolized to DHT in some peripheral tissues by two types of 5 α -reductase enzymes: 5 α -reductase type 1 (SRD5A1) and 5 α -reductase type 2 (SRD5A2). The irreversible conversion of testosterone to DHT in the urogenital tubercle, labioscrotal swellings and urogenital folds is necessary for the differentiation of male external genitalia (penis, scrotum and urethra).

4.1.3. 5 α -reductases

The 5 α -reductase isozymes family comprises five members. 5 α -reductase type 1 (SRD5A1) and 5 α -reductase type 2 (SRD5A2) are NADPH-dependent proteins responsible for the irreversible conversion of testosterone to 5 α -dihydrotestosterone. The cDNA of the 5 α -reductase type 2 gene (on 2p23), encodes for a protein of 254 amino acid residues with a predicted molecular mass of 28 kDa. The NH₂-terminal part of the protein contains a subdomain supposedly involved in testosterone binding, while the COOH-terminal region is involved in NADPH-binding (Zhu and Imperato-McGinley 2009). More recently, genome wide gene expression profile analysis identified a third isoform, 5 α -reductase type 3 and two other proteins exhibiting 5 α -reducing capabilities, glycoprotein synaptic 2 (GPSN2) and glycoprotein synaptic 2-like (GPSN2L) proteins. In adults, the three isoforms are expressed in skin, testis and prostate; while the third isoform seems ubiquitously expressed, with yet unknown roles in androgen signaling and prostate cancer, 5 α -reductases type 1 and 2 are differently expressed in liver, genital and non-genital skin, seminal vesicles, ovary, uterus, kidney, exocrine pancreas and brain (Azzouni, Godoy et al. 2012).

Both 5 α -reductase type 1 and type 2 are expressed in the rat testis (Killian, Pratis et al. 2003), where testosterone negatively regulates the type 1 isoform and human testis. In human testis 5 α -reductase type 1 is expressed in Sertoli and Leydig cells, while 5 α -reductase type 2 is expressed in spermatogonia and rarely in Leydig cells (Azzouni, Godoy et al. 2012; Aumuller, Eicheler et al. 1996). In humans, only mutations in the SRD5A2 gene, responsible for 5 α -reductase type 2 deficiency were reported as responsible for variate phenotypes (cryptorchidism, pubertal virilization in a 46, XY subject raised as female, with external female genitalia, pseudoscrotal perinovaginal hypospadias).

In a small village in the Dominican Republic 12 out of 13 families were affected resulting in an incidence of 1:90 males (Imperato-McGinley et al., 1974).

4.2. The Androgen Receptor

Androgenic actions are mediated by the androgen receptor (AR, also known as NR3C4; Nuclear Receptor subfamily 3, group C, gene 4). This ligand dependent transcription factor belongs to the superfamily of 48 known human nuclear receptors and 49 murine nuclear receptors (Zhang, Pakarainen et al. 2004). Studies of AR-deficient mice with different patterns of AR inactivation either global or only in Sertoli cells, revealed that AR has at least three functions with regards to spermatogenesis: 1) promotion of the progression of meiotic spermatocytes, 2) the maturation and/or survival of round spermatids, allowing the generation of elongating spermatids, and 3) the final maturation and release of elongated spermatids into the lumen of the seminiferous tubules (Chang, Chen et al. 2004; De Gendt, Swinnen et al. 2004; Bai, He et al. 2005; Abel, Baker et al. 2008).

4.2.1. The Androgen Receptor: genomic signalling and effects

The cDNA encoding AR was cloned in 1988-1989 by several groups (Chang, Kokontis et al. 1988; Lubahn, Joseph et al. 1988; Trapman, Klaassen et al. 1988; Tilley, Marcelli et al. 1989). The AR gene is located on X chromosome at Xq11-12 (Fig.3.3.a); it comprises eight exons and spans 180 kb. Structure of AR is typical for NRs and comprises the following regions: NTD, DBD, hinge region, and LBD (Fig.13.a). Recently, constitutively active AR splice variants have been described in human prostate cancer, contributing to the development of castration-resistant prostate cancer (Sun, Sprenger et al. 2010); moreover, different isoforms of AR may be present (Faber, Kuiper et al. 1989; Tilley, Marcelli et al. 1989; van Laar, Bolt-de Vries et al. 1989; Tilley, Marcelli et al. 1990; Sleddens, Oostra et al. 1993).

AR is a protein, generally containing 919 aminoacids (or 920 amino acid residues according to the new nomenclature, available on [www.http://androgendb.mcgill.ca/](http://androgendb.mcgill.ca/)(Gottlieb, Beitel et al. 2012)), while the number of amino acid residues in the AR protein might vary between individuals due to the polymorphic polyglutamine stretch and the less variable polyglycine stretch in the NH₂-terminal domain (NTD)(Faber, Kuiper et al. 1989).

AR protein is of about 98 kDa; AR migrates on SDS-PAGE electrophoresis gel mainly as a protein of 110 kDa, in relation with its phosphorylated state (van Laar, Bolt-de Vries et al. 1989)(Fig.13.b).

4.2.1.1. The Androgen Receptor Functional Domains

a) The NH₂-terminal Domain (NTD)

The NTD of AR is the least conserved part of the receptor compared to other NRs (Fig.13.a). This domain harbors the major transcription activation functions and several structural subdomains. Within its 538 aminoacids, two independent activation domains have been identified: activation function 1 (AF-1) (located between residues 101 and 370) that is essential for transactivation potential of full-length AR, also called TAU-1 and AF-5 (located between residues 360 and 485), also called TAU-5, that is required for transactivation of a constitutively active AR which lacks the LBD (Jenster, van der Korput et al. 1995; Claessens, Denayer et al. 2008) (Fig.14.). Interestingly, the AF-5 region interacts with a glutamine-rich domain of p160 cofactors like SRC-1 and SRC-2 and not with their LxxLL-like protein-interacting motifs (Claessens, Denayer et al. 2008).

The AR NTD also contains a dimerization surface involving the first 36 amino acids and a second region containing residues 370–494, mapped by Berrevoets (Berrevoets, Doesburg et al. 1998), both of which interact with the LBD in an intramolecular head-to-tail interaction. As a matter of fact, AR is the only nuclear receptor expressing its own LxxLL-like motifs located in the NTD (Fig.14) (He, Bowen et al. 2001; Steketee, Berrevoets et al. 2002). Particularly, the FQNLF motif (F = phenylalanine, Q = glutamine, N = asparagine) in the NTD facilitates binding of the AR NTD to the AR LBD. This so-called N/C interaction takes place after hormone binding, leading to stabilization of the AR dimer complex and in stabilization of the ligand receptor complex by decreasing the rate of ligand dissociation and receptor degradation(Langley, Zhou et al. 1995; Berrevoets, Doesburg et al. 1998).

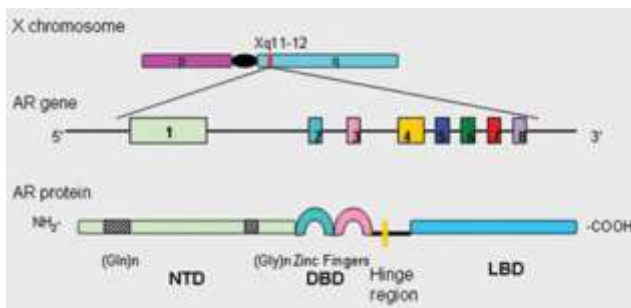
b) The DNA-Binding Domain

The DNA-binding domain is the most conserved among the members of the nuclear receptor superfamily (Fig. 13.a and b). It is characterized by a high content of basic amino acids and by nine conserved cysteine residues. DNA-binding domain has a compact, globular structure in which three α -helices can be distinguished; they are organized in two zinc finger modules, and a more loosely structured carboxy-terminal extension (CTE) (Fig.13.c). Both zinc substructures centrally contain one zinc atom which interacts via coordination bonds with four cysteine residues. Both the zinc coordination centres are C-terminally flanked by a α -helix (Jakob, Kolodziejczyk et al. 2007). The two zinc clusters are structurally and functionally different and are encoded by two different exons. The α -helix of the most N-terminally located zinc cluster interacts directly with nucleotides of the hormone response element in the major groove of the DNA. Three amino acid residues at the N terminus of this α -helix are responsible for the specific recognition of the DNA sequence of the responsive element. These three amino acid residues, the so-called P (proximal) box [Glycine-Serine-Valine], are identical in the androgen (AR), progesterone (PR), glucocorticoid (GR) and mineralocorticoid receptors(MR), and differ from the residues at homologous positions in the oestradiol receptor (ER). It is not surprising therefore that the androgen, progesterone, glucocorticoid and mineralocorticoid receptors can recognize the same response element (Brinkmann 2011). Upon ligand-binding, the AR is translocated to the nucleus, process controlled by a nuclear localization signal flanking the DNA-binding domain (DBD) and hinge region (Simental, Sar et al. 1991). In an AR dimer, two DBDs are capable of recognizing specific DNA sequences referred to as androgen response elements (AREs)(Verrijdt, Haelens et al. 2003). The consensus ARE consists of two palindromic half sites spaced by three base pairs (AGAACA_nnnTGTTCT). Mutational analysis of a wide variety of candidate AREs suggests two classes: the consensus, classical ARE and selective AREs that are more discriminatory for the AR (Denayer, Helsen et al. 2010). Whether type and sequence ARE are of influence for the next steps of transcription regulation and cofactor recruitment is not fully understood yet.

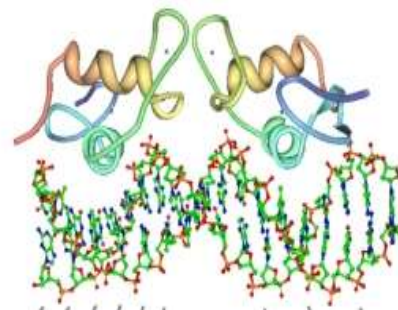
c) The Hinge Region

Between the DNA-binding domain and the ligand-binding domain, a non-conserved hinge region is located, which is also variable in size in different steroid receptors. The hinge region is important for nuclear localization, being considered as a flexible link between the ligand-binding domain and the rest of the receptor molecule. Androgens bind to the ligand-binding domain (LBD) after which the AR translocates to the nucleus via a nuclear localization signal at the border of the DNA-binding domain (DBD) and hinge region (H). The AR hinge is a major site for posttranslational modifications and interactions with nuclear coregulators; moreover, the crucial role of the hinge region of the AR has been highlighted by the AR mutations present in this region which are responsible in humans for AIS and prostate cancers. A review concerning the hinge region in androgen receptor control has been recently published (Clinckemalie, Vanderschueren et al. 2012).

a)



c)



b)



Human testis LNCaP cell line

d)

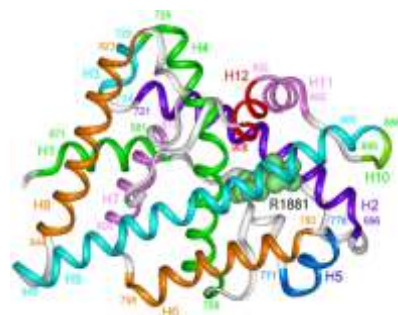


Fig.13. Molecular structure of the AR

a) *Structural organisation of the androgen receptor gene at the Xq11.2-q12 locus on the X chromosome and the derived protein size and structural organization* The human androgen receptor protein (919/920 amino acid residues) is encoded by 8 exons. Analogous to other nuclear receptors the protein consists of several distinct functional domains: the NH₂-terminal domain, the DNA-binding domain, the hinge region and the ligand binding domain (Reproduced from (Galani, Kitsiou-Tzeli et al. 2008))

b) *AR protein is detected by western blotting in the human testis and the LNCaP cell line as a band of 110 kDa.*

c) *The crystal structure of the AR-DBD (reproduced from (Claessens, Denayer et al. 2008))*

d) *The crystal structure of the AR-LBD (realised by Dr JH Wu, LDI Molecular Lab)*

d) The Ligand-Binding Domain

In order to be ligand-activated (testosterone (T) or dihydrotestosterone (DHT)), the AR contains the second-best conserved region, the C-terminal ligand-binding domain (LBD) or the hormone-binding domain, which is encoded by approximately 250 residues (Sack, Kish et al. 2001; Brinkmann 2011). The LBD is composed of twelve α -helices and two anti-parallel β -sheets arranged in a helical sandwich pattern, surrounding a ligand-binding pocket, represented by central hydrophobic cavity. In the agonist-bound conformation, the C-terminal helix 12 is positioned in an orientation allowing a closure of the ligand-binding pocket. The fold of the ligand-binding domain upon hormone binding results in a globular structure with an interaction surface for binding to specific FXXLF and LXXL motifs of coactivators. In this way the AR selectively recruits a number of proteins and can communicate with other partners of the transcription initiation complex.

The AF-2 (activation function 2) is a hydrophobic groove on the surface of AR-LBD, resulting from the repositioning of the LBD helix 12 over the pocket to stabilize the bound ligand. AF-2 conformation is strongly dependent on the presence of nuclear receptor coactivators, as the formation of the hydrophobic groove serves as a platform for high affinity interactions with LxxLL sequences (L = leucine, x = any amino acid) present in several coactivators such as the members of the p160 family (York and O'Malley 2010).

e) AR domains interactions

The AR NH₂/COOH-terminal interaction was shown to be facilitated by several coactivators and is important in stabilizing the bound ligand (Heinlein and Chang 2002). It is possible that in the full-length AR protein, the AF-1 domain functions to interact with coactivators and provides an interaction surface for the AR COOH terminus. Because AR has two separate NH₂-terminal transactivational domains, it is possible that each domain interacts with different coregulators or transcription factors, possibly in a promoter context-dependent manner. Point mutagenesis studies within the AR LBD suggest that the NH₂/COOH-terminal interaction positions or stabilizes helix 12 across the ligand-binding pocket, resulting in a reduced dissociation rate of bound androgen (He, Kempainen et al. 1999).

Recently, a combination of in silico modeling, pointed out a putative interface between AR-DBD and LBD. These interactions were confirmed by point mutation analysis, inspired by known AR mutations described in human pathologies such as androgen insensitivity syndromes and prostate cancer. This aspect will be further developed within the chapter on androgen receptor coregulators.

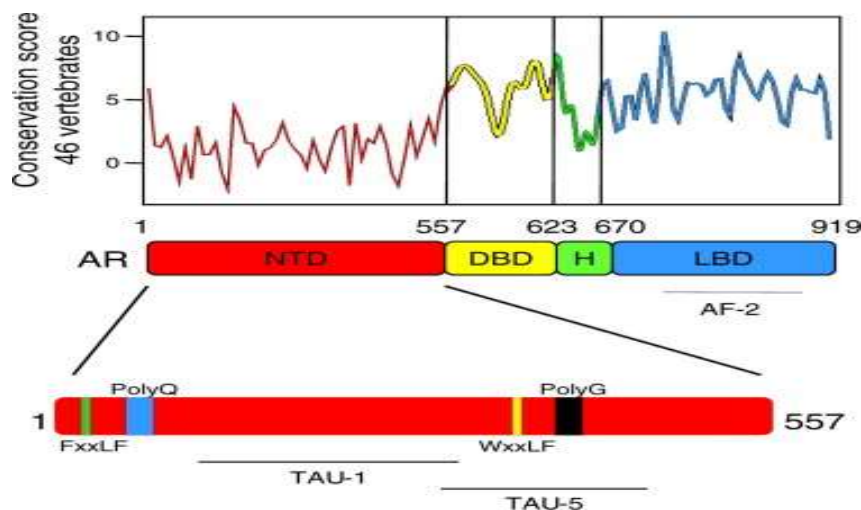


Fig.14. Functional domains of the androgen receptor. The N-terminal domain (NTD) is responsible for transcription activation and harbors two transcription activation units (TAU-1 and TAU-5). Within the AR NTD, two FxxLF-like motifs are present and capable of binding to LBD's AF-2, allowing cofactors recruitment (reproduced from (van de Wijngaart, Dubbink et al. 2012))

4.2.1.2. Transcriptional regulation of AR regulated target genes in testis

Genomic DNA is tightly packed in chromatin. The condensed organization of chromatin generates a barrier for transcriptional activities. The ultimate challenge of the AR in case of transcription activation, is to create an open chromatin structure to facilitate the assembly of the preinitiation complex (PIC), including AR, coactivators and recruitment of RNA polymerase II (pol II) to the promoter of target genes, mediated through the assembly of general transcription factors (GTFs)(Shang, Myers et al. 2002),(van de Wijngaart, Dubbink et al. 2012). The AR performs these tasks by binding specific proteins that are generally part of larger complexes that implement specific functions essential for the overall transcription process (Fig.12.a).

However, the molecular mechanisms underlying these events have not been yet fully elucidated – partly because there were definitively identified only a few androgen-responsive genes in the testis. Among the few genes reported to be AR regulated are *Rhox5(Pem)*(Rao, Wayne et al. 2003),*Myc* (Amir, Barua et al. 2003), members of the claudin junction complex protein family (*Cldn1*) (Gye 2003), *Gsta* (Dean and Sanders 1996), serine protease inhibitor-like protein with Kunitz and WAP domains 1 (Spinlw1, previously known as Eppin)(O'Rand M, Widgren et al. 2004), or the connexin-43/gap junction $\alpha 1$ (*Gja1*)(Dean and Sanders 1996) (Table 1.). Most androgen-regulated genes in the testis are probably not direct targets of the AR, but instead respond indirectly to androgen via activation or suppression of transcription by other factors that androgen regulates (Maclean and Wilkinson 2005).

4.2.1.3. Post-transcriptional and post-translational modifications of AR

Recent interest has been remarked on the complex roles of post-translational modifications of the AR. Modifications, such as phosphorylation, acetylation, SUMOylation, methylation and ubiquitination contribute to regulation of AR structure, activity and stability (Gioeli and Paschal 2012).

1) AR phosphorylation

Phosphorylation occurs either in the presence or absence of androgens and affects AR activity by increasing or decreasing protein interactions that occur proximal to the phosphosite. This feature could be highly relevant to both normal development and human disease given the

substantial body of evidence showing that kinase cascades can activate the AR in the absence of androgen, or sensitize the AR to low levels of androgen.

Two models of phosphorylation are described. Androgen binding increases phosphorylation of AR through a conformation-based change that increases solvent exposure or structure of the phosphosite making it more accessible to kinases. An alternative view is that phosphosites are constitutively accessible to kinases, androgen binding causing a net increase in phosphorylation by reducing phosphatase interactions with the AR. There is evidence for AR phosphorylation on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues (Gioeli and Paschal 2012).

Each of the major AR domains contains at least one phosphorylation site. The majority of the phosphosites are in the AR NTD, which contains the hormone-independent coactivation domain, AF1 (Gioeli 2005). AR phosphorylation sites are also represented by Ser 578 in the DNA binding domain and Ser 650 is in the hinge region (Ponguta, Gregory et al. 2008). Finally, in the LBD namely in the AF2 domain, which mediates androgen-induced transactivation, phosphorylation might occur on Ser 791. Phosphorylation of many sites increases in response to androgen treatment.

While the steady-state localization of the AR is predominantly cytoplasmic in the absence of androgen and nuclear in the presence of androgen, the AR is constantly shuttling from the nucleus to the cytoplasm (Black, Holaska et al. 2001; Gioeli, Black et al. 2006). The contribution of nuclear and cytoplasmic localization towards establishing the AR phosphorylation state was examined using nuclear localization signal (NLS) and nuclear export signal (NES) fusions with the AR (Kesler, Gioeli et al. 2007).

2) Dephosphorylation

Two abundant phosphatases, Protein phosphatase 1 and 2A (PP1 α , PP2A), have been shown to bind AR (Chen, Xu et al. 2006; Yang, Xin et al. 2007). The identification of AR phosphorylation sites by mass spectrometry and the development of phosphosite antibodies opened the door for analyzing phosphatase action on AR.

3) Acetylation

The AR is a direct substrate of histone acetyltransferases (HATs) and is acetylated on Lys 630, Lys 632, and Lys 633. The AR can be acetylated *in vitro* by several coactivators, such as p/CAF, p300, or by Tip60 on Lys 630 in the hinge region near the second zinc finger of the DNA binding domain (Gaughan, Logan et al. 2002). The histone deacetylases (HDACs) HDAC1 and SIRT1 deacetylate the AR and down regulate AR transcriptional activity (Gaughan, Logan et al. 2002). Acetylation regulates the affinity of the AR for cofactors and enhances transcription of reporter genes as well as cell growth. The acetylation-deficient mutants of the AR, K630A or K632/633A, show decreased AR transactivation of reporter genes and decreased co-activation by SRC1, TIP60, and p300 (Gong, Zhu et al. 2006).

Conversely, the acetylation-mimetic mutation K630Q can increase AR transactivation on PSA and MMTV reporter constructs (Zhou, Lane et al. 1995). In another study, the AR acetylation site mutants showed a delayed ligand-dependent nuclear translocation, suggesting that AR acetylation may be involved in regulating multiple aspects of AR function (Gioeli and Paschal 2012).

4) Methylation

The hinge region of AR contains a sequence (Lys–Leu–Lys–Lys) that is similar to the sites in proteins modified by the methyltransferase SET9 (Gaughan et al. 2011). SET9 was shown to bind and methylate AR *in vitro*, while IHC of human prostate cancer tissue microarrays revealed increased nuclear expression of SET9. Collectively, the data identify SET9 as a positive regulator of AR function that might be involved in prostate cancer (Gaughan, Stockley et al. 2011).

5) SUMOylation

AR can be modified by SUMOylation, which refers to the covalent attachment of the small ubiquitin-like modifier (SUMO) to lysine side chains (Geiss-Friedlander and Melchior 2007). Four SUMO isoforms are expressed in mammals, AR being preferentially modified by SUMO1. Analogous to ubiquitination, specific enzymes (termed E1, E2, and E3) are used in sequence to activate and conjugate SUMO to target proteins including AR.

There is a growing number of SUMO E3 “ligases” that present specificity to the SUMOylation reaction. PIAS1 and PIASx α are two AR coregulators which have SUMO E3 ligase activity towards AR (Kotaja, Karvonen et al. 2002; Nishida and Yasuda 2002). Androgens induce AR SUMOylation conformational changes that enhance the interaction of AR with SUMOylation enzymes. Under steady state conditions, only a small fraction of the total AR pool (10%) is conjugated with SUMO (Kaikkonen, Jaaskelainen et al. 2009). The biological effect of AR SUMOylation was explored by mutating one (or both) SUMOylation sites and measuring androgen-induced transcription. AR containing the Lys 386 to Arg substitution either alone, or together with a Lys 520 to Arg substitution, showed a 2–3-fold enhancement of androgen-dependent transcription on promoters containing multiple AREs (Kaikkonen, Jaaskelainen et al. 2009). The activity of AR containing the Lys 520 to Arg substitution alone was similar to WTAR. The data suggest that SUMOylation of AR primarily at Lys 386 reduces the transcriptional activity of AR. The underlying mechanism for this effect has not been defined.

6) Ubiquitination

Current models suggest AR-mediated transcription begins with androgen-induced nuclear import and concludes with ubiquitin-dependent degradation of AR by the proteasome (Shank and Paschal 2005). AR is ubiquitinated at two sites, Lys 845 and Lys 847, which were identified by mass spectrometry (Xu, Shimelis et al. 2009). Ubiquitin modification of substrates involves attachment of a single ubiquitin to an acceptor lysine; the ubiquitin chain that is subsequently formed (via ubiquitin–ubiquitin linkages) acts as a degradation signal. Ubiquitination is reversible through the action of deubiquitinating enzymes (DUBs)(Reyes-Turcu, Ventii et al. 2009). Additionally, there is a growing list of proteins that are regulated by monoubiquitination. Transcription factors including AR may be subject to biphasic ubiquitination whereby monoubiquitination (perhaps at multiple sites) promotes transcription; ubiquitin chain formation and proteasomal degradation would then reduce the pool of AR available for reinitiation of transcription.

Gene	Characteristics	Reference
Rhox 5 (reproductive homeobox gene on the X chromosome) (Pemb-Placentae and embryos oncofetal gene)	<ul style="list-style-type: none"> selectively expressed in Sertoli cells under the control of specific ARE located in its promoter region the only gene identified in all transcriptional profiling studies searching for androgen-regulated genes in Sertoli cells Rhox 5 null mice are subfertile presenting combined defects in both spermatozoa formation and maturation 	(Sadate-Ngatchou, Pouchnik et al. 2004; Maclean, Chen et al. 2005; Zhou, Shima et al. 2005; Denolet, De Gendt et al. 2006; De Gendt, McKinnell et al. 2009; Battaglia, Maguire et al. 2010)
c-myc	<ul style="list-style-type: none"> an androgen inducible gene in cultured Sertoli cells 	(Lim, Yoo et al. 1994)(Lim, Yoo et al. 1994)
Eppin (epididymal protease inhibitor) or Spinl1 (Serine protease inhibitor-like protein with Kunitz and WAP domains),	<ul style="list-style-type: none"> Eppin immunization of male monkeys results in complete, but reversible, contraception localized on the surface of ejaculated spermatozoa 	(O'Rand M, Widgren et al. 2004; O'Rand, Widgren et al. 2011) (AgoulNIK, Bingman et al. 2008; Macleod, Sharpe et al. 2010)
Gpd1 (glycerol-3-phosphate dehydrogenase 1)	<ul style="list-style-type: none"> enzyme involved in glycerol and lipid metabolism 	(Brown, Koza et al. 2002)
Drd4 (dopamine receptor 4)	<ul style="list-style-type: none"> G-protein coupled receptor 	(Denolet, De Gendt et al. 2006)
the claudin junction complex protein family (<i>Cldn1</i>)	<ul style="list-style-type: none"> Androgen regulated protein forming tight junctions 	(Gye 2003)
connexin-43/gap junction $\alpha 1$ (<i>Gja1</i>)	<ul style="list-style-type: none"> Androgen regulated protein forming gap junction 	(Dean and Sanders 1996)
β tubulin3 (<i>Tubb3</i>)	<ul style="list-style-type: none"> Androgen regulated proteins 	(De Gendt, Denolet et al. 2011)

Table 1. Selection of androgen receptor regulated genes in Sertoli cells

Crosstalk between different posttranslational modifications of AR

To date, however, there is relatively little and conflicting information regarding crosstalk between different posttranslational modifications on AR. Some studies link acetylation and phosphorylation. Mutating the AR sites that undergo phosphorylation and acetylation did not affect AR SUMOylation, suggesting cross-talk between these sites does not occur (Kaikkonen, Jaaskelainen et al. 2009).

4.2.2. The Androgen Receptor: non genomic signalling and effects

The biological activity of testosterone and dihydrotestosterone is thought to occur predominantly through binding to the androgen receptor (AR). However, androgens have also been reported to induce rapid activation of kinase-signaling cascades and modulate intracellular calcium levels.

These effects are considered to be non-genomic, because they occur in cells too rapidly to involve changes in gene transcription, in relation to rapid and prolonged increases in intracellular Ca^{2+} (Walker and Cheng 2005). Immunocytochemistry studies recently demonstrated that a population of classical AR in Sertoli cells localize to the plasma membrane, and that AR associates with, and activates the tyrosine kinase Src kinase after testosterone stimulation, which subsequently activates the MAPK pathway through the EGF receptor (Cheng, Watkins et al. 2007). Moreover, it has been recently demonstrated that short-term inhibition of p42/p44 MAPK activity either by a MAPK kinase inhibitor, or by depletion of kinase with small interfering RNA, caused target gene-specific reductions in AR activity, underlining the importance of the non-classical effects of testosterone in the regulation of the transcription factor activity of AR (Agoulnik, Bingman et al. 2008). In addition, androgens may function through the sex hormone binding globulin receptor and possibly a distinct G protein-coupled receptor to activate second messenger signalling mechanisms (Heinlein and Chang 2002; Rahman and Christian 2007). Non-classical effects of androgens have also been demonstrated in the reproductive system (Heinlein and Chang 2002; Rahman and Christian 2007).

4.3. The androgen receptor and the molecular biology of androgen insensitivity

Human disorders related to androgen sensitivity (the androgen insensitivity syndrome, AIS) are the main category of the 46, XY disorders of sex development (DSD) (Hughes, Davies et

al. 2012). The DSD gather another two classes: the disorders of gonad development such as gonadal dysgenesis and the disorders of androgen biosynthesis and metabolism. In DSD, related to androgen insensitivity, androgen receptor (*AR*) mutations have been identified in patients with mild, partial or complete androgen insensitivity syndromes (AIS) (Hughes, Davies et al. 2012). Among DSD related to the disorders of androgen biosynthesis and metabolism, genetic causes have been identified, such as loss of function mutations of the LH receptor gene (*LHCGR*), 17β hydroxysteroid dehydrogenase deficiency type 3, related to mutations of the *HSD17B3* gene and loss of function mutations of the *SRD5A2* gene, responsible for 5α -reductase type 2 deficiency (5α -R2 deficiency).

4.3.1. Androgen insensitivity syndrome in humans

The pivotal role of androgens in male reproduction was proven by clinical and genetic studies in humans with androgen insensitivity syndromes (AIS). In 1953, Morris (Morris 1953) described the pubertal clinical features of this inherited pathology (female external genitalia with absent uterus and blind-ended vagina associated with undescended testes producing androgens). In 1975, Keenan established the androgen binding assays in genital skin fibroblasts (Keenan, Meyer et al. 1975), allowing to study the etiology of the AIS at the cellular level, while the molecular analysis of the AIS started in 1988, when human AR cDNA (Lubahn, Joseph et al. 1988) and the first AR mutation associated with CAIS have been reported (Brown, Lubahn et al. 1988).

AIS prevalence varies between reports with estimates of complete AIS (CAIS) of 1:20,400 genetic males primarily diagnosed with the disorder, as reported in Denmark (Bangsboll, Qvist et al. 1992), to a minimal incidence of complete AIS patients with molecular proof of the diagnosis of 1:99,000 in The Netherlands (Boehmer, Brinkmann et al. 2001). Partial AIS was estimated to have a prevalence of 1:130,000.

The clinical characteristics of AIS vary from completely female external genitalia to variable degrees of masculinization impairment covering a broad spectrum of 46, XY phenotypes (Table 2). AIS can be divided into three categories in function of the degree of genital masculinization: complete androgen insensitivity syndrome (CAIS) which is characterized by normal female external genitalia and a history of inguinal hernia or labial swelling in a postpubertal primary amenorrheic patient; partial androgen insensitivity syndrome (PAIS) when the external genitalia are partially, but not fully, masculinized, and a degree of sexual

ambiguity exists (clitoromegaly, hypospadias) (Hughes, Davies et al. 2012), and mild androgen insensitivity syndrome (MAIS) when the external genitalia are normal in adolescents or adults presenting with gynecomastia and infertility. Diagnostic evaluation is based on clinical findings, karyotype analysis, hormonal assays (including basal and hCG stimulated serum testosterone (T), DHT, LH, FSH and eventually AMH) and AR mutation analysis, preferentially with functional characterization.

Children and postpubertal patients have normal or high basal testosterone, high LH concentrations and high androgen sensitivity indexes (TxLH) (Gottlieb, Lombroso et al. 2005), as well as high AMH levels.

The recently (1st of July 2014) reviewed updated AR mutation database (ARDB) (available at <http://androgendb.mcgill.ca/>) contains 906 entries of mutations in patients declared with AIS. In November 2010, from a total of 421 different AR mutations, 28% were nonsense point mutations, small deletions or insertions leading to a premature stop codon; large deletions and splice site mutations covered 10% of all reported mutations (Jaaskelainen 2012).

In July 2014, the McGill ARDB included about 566 different AR mutations in patients with CAIS, while in PAIS and MAIS the AR mutations described as causing the disease are less numerous: 285 (for PAIS) and 55 (for MAIS).

Generally, AR mutations are identified in about 83% of the patients with a CAIS phenotype and biochemical characteristics, while in PAIS, the mutation detection rates are varying between 28-73%, upon studies (Jaaskelainen 2012).

Conversely, phenotypic variability was registered in several cases presenting identical AR mutations and different PAIS phenotypes, or even in phenotypically normal males with preserved fertility, who were siblings of PAIS presenting the same AR mutation (Jaaskelainen 2012).

These aspects could possibly be explained by somatic mosaicism, variable expression of other genes involved in androgen biosynthesis such as 5 α -reductases or 17- β -HSD type3 and variable tissue expression of AR coregulators. Moreover, I shall detail in the chapter related on AR coregulators, some aspects related on AR mutations responsible for AIS that do not alter AR ligand binding, but present deleterious consequences related to altered AR-coregulator interactions.

Type	External Genitalia/ (Synonyms)	Clinical Phenotype
CAIS	Female ("testicular feminization"/Morris syndrome)	Absent/ rudimentary Wolffian duct derivatives Absence or presence of epididymides and/or vas deferens Pelvic/Inguinal / Labial testes Short blind-ending vagina Scant / absent pubic and axillary hair
	Predominantly female ("incomplete AIS")	Inguinal /labial testes Clitoromegaly and labial fusion Distinct urethral and vaginal openings/ presence of an urogenital sinus
PAIS	Ambiguous	Microphallus (<1 cm) with clitoris-like underdeveloped glans; labia majora-like bifid scrotum Descended/ undescended testes Perineoscrotal hypospadias / urogenital sinus Gynecomastia in puberty
	Predominantly male	Simple (glandular or penile) or severe (perineal) hypospadias with a normal-sized penis and descended testes severe hypospadias with micropenis, bifid scrotum, and either descended or undescended testes Gynecomastia in puberty
MAIS	Male ("undervirilized male syndrome")	Impaired spermatogenesis and/or impaired pubertal virilization ; rarely, micropenis. Gynecomastia in puberty

Table.2. Clinical classification of AIS (adapted from (Gottlieb, Beitel et al. 1993))

Another challenging aspect related on AIS is that despite the clinical phenotype and after exclusion of other genetic mutations (such as mutation of *SRD5A2* or *17βHSD3* genes), several groups having performed mutational analysis, did not find any AR mutation in about 17% of reported CAIS cases and 72% of PAIS cases (Ahmed, Cheng et al. 2000), including 85-90% of sporadic PAIS cases (Sultan, Lombroso et al. 2002). Other aspects, such as identification of AR mutations with both gain of function and loss of function character, and multiple mutations within same tissues suggest that AR signaling perturbations and their consequences are very complex.

The identification of AR genotype is certainly not sufficient to conclude on the patient's phenotype and management as several other epigenetic modifications not detected by DNA sequencing might be responsible for AR signaling alterations. Indeed, recently, it is

considered that AR mutations are detected in less than 75% of CAIS and less than 25% of PAIS patients.

4.3.2. Androgen insensitivity syndrome in spontaneous and generated AR-knockout models

As experimental studies of androgen action and AR signaling in human reproductive tissues from AIS patients is not always possible in vitro, the majority of research performed regarding AIS pathology and the androgen signaling particularities was performed in AIS animal models (either naturally AR mutated mice (*tfm*) or in generated AR-knock-out animal models, using gene-targeting approaches such as the Cre/LoxP system).

In 1970's the first AIS animal model described was represented by *tfm* (testicular feminization mouse) mice, which presented an exon 1 single nucleotide deletion in AR gene. Genetic *tfm* males exhibit a normal female somatic and external genital morphology; they are sterile with small, intra-abdominal testes, with spermatogenesis arrested at the late pachytene spermatocyte stage as well as reduced testosterone secretion in adults, related to insufficient and defective adult Leydig cells (Lyon and Hawkes 1970).

Generated models of global ARKO mice recently exhaustively presented in several reviews (Wang, Yeh et al. 2009; Walters, Simanainen et al. 2010) reproduce the *tfm* phenotype with high fidelity. Conversely, all these mice present cryptorchidism; we believe it is difficult to evaluate the relative contribution of cryptorchidism to the particularities of the phenotype.

Sertoli cell ARKO mouse models present alterations of spermatogenesis (arrest at the level of spermatocytes) and small size gonads; however, testis development or descent, development of internal and external genitalia or sexual differentiation is not altered when AR activity in Sertoli cells is impaired (Welsh, Saunders et al. 2009). Leydig cell ARKO mice are also infertile, but with normal testis descent, while peritubular myoid cell ARKO mice are fertile (Zhang, Yeh et al., 2006) or infertile, depending on the model, the infertile phenotype presenting impaired Sertoli cell function (Welsh, Saunders et al. 2009).

In conclusion AR inactivation in mouse models offers interesting insights for better understanding of AR roles and implications in male fertility.

Chapter 5.

Androgen receptor coregulators

5.1. Nuclear receptor (NR) coregulators

5.1.1. History of coregulators

Nuclear receptor coregulators belong to a complex and diverse family of molecules involved in the proficient function of nuclear receptors, by modulating nuclear receptor effects on activation (coactivators) or repression (corepressors) of gene transcription (McKenna, Lanz et al. 1999; Heinlein and Chang 2002).

Coregulators have large physiological implications in genome wide effects on the control of nuclear receptor and other types of transcription factors.

Starting with the early 1990s, when coregulators have been first cloned, until 2012, more than 300 nuclear coregulators that perform complex biological functions have been well described (Lonard and O'Malley 2012).

As early as the 1970s, receptor-associated nonhistone proteins, called “nuclear acceptor” molecules were found to interact with and support the function of nuclear receptors (Puca, Nola et al. 1975; Yamamoto and Alberts 1975; Yamamoto 1985).

Conversely, the observation of indirect inhibition of transcriptional properties of one NR by the activation and overexpression of another NR in a dose and ligand dependent manner (Meyer, Gronemeyer et al. 1989) brought into discussion the existence of other factors than the basal transcription machinery, which contribute to the transcriptional regulation.

Evidence of new molecules that do not bind to DNA, but control transcription by binding to NRs, were brought starting from 1992. O'Malley's research team defined a ligand-controlled repressor domain in NRs that suppresses transcriptional activity (McDonnell, Vegeto et al. 1992; York and O'Malley 2010). Yamamoto's laboratory reported the same year that GR interacted with regulatory protein complexes (Yoshinaga, Peterson et al. 1992).

However, the first direct evidence for the existence of coregulators was brought two years later, by Myles Brown's research team, who discovered that ligand binding resulted in the recruitment by ER of associated proteins called ERAP140 and ERAP160 (named according to their size in kilodaltons), capable of mediating hormone induced transcription in a human breast cell line, MCF-7 (Halachmi, Marden et al. 1994).

The use of yeast two-hybrid protein-protein interaction assays led to the identification in mid 1990s of large series of clones encoding nuclear receptor coregulator molecules (See Table 3.). Few of the most known coregulators are briefly presented in table 3.

The first nuclear receptor coactivator called steroid receptor coactivator 1 (**SRC-1**)(Onate, Tsai et al. 1995), was cloned in Bert O'Malley's laboratory, in 1994. This discovery was followed by two other related 160 kilodalton proteins sharing structural homology and conserved structure between species; altogether, the three proteins composed the SRC/p160 family of coactivators. The other two members are **SRC-2** (steroid receptor coactivator-2, also named glucocorticoid receptor-interacting protein 1(GRIP-1), transcriptional intermediary factor 2 (TIF2) or nuclear receptor coactivator-2 (NCoA2)),first cloned by Michael Stallcup (Hong, Kohli et al. 1996) and **SRC-3** (p300/CBP-interacting protein, receptor-associated coactivator 3, acetyltransferase ACTR, amplified in breast cancer 1, or thyroid hormone receptor activator molecule1), initially identified in Ron Evans's team(Chen, Lin et al. 1997; McKenna and O'Malley 2002; Xu and Li 2003). These three AR coactivators will be named hereafter as SRC-1, SRC-2 and SRC-3.

At present, there is a myriad of coregulators fulfilling a wide array of complexes, with specific cellular and tissue functional impacts.

Recently, a large-scale proteomics study has been initiated, to characterize the coregulator complexome, which includes a network of transcription factors, coregulators, and their regulatory enzymatic molecules. The results are available online on the www.NURSA.org website (McKenna, Cooney et al. 2009).

5.1.2. General factors involved in coregulators functionality

Coregulators form complexes which can be recruited by ligand-bound NRs to specific chromatin regions, facilitating the assembly of general transcription factors. These multi subunit complexes, including coregulators, play important roles in the transcription processes, either enhancing gene transcription by inducing chromatin remodeling, histone acetylation and methylation and recruitment of RNA polymerase type II(Xu and Li 2003)(coactivators), or enhance chromatin condensation, by histone deacetylation and methylation, leading to gene silencing (corepressors)(Suganuma and Workman 2008) (Fig.15.)

Most coregulators are either enzymes needed for gene expression, or molecules that regulate the enzymatic activities of other coregulators from the transcriptional complexes (McKenna, Lanz et al. 1999; McKenna and O'Malley 2002; McKenna and O'Malley 2002; O'Malley, Qin et al. 2008; O'Malley and Kumar 2009).

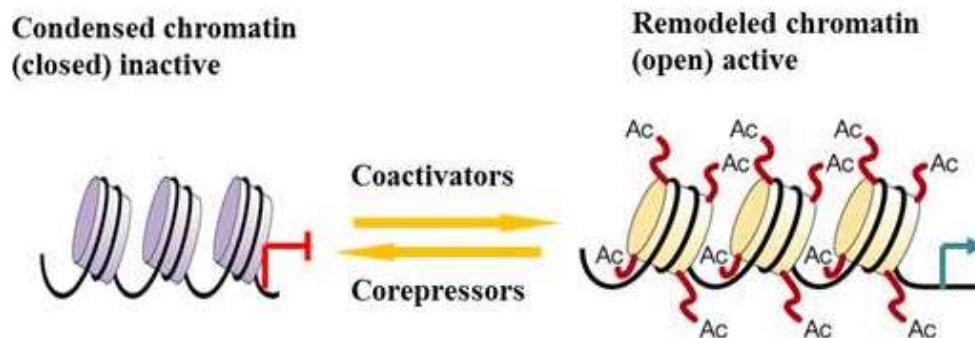


Fig.15. Coregulators are involved in chromatin remodeling. Corepressors enhance chromatin condensation leading to gene silencing, whereas coactivators acetylate histones, loosening the nucleosomal structure, so that DNA specific response elements might be revealed and transcription might be initiated.

Histone acetyl transferase (HAT) coactivators are enzymes that acetylate internal lysine residues of histone N-terminal domains, which is associated with loosening of the nucleosomal structure, opening the nucleosome to transcription factors (Kuo and Allis 1998). Conversely, histone deacetylases recruited by corepressors reverse this reaction, silencing transcription of the target gene (Nagy, Kao et al. 1997). Other histone modifications have similar or opposite effects on transcription. The term “histone code” defines the combination of these modifications.

In addition, some coactivators can also bind to a secondary coactivator, which in turn binds to transcription factors, but must rely on the coregulators complex and other factors for enzymatic chromatin modifications (Spiegelman and Heinrich 2004).

Coregulators are not specific for a particular receptor, forming molecular complexes where many coregulators contribute and might compensate for NRs modulation of transcription (Gehin, Mark et al. 2002). The variation in cellular levels of coactivators and corepressors determines the activation pathways of gene transcription.

Coregulators		References
Coactivators		
SRC-1/NCoal	Interacts with CBP/p300, PCAF; possesses acetyltransferase activity; coactivates nuclear receptors, such as PPAR γ ; targeted deletion causes partial insensitivity;	(Onate, Tsai et al. 1995; Onate, Boonyaratanakornkit et al. 1998; Heinlein and Chang 2002; Lonard and O'Malley 2012)(Wang, Stafford et al. 1998; Xu, Qiu et al. 1998)
SRC-2 /GRIP1/TIF2	Initially identified as a coactivator, might also mediate promoter-dependent corepression; mice invalidated for SRC-2 are hypofertile	(Wang, Stafford et al. 1998; Rogatsky, Zarembek et al. 2001);(Gehin, Mark et al. 2002)
SRC-3 /TRAM1/RAC3/AIB-1/pCIP	Coactivates PR, RAR, TR; coactivates ER α over beta Overexpressed in breast tumours and ovarian cancer	(Anzick, Kononen et al. 1997; Takeshita, Cardona et al. 1997)(Osborne, Bardou et al. 2003)
PGC-1	Transduces GR and CREB-mediated hepatic gluconeogenesis	(Herzig, Long et al. 2001)
RIP140	Coactivator of ER At high receptor/coregulator ratio functions as a AR, GR, ER, TR, PPAR α and PPAR γ corepressor	(Cavailles, Dauvois et al. 1995; Lee, Chinpaisal et al. 1998; Heinlein and Chang 2002; Fritah, Christian et al. 2010)
ERAP 160	ERAPs bind ER in ligand dependent manner	(Halachmi, Marden et al. 1994)
ARA54 ARA55 ARA-70	AR coactivators in prostate cells	(Yeh and Chang 1996; Sampson, Yeh et al. 2001)
PIAS3	AR and GR coactivator	(Kotaja, Karvonen et al. 2002; Kotaja, Vihinen et al. 2002)
Corepressors		
NCoR	Corepresses TR α , RAR α , DAX1; contains autonomous repression domains; reduces RU486/PR partial agonist activity	(Horlein, Naar et al. 1995; Zamir, Harding et al. 1996; Shibata, Nawaz et al. 1997)(Crawford, Dorn et al. 1998)
SMRT	Interacts with and corepresses unliganded TR and RAR; presents sequence similarity with NCoR; reduces tamoxifen/ER and RU486/PR	(Chen and Evans 1995; McKenna and O'Malley 2002)
HBO1	ER α , PR, GR, MR coactivator AR corepressor	(Sharma, Zarnegar et al. 2000; Georgiakaki, Chabbert-Buffet et al. 2006; Wang, Liu et al. 2010)
PIAS1	AR, corepressor but also	(Tan, Hall et al. 2000; Kotaja,

	coactivator GR and PR coactivator	Karvonen et al. 2002; Kotaja, Vihinen et al. 2002; Tan, Hall et al. 2002; Yan, Santti et al. 2003)
PIASx	AR corepressor	(Nishida and Yasuda 2002)

Table 3. Selected recent reports on several most known receptor coregulators (adapted from (McKenna, Lanz et al. 1999; McKenna and O'Malley 2002; McKenna and O'Malley 2002; O'Malley and Kumar 2009)). Certain coactivator proteins, contain HAT activity and bind directly to transcription factors (TF), as well as recruit other HATs (Fig. 16.).

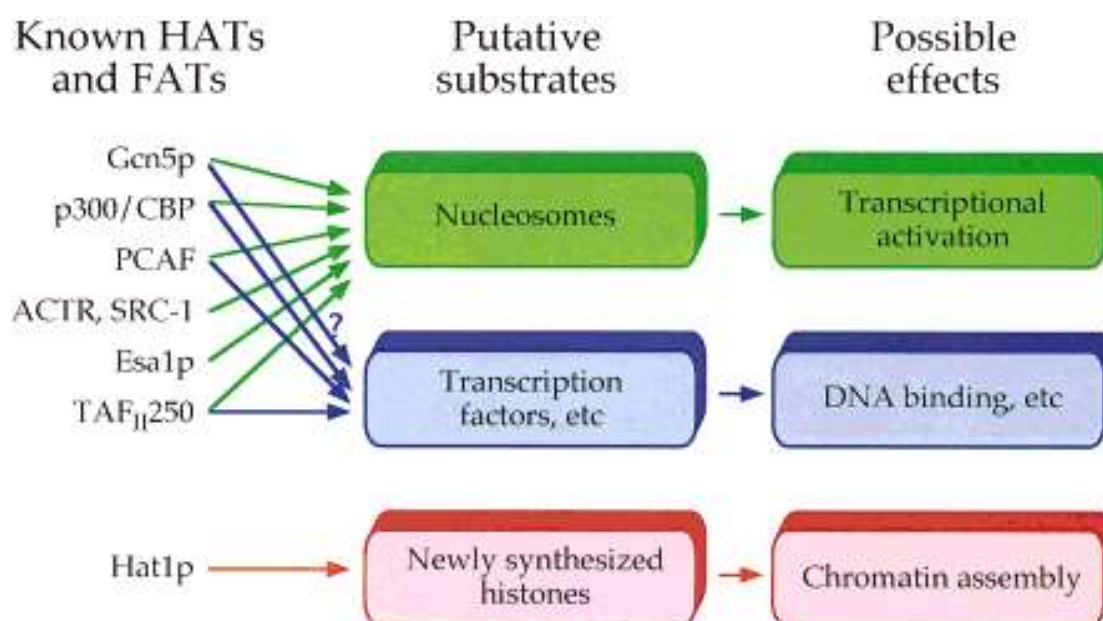


Fig.16. Representative scheme of *in vivo* putative substrates for HAT and possible effects of their enzymatic activities (reproduced from (Kuo and Allis 1998)).

Moreover, as coregulators are not primarily regulated at the level of DNA transcription, their functions are mainly dictated by a combination of posttranslational modifications (PTMs), including phosphorylations, methylations, acetylations, ubiquitinations and SUMOylations.

These PTMs are essential for coregulator activation/inactivation, assembly of specific partners in the functional complex, targeting specific transcription factors for regulation, and even for the degradation of the coregulator proteins. Indeed, acetylation and ubiquitination alter the half-life of coactivator complexes (O'Malley, Qin et al. 2008), whereas phosphorylation of some coactivators (SRC-1, PGC1 α) increases promoter recruitment and

interaction with NRs (Han, Lonard et al. 2009). Moreover, protein kinase C can mediate SRC-3 phosphorylation, increasing the cellular SRC-3 protein level in a selective ER-dependent manner, making it more resistant to proteasomal degradation, thus determining SRC-3 stabilization and effect on tumorigenesis (Yi, Feng et al. 2008).

Furthermore, tissue-specific metabolic pathways are regulated by tissue-specific posttranslational modifications of coactivators; for example, acetylation and phosphorylation of coregulator PGC-1 α is involved in gluconeogenesis and fatty acid metabolism in liver (Lerin, Rodgers et al. 2006; Han, Lonard et al. 2009).

5.1.3. Coactivators

In 2002, Neil McKenna (McKenna and O'Malley 2002), classified coactivators in several classes based upon their functional properties, such as:

- Acetyltransferases- represented mainly by the members of the SRC family (Leo and Chen 2000)
- Ubiquitin ligases (Nawaz, Lonard et al. 1999),
- ATP-coupled chromatin remodeling complexes (Fryer and Archer 1998)
- Protein methylases, such as CARM-1 (Chen, Ma et al. 1999)
- RNA transcripts
- Cell cycle regulators
- RNA helicases
- Members of the TRAP/DRIP complex, which foster direct contact with components of the basal transcription machinery (McKenna, Lanz et al. 1999; McKenna and O'Malley 2002; McKenna and O'Malley 2002).

The first discovered and most studied nuclear receptor coactivators, with important implications in human physiology and pathology, belong to the SRC/p160 family. These coactivators are characterized by the presence of common structural domains and motifs (Fig. 17), such as:

- N-terminal tandem PAS (Per-ARNT-Sim domain) and basic, beta helix-loop-helix (bHLH) motifs, involved in protein recognition and homodimerization;
- A central domain which binds to the coactivators CBP and p300;
- Single or multiple copies of central amphipathic alpha-helical LXXLL motifs (a contiguous sequence of five amino acids, where L=leucine and X=any amino acid),

also named “nuclear receptor boxes”(NR boxes); these motifs are involved in ligand dependent recruitment by the AF-2 of the nuclear receptors (Heery, Kalkhoven et al. 1997).

- Two intrinsic activation domains (AD1 and AD2) located C-terminal to the receptor-interacting domain. Interestingly, the AD1 domain also contains three additional LXXLL motifs representing NR boxes iv, v, and vi, that interact with coactivators CBP/p300, so that AD1 plays a role in recruiting acetyltransferases (Leo and Chen 2000; Xu and Li 2003). The second activation domain (AD2) is responsible for interaction with histone methyltransferases to a promoter/enhancer, which may be critical for NR-mediated chromatin remodeling and assembly of the transcriptional machinery around the promoter (Chen, Ma et al. 1999).
- A C-terminal region with histone-actyltransferase activity;
- A C-terminal region which mediates interaction with the CARM-1 coactivator.

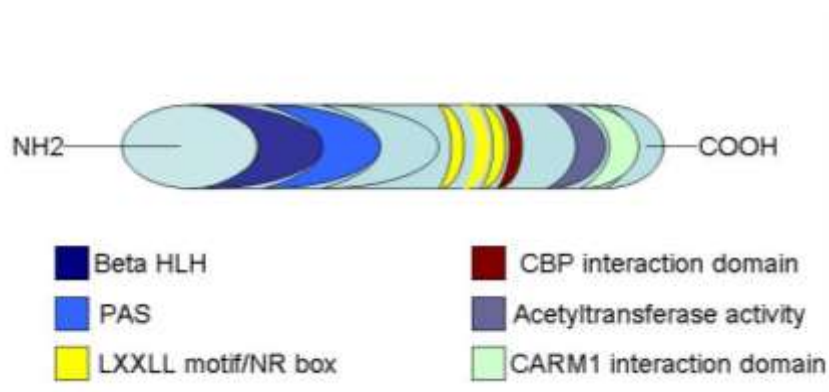


Fig.17. General structure of the SRC/p160 family of coactivators (adapted from McKenna and O'Malley 2002).

5.1.4. Corepressors

The first two corepressors: nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT), were simultaneously identified by Ron Evans' team (Chen JD Nature 1994) and in Geoff Rosenfeld's laboratory (Horlein, A.J.,1995, Nature) thanks to their ability to interact with unliganded NRs.

Analogous to coactivators, recruitment of corepressors, generally occurring in the absence of ligand, depends on a critical conformation of the nuclear receptor AF-2 domain, as well as upon the presence of specific nuclear receptor box-like helical motifs in the corepressor (Li, Wang et al. 2000). Moreover, corepressors themselves recruit ancillary enzyme activities which help to establish or maintain the repressive state at their target promoters. More recently, it has been shown that ligand-activated transcription factors can recruit corepressors leading to gene silencing (Battaglia, Maguire et al. 2010).

The interaction between NCoR/SMRT and NRs is dependent on CORNR boxes (nuclear receptor interaction motifs exhibiting a consensus sequence of LXX I/H I XXX I/L), expressed on the corepressor and on the nuclear LBD-receptor pocket also required for coactivator binding, formed by helices 3/5/6 of the NR LBD (Hu and Lazar 1999; Perissi, Staszewski et al. 1999; Xu, Stanley et al. 2002). Steroid receptors display low corepressor binding in the absence of hormone but gain an increased ability to bind corepressors in the presence of hormone antagonists; ligands such as tamoxifen or RU486 (selective hormone response modulators or SHRMs) induce helix 12 conformations that favor corepressor binding and are distinct from the conformations assumed in the absence of hormone or in response to hormone agonist (Shiau, Barstad et al. 1998; Farboud, Hauksdottir et al. 2003; Privalsky 2004; Grinspon and Rey 2011).

NCoR and SMRT recruit proteins that have histone deacetylase activity, converting chromatin to an inactive form (Guenther, Barak et al. 2001). In addition, receptor isoform and heterodimer composition, as well as DNA binding sequence and interactions with other transcription factors at the promoter influence the ability of NCoR and SMRT to bind to corepressor docking sites on the NR (Privalsky 2004).

Although NCoR and SMRT have structural similarities, genetic deletion of either protein results in embryonic lethality, indicating that they cannot fully compensate for each other during development. Moreover, gene knockout studies reinforce the different and nonredundant roles of the two corepressors, as NCoR is required for CNS, erythrocyte, and thymocyte development, while SMRT is required for cardiac development and maintenance of neural stem cells (Privalsky 2004; Ghisletti, Huang et al. 2009).

At present, a large panel of corepressors has been identified, with different implications, expression and roles in human tissues, such as REA (repressor of estrogen receptor activity), RIP140, HBO1 or PIAS proteins (Table.3.).

5.1.5. Mechanism of interaction between nuclear receptors (NR) and coregulators

The conformation of the LBD domain of the unliganded receptor allows constitutive interaction with coactivators and corepressors (Fig.18). Specifically, there are interactions between the hydrophobic groove of the LBD region with either LXXLL/FXXLF motifs of coactivator NR boxes(van de Wijngaart, Dubbink et al. 2012) or the LXX I/H I XXX I/L motif of corepressor nuclear receptor box (CoRNR) present in the corepressors(Hu and Lazar 1999). Ligand binding induces conformational changes in the nuclear receptors LBDs, repositioning the hydrophobic residues in helices 3, 4, 5, and 12 of the LBD in such a way that the size of the LBD groove can now accommodate shorter LxxLL-motifs of coactivators (also named NR boxes), but not the larger CoRNR box of corepressors(Glass and Rosenfeld 2000).

Transcriptional regulation of NRs by coregulators is very complex and largely depends on the cell type, the receptor, the coregulator and the promoter context. Moreover, a coregulator can have dual roles and variable effects depending on the cellular and promoter contexts, as well as the receptor subtype. For example, HBO1 can increase the transcriptional response for the progesterone receptor (PR)(Georgiakaki, Chabbert-Buffet et al. 2006), while inhibiting transactivation properties of the androgen receptor (AR) (Sharma, Zarnegar et al. 2000). Depending on cell type, SRC-1 might increase PR transcriptional activity in response to progesterone in uterine stroma and myometrium, while it decreases PR transcription in the luminal and glandular compartments (Han, DeMayo et al. 2006). RIP140 is another coregulator with a dual role, since it can serve as a co-activator as well as a co-repressor in liver cells (Herzog, Hallberg et al. 2007).

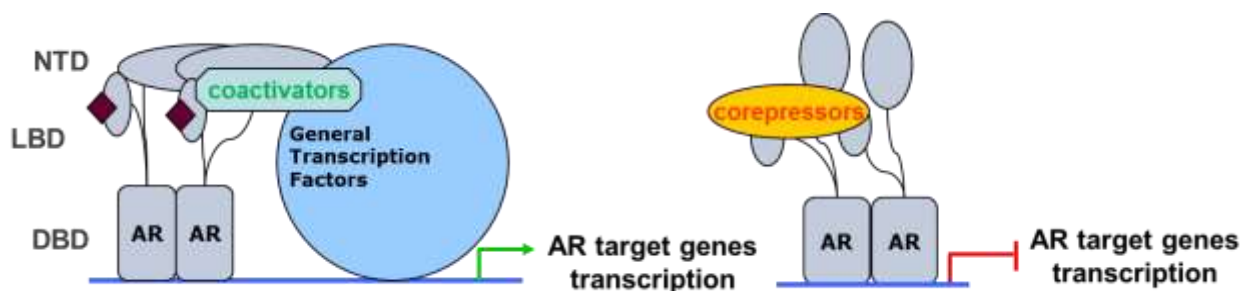


Fig.18. Model of coregulator recruitment by nuclear receptors. Corepressors recruited by unliganded steroid receptor enhance chromatin condensation leading to gene silencing; in the presence of the ligand (agonist), a conformational change of the receptor provides a steric environment which allows recruitment of transcriptional coactivators; this leads to chromatin decondensation. Coactivators are grouped in multimolecular complexes, as they serve as scaffolding protein for other coregulators, facilitating assembly of coactivator complexes and gene transcription factors, ultimately activating gene transcription. NTD, amino terminal domain; DBD, DNA binding domain, LBD, ligand binding domain.

5.1.6. Coregulators involvement in biological processes

Using sequences from cloned coregulator genes, several research teams, such as those coordinated by Bert O'Malley (SRC-1)(Xu, Qiu et al. 1998), Geoff Rosenfeld (NCoR) (Jepsen, Hermanson et al. 2000) and Pierre Chambon (SRC-2) (Gehin, Mark et al. 2002) developed knockout mouse models which brought light on a series of physiological roles of some coregulators. The effects of the lack of expression of certain coregulators might range from embryonic lethality as revealed in 53 out of 92 knockout models (Lonard, Lanz et al. 2007), concerning coregulators such as TRAP220 (Landles, Chalk et al. 2003), HBO1(Kueh, Dixon et al. 2011) to the more subtle developmental and metabolic phenotypes associated mainly with members of the SRC family.

Mouse genetic studies have demonstrated distinct roles for each SRC in reproduction, cancer and energy metabolism. The effects on reproduction and cancer shall be discussed later on. Regarding SRC roles in metabolism, each coactivator exerts particular roles in adipogenesis and energy expenditure. SRC-1 knockout mice become obese due to decreased energy expenditure while SRC-2 knockout mice are lean due to the reduced transcriptional capacity of PPAR γ 2, a nuclear receptor involved in adipocyte differentiation (Picard, Gehin et al. 2002) Conversely, SRC-3 promotes white adipose cell differentiation, and adult SRC-3 null mice have a decrease in the adipose tissue mass (Louet, Coste et al. 2006).

Present studies showed that both coactivators and corepressors have crucial roles in the development of certain organs and for the normal functioning of thyroid and steroid hormones.

5.1.7. Coregulators and human disease

The multitude of nuclear receptor coregulators, with their wide implications in a variety of molecular functions are involved in several human pathologies, such as cancer, metabolic syndromes (obesity, diabetes) and heritable syndromes like Rubinstein-Taybi syndrome (CBP/p300), Von Gierke's disease (SRC-2) or Angelman syndrome (E6-AP). Recent reviews of the role of coregulators in human disease, presented that more than 165 of the known coregulators are involved in human pathologies (Lonard, Lanz et al. 2007; Lonard and O'Malley 2012).

During the last 18 years (since the cloning of the first nuclear coregulator), several studies linked coregulators to cancer. Expression profiling in solid tumors revealed specific patterns of altered expression and localization of several coregulators, such as the expression of coactivators like SRC-1, SRC-2 and mainly SRC-3, or corepressors, such as NCOR1 and NCOR2/SMRT in breast, bladder, and prostate cancers (Battaglia, Maguire et al. 2010). Coregulators with dual functions, such as MTA1 (metastasis-associated gene) (O'Malley and Kumar 2009) play also important roles in the biology of cancer. The cancer profiling Oncomine database provided analysis of more than 285 coregulators and reported a preponderance of coregulators overexpression rather than underexpression in human cancers especially for breast, prostate, lung cancer, leukemias and lymphomas (Lonard, Lanz et al. 2007). Moreover, coregulators might act as proto-oncogenes, such as SRC-3 (Anzick, Kononen et al. 1997), or MTA (metastasis-associated gene) (Toh and Nicolson 2009), while some corepressors have been identified as tumor suppressors (RIP140 and BRMS1) (Toh and Nicolson 2009; Battaglia, Maguire et al. 2010).

The overexpression of several coactivators of the p160/SRC family such as SRC-3/ACTR/AIB1 is critical for the proliferation of hormone-dependent and independent breast (ER positive and ER negative), prostate cancer cells and for tamoxifen resistance (Agoulnik, Vaid et al. 2006; O'Malley and Kumar 2009; Spears, Oesterreich et al. 2012).

The two corepressors NCoR and SMRT have been linked not only to resistance to thyroid hormone (Safer, Cohen et al. 1998), but also to acute pro-myelocytic leukaemia and acute myeloid leukaemia (O'Malley and Kumar 2009).

The complex implications of the androgen receptor coregulators in human prostate cancer, in androgen insensitivity syndromes and infertility will be detailed below.

The diversity of coregulators and their complex mechanisms of regulation may explain their diverse roles in human physiology and pathology, as well as distinct differences between individuals in response to specific genetic, environmental and dietary aspects (Table 4.).

5.2. Androgen receptor (AR) coregulators

5.2.1. Introduction

Androgens mediate a wide range of developmental processes being especially important in testicular differentiation, pubertal sexual maturation and the maintenance of spermatogenesis. The effects of androgens are mediated through the androgen receptor (AR), a 110kDa ligand-inducible nuclear receptor that regulates the expression of target genes through binding to androgen response elements (Keller, Ershler et al. 1996) The interaction of the androgen receptor (AR) with specific coregulatory proteins is a potential mechanism for modulating specificity of androgen actions.

Androgen receptor interactions with the RNA polymerase II holocomplex and the chromatin environment depends upon AR coregulators, which influence the ligand selectivity, the DNA binding capacity and the transcriptional activity of AR(Heinlein and Chang 2002; Heinlein and Chang 2002; Urbanucci, Waltering et al. 2008; van de Wijngaart, Dubbink et al. 2012).

5.2.2. Classification of AR coregulators

In May 2007, Heemers and Tyndall listed 169 AR coregulators, while in August 2013, in the Androgen Receptor Gene Mutations Database (<http://androgendb.mcgill.ca>) there were reported more than 350 AR coregulators and interacting proteins, including 168 AR coactivators and 89 AR corepressors. This large list of AR coregulators can be classified in many ways, starting from the division in AR coactivators and corepressors, having in consideration their effect on the AR-mediated transcriptional activity, to the classification upon primary functions, the last type of classification being preferred (Heinlein and Chang 2002; Heemers and Tindall 2007; Lonard and O'Malley 2012).

Initially, Heinlein classified AR coregulators as Type I coregulators, (that function primarily with the nuclear receptor at the target gene promoter to facilitate DNA occupancy, chromatin remodeling, or the recruitment of general transcription factors associated with the RNA polymerase II holocomplex) and Type II coregulators that primarily modulate ligand binding or facilitate NH₂/COOH-terminal interaction (N/C interaction), events which may contribute

to AR protein stability in the presence of agonistic ligands or influence the subcellular distribution of AR. Principal type I AR coregulators are the three members of the SRC family - SRC-1 (NCoA-1), SRC-2 (TIF2/GRIP1/NCoA-2), SRC-3 (NCoA-3), while one example of type II coregulator might be ARA70 that facilitates the translocation of the ligand-bound receptor to the nucleus (Heinlein and Chang 2002).

Later on, Heemers and Tyndall provided a new and exhaustive classification of AR coregulators, upon their functions, such as the organization of AR coregulators related to their mechanisms involved in altering the chromatin structure (this is realized either by covalent modification of histone molecules by (de)acetylation, (de)methylation, (de)phosphorylation, ubiquitination, and sumoylation, or by chromatin remodeling), related to their involvement in RNA metabolism, DNA repair, to their roles as chaperones, cytoskeletal proteins, proteins involved in endocytosis, as well as their roles as adaptors, cell cycle and apoptosis regulators (Heemers and Tindall 2007).

Given the multitude of these diverse functions, I shall not detail this classification, and rather prefer to present a brief overview of the AR coregulators mechanisms of modulation of AR transcription, dividing them in coactivators and corepressors. I shall also focus on some AR coregulators and their implications in human physiology and pathology. A short list of AR coregulators and their expression in human tissues is presented in Table 4.

5.2.3. Modulation of AR transcriptional activation by androgen receptor coactivators

AR modulation of transcription activation implies the creation of an open chromatin structure. In this respect, AR recruits different proteins that facilitate transcription activation, called coactivators. They are a component of, or contribute to the recruitment of different complexes, such as the histone modifying enzymes (HME), the chromatin remodeling complex (SWI/SNF), the Mediator complex and the preinitiation complex (PIC) necessary for AR transcription activation (Fig.19).

Upon ligand binding, AR forms a homodimer that binds to specific DNA sequences, named androgen-response elements (ARE), located in the promoter regions of target genes. Ligand induces conformational changes in the ligand-binding domain that enable AR to interact with coactivator proteins and to activate transcription. Insertion of the agonist (testosterone/dihydrotestosterone) in the AR LBD pocket changes conformation and stabilizes

the α -helix 12 of the AF-2 domain which, together with the hydrophobic residues 3,4 and 5, serves as interaction surface with the LXXLL-motif containing coactivators (Heemers and Tindall 2007; van de Wijngaart, Dubbink et al. 2012).

AR differs from the other nuclear receptors by its specific interactions with coactivators. First, the AR LBD displays weak or no AF2 activity, due to different affinities for the coregulators LXXL binding motifs. Unique for the AR, it harbors on the AR N-terminal domain its own FXXLF (FQNLF) motifs (He, Kempainen et al. 2000). AR LBD AF2 binds to LXXL motifs of the SRC/p160 family of coactivators with an affinity 5-10 times weaker than that of other NRs (He, Kempainen et al. 1999; He, Kempainen et al. 2000; He, Gampe et al. 2006). Moreover, the hydrophobic groove of the AR LBD AF2 facilitates intra and intermolecular interactions with the FXXLF and WXXLF-binding motif (⁴³³WHTLF⁴³⁷) present on the AR N-terminal domain, creating the N/C interactions (He, Kempainen et al. 2000; He, Bowen et al. 2001; He, Lee et al. 2002), which make AR not available for coactivator binding.

When the homodimer-ligand complex binds to DNA androgen response elements (ARE), the N/C interaction is disrupted (Fig.19).

Name	Function	AR Coactivator/ Corepressor	Human tissue distribution/ Implications in human physiology/pathology
TRAP220 /MED1	Recruitment preinitiation complex	Coactivator	Ubiquitous expression in human tissues Overexpressed in prostate adenocarcinoma and in breast and ovarian ER positive cancers (Zhu, Qi et al. 1999; Vijayvargia, May et al. 2007) Its absence leads to embryonic lethality
SRC-1	Histone acetyltransferase	Coactivator	Expressed in Purkinje cells, hepatocytes, adipocytes, muscle breast, prostate, gastric epithelium, testis, normal and ectopic endometrium (Heemers and Tindall 2007; Shi, Xu et al. 2014) Overexpressed in breast and prostate cancer; associated with early resistance to cancer therapy and poor disease-free survival (Kurebayashi, Otsuki et al. 2000; Myers, Fleming et al. 2004; O'Malley and Kumar 2009) Underexpressed in urothelial bladder cancer (Boorjian, Heemers et al. 2009)
SRC-2	Histone	Coactivator	Involved in brain, ER/PR dependent breast

	acetyltransferase		cancer, in prostate and urothelial bladder cancer(Carapeti, Aguiar et al. 1998; Kurebayashi, Otsuki et al. 2000; Deguchi, Ayton et al. 2003; Boorjian, Heemers et al. 2009; O'Malley and Kumar 2009; Heemers, Schmidt et al. 2010) Chromosomal rearrangements involving SRC-2 correlates with acute myeloid leukemia (Carapeti, Aguiar et al. 1998)
SRC-3	Histone acetyltransferase	Coactivator	High expression in heart, skeletal muscle, pancreas and placenta. Low expression in brain, and very low in lung, liver and kidney (www.UniProtKB). Also expressed in normal endometrium, prostate. Involved in breast and tamoxifen resistance, ovarian, endometrial, digestive, urothelial bladder cancers, but also in polycystic ovary syndrome. Overexpression correlates with prostate cancer high tumour grade and stage. (Villavicencio, Bacallao et al. 2006; Boorjian, Heemers et al. 2009; O'Malley and Kumar 2009; Battaglia, Maguire et al. 2010)
ARA 54	E3-ubiquitin protein ligase	Coactivator	Expressed in normal human spleen, thymus, prostate, testis (immunodetected in round spermatids)(Lan, Hseh et al. 2008); expressed in colorectal carcinoma and prostate cancer (Fujimoto, Yeh et al. 1999; Kang, Yeh et al. 1999; Kikuchi, Uchida et al. 2007)
ARA 55	E3-ubiquitin protein ligase	Coactivator	Expressed in normal human testis (peritubular myoid cells)(Lan, Hseh et al. 2008) and in prostatic stromal compartment(Heitzer and DeFranco 2007) Involved in prostate cancer progression free survival(Miyoshi, Ishiguro et al. 2003).
ARA 70	E3-ubiquitin protein ligase	Coactivator	Expressed in human testis, endometrium (Villavicencio, Bacallao et al. 2006; Lan, Hseh et al. 2008) Overexpressed on polycystic ovarian syndrome and in prostate cancer(Hu, Yeh et al. 2004; Villavicencio, Bacallao et al. 2006) Involved in androgenic alopecia(Lee, Zhu et al. 2005)
PIAS1	SUMO E3-protein ligase	Corepressor/ Coactivator	Predominates in human testis, in PTM, Sertoli, Leydig and germ cells(pachytene spermatocytes and round spermatids)(Lan, Hseh et al. 2008; Tan, Hall et al., 2000)

PIAS3	SUMO E3-protein ligase	Corepressor	Expressed only in brain, heart, thymus, muscle, lung, testis, lactating breast and embryonic stem cells(www.UniProtKB)
NCoR	Recruitment of histone deacetylases	Corepressor	Responsible for the syndrome of thyroid hormone resistance(Yoh and Privalsky 2000) Involved in lipid disorders related to hypothyroidism(Zhu, Kim et al. 2011) Overexpressed in colorectal carcinomas and endometrial cancers Also linked to acute promyelocytic leukemia and myeloid leukemia(Privalsky 2004; Petrie, Prodromou et al. 2007)
RIP140	Histone deacetylase	Corepressor/ Coactivator	Expressed in human adipose tissue(Otway, Mantele et al. 2011) Expressed in breast cancer tumors(Docquier, Harmand et al. 2010)
HBO1	Histone acetyltransferase	Corepressor	Abundantly expressed in human testis, ovary. Detectable in thymus, prostate, colon, intestine and peripheral blood leukocytes (Sharma, Zarnegar et al. 2000). Overexpressed in carcinomas of the testis, ovary, breast, stomach/esophagus, and bladder (Iizuka, Takahashi et al. 2009; Wang, Liu et al. 2010).

Table 4. List of some coregulators that modulate AR transcriptional activity and that are identified in normal human tissues and certain human pathologies. More extensive lists have been consulted (Heinlein and Chang 2002;Heemers and Tindall 2007; van de Wijngaart, Dubbink et al. 2012)

AR transactivation through AF2 in the presence of androgens seems to be limited not only by the weaker binding affinity to the LXXL motifs, but also through competitive inhibition of coactivator binding to the AR FXXLF motifs (He, Gampe et al. 2006).

Recently, the structural basis for this selectivity was brought to light by X-Ray crystallography studies showing that the AR-NTD FxxLF motif presents a specific conformation so that the coregulator hydrophobic residues interact better with the glutamic acid residue from the LBD helix 12. In contrast, an LxxLL motif peptide fails to make hydrogen bond contacts with the glutamic acid residue in helix 12 and makes fewer

hydrophobic contacts on the surface pocket on the LBD (Hur, Pfaff et al. 2004; Duff and McEwan 2005).

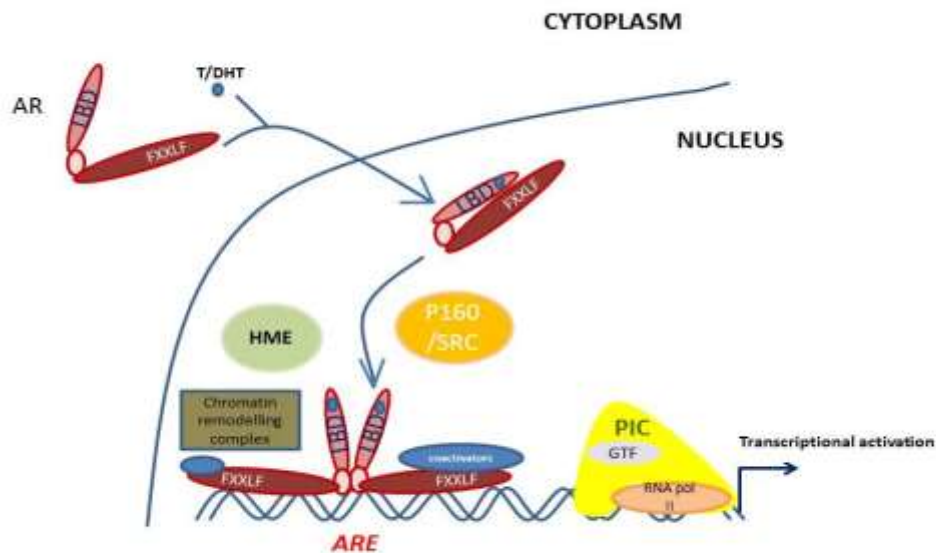


Fig.19. Schematic presentation of the AR transcription modulation by coactivators. After ligand binding, N/C interaction is initiated rapidly; when the homodimer-ligand complex binds to DNA androgen response elements (ARE), the N/C interaction is disrupted, allowing interactions with other molecules. At this moment, coregulators forming diverse complexes are recruited in order to modulate chromatin and initiate transcription regulation.

AR, androgen receptor; ARE, androgen response element;

T/DHT, testosterone/dihydrotestosterone; HME, histone modifying enzymes; GTF, general transcription factors; RNA pol II, RNA polymerase II; schema adapted from (Heemers and Tindall 2007; van de Wijngaart, Dubbink et al. 2012)

Zacharov demonstrated by an elegant computational modeling that in the presence of the same ligand, AR-LBD can occupy distinct conformational states depending on its interactions with specific coactivators, as different coactivators (such as SRC-3) induce specific

alterations in the backbone flexibility of AR-LBD even distant from the site of coactivator binding (Zakharov, Pillai et al. 2011).

Specifically, SRC-1 and SRC-2 primarily interact with the AR N- terminal domain and possibly the DBD, without stabilizing the N/C terminal interaction of AR (He, Kemppainen et al. 1999). Moreover, SRC-1 potentiates receptor activity via recruitment of other coregulators with histone acetyltransferase activity, such as CBP/p300 (Powell, Christiaens et al. 2004).

Unlike other NRs that interact with coregulator LXXLL motifs, AR and PR exhibited an interaction with a subset of FxxLF motifs. Phenylalanines are essential for interaction with AR LBD, as substitution of the phenylalanine residues by leucines in the FxxLF motifs of ARA54 and ARA70 entirely abolish AR LBD interaction, while substituting the leucine residues at positions +1 and +5 of LxxLL motifs by phenylalanines increases AR LBD affinity (He, Lee et al. 2002; Dubbink, Hersmus et al. 2004; Dubbink, Hersmus et al. 2006). Dubbink has also assessed FxxLF motifs and their interaction with other NRs LBD.

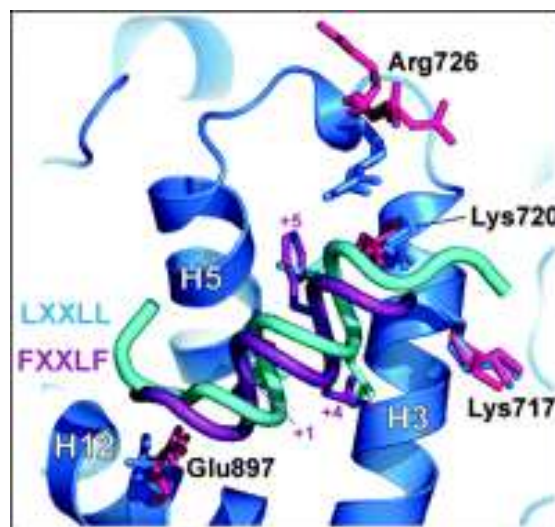


Fig. 20. AR FxxLF and LXXLL binding modes to the AR LBD as Revealed by x-Ray Crystallography (image reproduced from (Dubbink, Hersmus et al. 2006)). Helices H3–5 and H12 of AR LBD are drawn schematically in blue. FXXLF and TIF2 NR box III LXXLL peptides are presented as purple and cyan coils. Orientations of LBD residues analyzed were shown from five independent structures of FXXLF (pink) and LXXLL (blue) peptide complexes. The FXXLF peptide fully engages the charge clamp (K720/E897) whereas the TIF2 NR box III LXXLL peptide is shifted toward the K720 end of the cleft. The coordinates of AR LBD in complex with TIF2 box III LXXLL (Protein Data Bank codes: 1XQ2, 1T63),

ARA70 FXXLF (1T5Z), AR FXXLF (1XOW), and phage display FXXLF (1T7R) were superposed using the LBD residues alone for this comparison.

The sequences were illustrated by F/L swapping experiments of FxxLF and LxxLL-based peptide motifs, as shown in Fig.20.

In conclusion, phenylalanine residues at positions +1 and +5 strongly contribute to preferential and strong interaction with AR LBD and prevent binding to most other NR LBDs, allowing specific cofactor-AR LBD interactions.

There are also FxxLM and FxxLW as well as FxxFF or FxxMF variants (M = methionine; W = tryptophan) that are compatible with AR LBD binding although these motifs interact weaker with AR LBD than the wild type FxxLF motif (Dubink, Hersmus et al. 2004).

Coactivators binding to AR LBD via FxxLF motifs are AR-specific coregulators (such as ARA54 or ARA70).

5.2.4. Modulation of transcriptional repression by androgen receptor corepressors

Data related to potential AR corepressors have been brought by the study of the genomic profile of prostate cancers, as in human prostate cancer and androgen-dependent prostate cancer cells, 20–40% of genes are down-regulated by AR activation (Hendriksen, Dits et al. 2006). Van de Wijngaart's team showed that protein translation inhibition with cycloheximide abolishes the androgen-induced down-regulation of most of the repressed genes, showing that this was mainly an indirect effect, probably modulated by AR corepressors (van de Wijngaart, Dubink et al. 2012). AR corepressors either inhibit general functions such as DNA-binding or coactivator binding, but also might be involved in transcription repression, acting as histone deacetylases which revert the open chromatin state back to a condensed form (Table 4.).

The first and best-studied AR corepressors are NCoR and SMRT. These corepressors were identified as interacting proteins of unliganded thyroid hormone and retinoic acid receptors (Perissi, Staszewski et al. 1999).

The silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the related nuclear receptor corepressor (N-CoR) form complexes with the unliganded receptors to facilitate transcriptional repression of gene expression (Chen and Evans 1995). SMRT inhibits

ligand-dependent transcriptional activation by AR, also leading to disruption of AR N/C interaction and/or competition with the p160 coactivators (Liao, Chen et al. 2003). Liao et al (Liao, Chen et al. 2003) revealed that SMRT interacts directly with both the unliganded and liganded AR both in vivo and in vitro (HEK cell line) through the C-terminal NR-interaction domain (ID2) of SMRT. The minimal interacting surface on AR is mapped to the LBD/E domain, while the presence of DBD/hinge region of AR enhances this interaction substantially.

Knocking down SMRT and N-CoR using siRNA, enhanced the recruitment of the coactivators SRC-1 and p300 by agonist-bound AR and led to hyperacetylation of histone H3 and H4, suggesting that the corepressors actively compete with coactivators for binding to agonist-bound AR (Yoon and Wong 2006).

NCoR and SMRT transcription repression is mediated through Sin3 and recruitment of histone deacetylases (HDACs). HDACs perform the opposite function of histone acetyltransferases (HATs) and deacetylate histones, thereby compacting nucleosomes into tight and inaccessible structures.

In its unliganded state, the AR seems not to interact strongly with NCoR or SMRT, but these corepressors are recruited when the AR is occupied by antagonists such as cyproterone acetate, hydroxy-flutamide or bicalutamide (Liao, Chen et al. 2003; Hodgson, Astapova et al. 2005; Hodgson, Astapova et al. 2007). Moreover, the AR can be inhibited by expression of NCoR and SMRT through direct AR binding, when AR is in agonist-activated state (Cheng, Brzostek et al. 2002).

Both the AR NTD and LBD are required for corepressor binding, which is dependent on specific corepressors nuclear receptor (CoRNR) boxes which are located in the C-terminal part of NCoR and SMRT (Cohen, Wondisford et al. 1998; Perissi, Staszewski et al. 1999).

HBO1 (KAT7) belongs to the MYST family; it is characterized by a highly conserved histone acetyltransferase domain. Sharma mapped a transcriptional repression domain within the N-terminal region of HBO1 and found that HBO1 associates with the LBD, but not the NTD of AR to inhibit transcriptional coactivation by the ARA70 cofactor in mammalian cells (Sharma, Zarnegar et al. 2000).

The PIAS family (PIAS1, PIAS3, etc) comprises SUMO-E3 protein ligases that bind to AR DBD, presenting dual and dose-dependent bi-directional effects on AR transactivation (Tan, Hall et al. 2002).

5.2.5. Animal knockouts and the biological roles of AR coregulators

Coregulator knock-out mouse models provided precious information on the physiological impact of AR coregulators on the living organisms. Lonard, in a review on coregulator involvement in human disease, identified 92 coregulator knockout mouse models; 53 of which die during embryogenesis, while the others develop a wide spectrum of phenotypes with alterations in fertility and metabolism.

The pattern varies from hormone-resistant phenotypes with varying degrees, to compromised fertility related to hypospadias and changes in the morphology and degree of maturation of prostate, seminal vesicles, and testis (Heemers and Tindall 2007).

SRC-1 null mice present with partial steroid hormone resistance in reproductive organs such as uterus, prostate, testis, and mammary gland, as well as smaller testicular and prostatic sizes, without fertility impairment (Xu, Qiu et al. 1998).

In contrast, complete invalidation of SRC-2 in mice (Gehin, Mark et al. 2002) was compatible with survival, but the fertility of both sexes was impaired. Starting from 3 months of age, SRC-2 null male mice become hypofertile and exhibit testicular abnormalities. Gehin et al have shown that SRC-2 expression, which is exclusively confined to Sertoli cells, is essential for spermatogenesis, as SRC-2 null male mice develop alterations in cell adhesion between Sertoli cells and germ cells, and morphologic anomalies in Sertoli cells similar to those encountered in humans with teratozoospermia related to aging (Gehin, Mark et al. 2002).

The SV40-induced transgenic adenocarcinoma of the mouse prostate (TRAMP) model presents an increased SRC-3 expression in prostatic luminal epithelial cells during tumorigenesis, while SRC-3 null mice show resistance to induced prostate cancer progression (Tien, Zhou et al. 2009), suggesting that SRC-3 might play an important role in prostate cancer. Moreover, knockdown of SRC-3 with inducible short hairpin RNA expression in prostate cancer PC3 cells injected into nude mice to induce tumors growth, inhibited tumoral growth (Zhou, Yan et al. 2005).

In the female mouse models, SRC coregulators (especially SRC-2) contribute to uterine growth, blastocyst implantation and mammary gland development (Gehin, Mark et al. 2002; Mukherjee, Amato et al. 2006; Mukherjee, Soyala et al. 2006; Mukherjee, Amato et al. 2007).

The steroid receptor coactivator SRC-3 is required for normal growth, puberty, female reproductive function, and mammary gland development in the female (Xu, Liao et al. 2000).

5.2.6. Androgen receptor coregulators in human physiology and pathology

As previously mentioned, AR coregulators are important modulators of AR transcriptional activity, suggesting their potential involvement not only in the development and maintenance of androgen-responsive tissues but also in pathologies that are associated with impaired androgenic response or AR function. AR coregulators have specific expression in human tissues and organs. For example, AR coregulators display specific expression in androgen-dependent tissues such as prostate and testis. FHL2, a specific AR coactivator, is selectively overexpressed in epithelial cells of the prostate, whereas hic5/ARA55 is preferentially expressed in the prostatic stromal compartment (Muller, Isele et al. 2000; Heitzer and DeFranco 2007).

Bebermeier et al applied the strategy of cDNA microarray analysis of AR coregulators on 35 different normal human tissue types and observed different expression patterns of some AR coregulators (CAV1, ESR1, NCOA3, TP53, and PCAF), with abundant expression in the reproductive tract, brain and the lymphatic tissues (Bebermeier, Brooks et al. 2006). Aberrant co-regulator function or altered expression may be contributing factors in the progression of AR-mediated pathologies, such as androgen insensitivity syndromes, prostate cancers, male infertility and androgenic alopecia

5.2.6.1. Androgen receptor coregulators in androgen insensitivity syndromes (AIS) and male infertility

Androgen insensitivity syndrome (AIS) is the most common form of 46, XY disorder of sex development (DSD), with an incidence that varies between 1/20,000 and 1/99,000 live male births (Boehmer, Brinkmann et al. 2001) (Orphanet) and a prevalence that varies between 1/20,400 (Bangsboll, Qvist et al. 1992) to 1/62,400 genetic males (Jaaskelainen 2012). AIS can be divided into three categories in function of the degree of genital masculinization: complete androgen insensitivity syndrome (CAIS) which is characterized by normal female external genitalia and a history of inguinal hernia or labial swelling in a postpubertal primary amenorrheic patient; mild androgen insensitivity syndrome (MAIS) when the external

genitalia are normal in adolescents or adults presenting with gynecomastia and/or infertility, and partial androgen insensitivity syndrome (PAIS), when the external genitalia are partially, but not fully, masculinized, and a degree of sexual ambiguity exists (clitoromegaly, hypospadias)(Hughes, Davies et al. 2012).

Androgen insensitivity syndromes, usually caused by mutations in the X-linked AR gene, may also be related to altered AR-coregulator interactions (Ghadessy, Lim et al. 1999; Adachi, Takayanagi et al. 2000; Lim, Ghadessy et al. 2000; Quigley, Tan et al. 2004; Umar, Berrevoets et al. 2005; He, Gampe et al. 2006).

From the more than 800 AR mutations in patients with AIS phenotype registered in the McGill Androgen Receptor Gene Mutation Database July 2014 (<http://androgendb.mcgill.ca>), the most common mutations are located within the ligand binding domain, with functional defects resulting from disruption of the hydrophobic ligand binding pocket, which is necessary for repositioning of helix 12 to form the AF2-coregulator interaction surface.

Analysis of AR mutations in the region of the ligand binding pocket and AF2 binding surface for AR coactivator LXXLL motifs in patients with androgen insensitivity syndrome (AIS), involved effects on the N/C interaction, androgen dissociation rates and coactivator recruitment (He, Gampe et al. 2006).

Indeed, some AR mutations in AF2, responsible for AIS (E897K, V889M, I898T, Q733H, G743V, I737T), increased the androgen dissociation rate, while decreasing the AR coregulator FXXLF and LXXLL motifs binding to AR LBD AF2. The V889M mutation, responsible for a complete form of AIS, precedes by four amino acids the AF2 activation core region and corresponding to the 11/12 helix loop, alters the N/C interaction and is associated with a rapid dissociation rate of the ligand (Langley, Kempainen et al. 1998). He et al provided a functional and crystallographic analysis of the previously mentioned mutation, demonstrating that a specific conformation of the AF2 helices was required for proficient ligand binding, where valine in position 889 served as a gatekeeper (He, Gampe et al. 2006).

Ghali et al (Ghali, Gottlieb et al. 2003) identified AIS cases with 5 AR mutations related to a decrease in the AR N/C interaction correlated with the severity of the AIS phenotype, associated in three mutants with a reduction in coactivator binding; moreover, SRC-2 overexpression partially rescued N/C interaction for R871G, S814N and V866M AR mutations.

AR Coactivator	Knockout phenotype
SRC-1	Reduced testis weight, decreased growth and development of the prostate (Xu, Qiu et al. 1998) Moderate motor dysfunction, delayed development of Purkinje cells, control of energy balance (obese due to decreased energy expenditure), partial resistance to ER, PR, AR,TR hormones (Weiss, Xu et al. 1999; Nishihara, Yoshida-Komiya et al. 2003; Xu and Li 2003)loss of p/CIP and SRC-1 results in resistance to age-related obesity and glucose intolerance(Wang, Shah et al. 2012)
SRC-2	Defective spermatogenesis-teratozoospermia; testicular degeneration-Sertoli cell vacuolization, loss of Sertoli-germ cell adhesion; male and female hypofertility, female mice have placental hypoplasia (Gehin, Mark et al. 2002); progesterone-dependent uterine and mammary morphogenesis (Mukherjee, Amato et al. 2006; Mukherjee, Amato et al. 2007) higher lipolysis in white fat; higher energy expenditure in brown fat; resistance to obesity (Picard, Gehin et al. 2002) ; G6PD liver deficiency-Von Gierke disease phenotype (Chopra, Louet et al. 2008).
SRC-3	Somatic growth retardation; delay in puberty and mammary growth; lower IGF-1 and estrogen levels; lower female fertility; reduced ER-dependent vascular protection, reduced adipogenesis (Wang, Rose et al. 2000; Xu, Liao et al. 2000; Yuan, Liao et al. 2002).
FKBP52	Hypospadias, size reduction of seminal vesicles with normal testes and epididymis(Yong, Yang et al. 2007)
AR Corepressor	Knockout phenotype
N-CoR	Die during embryogenesis with defects in erythrocyte and thymocyte and loss of astrocyte differentiation (Hermanson, Jepsen et al. 2002)
HBO1	Die during embryogenesis with defects in blood vessels, mesenchyme, and somites organization (Kueh, Dixon et al. 2011)
RIP140	Females present decreased fertility due to impaired folliculogenesis and defective corpora lutea (Leonardsson, Steel et al. 2004) RIP140-null mice are lean, resistant to obesity and hepatic steatosis with enhanced glucose tolerance and enhanced responsiveness to insulin compared with matched wild-type littermates fed a high-fat diet(Leonardsson, Steel et al. 2004); they develop cardiac hypertrophy as they express more oxidative fibres in skeletal muscle(Fritah, Steel et al. 2010)

Table.5. Table resuming androgen receptor coregulator animal knockout models (phenotype is related to disruption of coregulator genes) presenting complex alterations in reproductive, metabolic and developmental functions.

Similar results were obtained by Thompson (Thompson, Saatcioglu et al. 2001) or by Quigley (Quigley, Tan et al. 2004), who described mutations located between helices 3-11 in the LBD in PAIS, with normal or slightly reduced androgen binding activity but severely impaired N/C interaction, decreased coactivator response and decreased transactivation.

Umar (Umar, Berrevoets et al. 2005) described a C-terminal AR mutation (Q902K) responsible for PAIS in a phenotypically male individual presenting with hypospadias; this AR mutation was associated with decreased N/C interaction and decreased transactivation by the coactivator SRC-2.

Lim identified a nonconservative N727K AR mutation in the region of the LBD between α helices 3 and 4, in a subfertile man with severely depressed sperm production who restored a normal sperm count after treatment with an androgen analogue, mesterolone, which allowed him to father a child (Lim, Ghadessy et al. 2000). This mutation didn't alter the androgen binding kinetics, but reduced N/C interactions and SRC-2 coactivation.

In a study of 173 infertile men, Ghadessy identified 3 non related individuals with impaired spermatogenesis (associated with different features such as gynecomastia in one case) who had M886V germline AR mutation, that interfered with both N/C interaction and SRC-2 binding (Ghadessy, Lim et al. 1999).

As several AR coregulators (ARA54, SRC-1, HBO1) are highly expressed in human testis (Table 5.2.), Lan et al performed a study on 27 men with obstructive azoospermia and 24 men with nonobstructive azoospermia to reveal the immunohistochemical and transcript expression of two AR coregulators (ARA54 and ARA55) in relation with spermatogenesis (Lan, Hseh et al. 2008). They identified a differential localization and transcript levels of the two coregulators in the human testis, as mentioned in table 4 .

All the above mentioned findings allow better understanding of the expression and the potential function of androgen receptor coregulators in male fertility. However, these results need to be interpreted carefully, as altered coactivator binding had not been proven to have a more important impact on phenotype rather than the defect in the N/C interaction. Moreover, as Quigley revealed, family members carrying the same genetic disorder responsible for a partial defect in AR function present with high phenotypic variations (Quigley, Tan et al. 2004).

5.2.6.2. Androgen receptor coregulators in prostate cancer

In 2011, prostate cancer was the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males worldwide (Jemal, Bray et al. 2011).

Prostate cancer develops initially as an androgen dependent disease that relies on the androgen receptor for growth and progression, which explains the initial effectiveness of androgen deprivation therapies, or hormonal castration therapies (using antiandrogens in combined therapy with gonadotrophin–releasing hormone analogues). Most hormone dependent cancers become refractory after one to three years and as they are no longer responsive to castration treatment, they develop recurrent growth and metastatic progression despite low levels of circulating androgen. This form of prostate cancer, also called castration-resistant prostate cancer (CRPC) is associated with high mortality due to lack of long–term effective therapeutic strategies.

AR transcriptional activity is the most important factor explaining tumor growth of both androgen-dependent and castration-recurrent prostate cancer. In CRPC, AR is no longer dependent on circulating androgens, but on local, AR gene amplification(Visakorpi, Hyytinen et al. 1995), increased mitogen signaling(Gregory, He et al. 2001; O'Malley and Kumar 2009), associated with high prevalence of AR somatic gene mutation and increased levels of AR coregulators(Wilson 2010).

Several interesting publications revealed the potential involvement of AR coregulators with prostate cancer. Overexpression of several AR coactivators correlate with tumor dedifferentiation, with aggressive forms and poor prognosis which makes from targeting AR-coregulators interactions with small molecule inhibitors, a challenging therapeutic approach (O'Malley and Kumar 2009).

SRC1 and CBP expression in human prostate have been studied by immunohistochemistry; like AR, they are both expressed in the luminal epithelial cells, where over 90% of prostate tumors arise (Powell, Christiaens et al. 2004). Another study based on tissue microarray analysis revealed an increased SRC-1 immunoexpression in androgen dependent metastatic prostate tumors compared to localized prostate tumours (Agoulnik, Vaid et al. 2005).

With a similar immunohistochemical approach, SRC-1 and SRC-2 expression were found to be more important in hormone-refractory prostate tumors compared with benign prostatic hyperplasia or androgen-dependent tumors (Gregory, He et al. 2001).

SRC-3 is an important regulator of prostate cancer proliferation and survival. Immunohistochemistry performed on tissue microarray of 480 clinically localized prostate cancers showed that SRC-3 is overexpressed proportionately to prostate cancer proliferation (Zhou, Yan et al. 2005).

In some AR mutations in prostate cancer, occurring in the AR AF2 and ligand binding region, such as V715M or H874Y, AR transcriptional activity was found to be increased through mechanisms that included increased binding of AR FXXLF to LXXLL motifs of SRC/p160 coactivators (mainly SRC-2 and SRC-3) and slower dissociation rates of bound androgen (testosterone and dihydrotestosterone) (He, Gampe et al. 2006). Moreover, Gregory demonstrated that AR mutations T877A of the LNCaP prostate cancer or the H874Y mutation of the CWR22 prostate cancer xenograft increase androgens ability to activate AR transcription when SRC-2 is overexpressed (Gregory, He et al. 2001).

Heemers and her team developed an RNA profiling array that probed the expression of 186 coregulators in order to study the androgen modulation of coregulator expression between androgen-dependent and castration-recurrent PCa cells and showed that 30% of coregulators displayed some level of androgen regulation. Heemers also identified some coregulators (SRC-2, PB1, CBP) that were overexpressed in castration-recurrent PCa cells (Heemers, Regan et al. 2009).

Furthermore, Heemers et al (Heemers, Schmidt et al. 2010), explored the effects of androgenic stimulation or deprivation on the expression of AR coactivators SRC-1, SRC-2, SRC-3, CBP, and p300 in multiple xenograft models, prostate cancer lines, versus nonmalignant prostate epithelial cell lines, as well as prostate tissues from castrated and androgen treated rat models, revealing different patterns of coregulator expression between normal and neoplastic prostate cells or tissues. Androgen stimulation of these models decreased coactivator expression, while androgen deprivation induced the expression of coactivators. This model might explain some implications of AR coregulators in the development of the CRPC.

Several other AR coactivators might be involved in proliferation of prostate cancer cells, such ARA54, ARA55 and ARA70 (Yamamoto, Hashimoto et al. 2000; Culig, Comuzzi et al. 2004).

Urbanucci et al, identified other four AR coactivators *MAK*, *BRCA1*, *SRC-3* and *CBP*, whose expression was more than 2-fold enhanced after DHT-stimulation in AR cDNA-transfected LNCaP cells, as well as 3 potential AR corepressors (β -catenin, cyclinD1 and gelsolin),

showing reduced expression in AR transfected LNCaP cells (Urbanucci, Waltering et al. 2008).

A recently identified AR coregulator that binds to the AR N-terminal FXXLF motif, and to other AR coactivators, known as the melanoma antigen gene protein A11 (MAGE-11)(Bai, He et al. 2005) was found to be overexpressed in CRPC and considered a new potential target in the treatment of advanced recurrent prostate cancer(Wilson 2010).

5.2.6.3. Androgen receptor coregulators in other pathologies

Inhibition of AR acetylation by SIRT1, a nuclear protein and deacetylase of the AR, was recently revealed to offer protection against DHT-dependent polyglutamine (polyQ)-expanded AR, responsible for proteotoxicity in spinal and bulbar muscular atrophy (SBMA)(Montie, Pestell et al. 2011).

Viral oncoproteins, such as the papilloma virus E2, or HBx, which is a nonstructural hepatitis B virus protein, have been suggested to regulate the transcriptional activity of the AR (Chiu, Yeh et al. 2007; Wu, Huang et al. 2007); coregulators were proposed as an explanation for the vulnerability of males to microbial infections and the subsequent risk for development of cancer. Finally, ARA70 (AR coactivator) may also be involved in androgenic alopecia (Lee, Zhu et al. 2005).

5.2.6.4. Androgen receptor coregulators: future directions

Despite the intensity of research activity in this area, many interesting questions regarding the fundamental mechanism of AR and its coregulators on androgen-related diseases, such as osteoporosis, androgen insensitivity syndrome, hypogonadotropic hypogonadism, etc, remain unsolved.

As coregulators have been shown to be related to prostate cancer progression and prognostic, efforts are now being directed towards better defining AR regions that can mediate critical interactions with clinically relevant cofactors and to identify new therapeutic targets that might interrupt the interaction between AR and these proteins.

Promising research is directed towards the development of small molecule inhibitors (SMIs) that interfere to coregulator actions in order to suppress cancer proliferation. For example, in a proof-of-principle study, gossypol was characterized as a small molecule inhibitor of SRC-1 and SRC-3 that binds directly to the coregulator interacting domain with the receptor and can

block cancer growth (Wang, Lonard et al. 2011). Andersen described another small molecule EPI-001, that affects AR N/C interaction and coregulators binding and inhibits in vivo, the developments of a transplanted LNCaP tumor (Andersen, Mawji et al. 2010).

SIMs could be not only potential new chemotherapeutics, but also new treatment strategies for metabolic diseases related to the modulation of the PGC-1 α , RIP140 or SRC-1 or SRC-2 expression and metabolic effects in the liver and adipose tissues.

Further studies are needed in order to expand knowledge of the precise expression and role of coregulators in physiology and pathology and to provide information on how to develop and apply coregulator-targeting drugs in various diseases.

Androgenic regulation in Sertoli cells

Objectives

Testis is responsible for many essential processes, such as steroidogenesis and hormonal regulation of spermatogenesis. Androgens, activating the androgen receptor (AR) in Sertoli cells (SC) are known to play crucial roles in fetal gonadal development and postnatal spermatogenesis. SC display distinctive expression patterns of the androgen receptor (AR) during pre- and postnatal testicular development, contrasting the constant AR expression in prepubertal and adult Leydig and peritubular myoid cells.

We and others, have studied AR patterns of expression in Sertoli cells during ontogeny and at different pre and postpubertal stages (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009), suggesting that the acquisition of androgen sensitivity by SC represents a process that develops in a carefully orchestrated manner in well-defined stages of early postnatal development (during the “mini-puberty” between 1-4 months of postnatal life, respectively) and postpubertal as well as adult spermatogenic development.

The main objective of the thesis was the better comprehension of the physiological molecular mechanisms of AR regulation in Sertoli cells during murine and human testicular ontogenesis.

Indeed, this research project and my personal contributions focused on three different problems related to the androgenic regulation of Sertoli cells. Therefore, the results obtained are structured and organized in three parts, in an attempt of raising several main questions.

In order to provide better comprehension and study the molecular mechanisms regarding physiological and developmental aspects of androgenic regulation of AR in Sertoli cells, I have performed a series of in vitro experiments using various molecular biology approaches. Indeed, due to the lack of a human immortalized Sertoli cell model and because all other Sertoli cell lines developed in various species were not appropriate for the study of androgenic regulation, as they lacked an endogenous functional AR capable of maintaining a stable expression for serial passages, a new cellular model was generated and partially characterized, a year before my arrival, in the INSERM Unit U693, by Kahina Boukari and her colleagues.

Therefore, the first part of this section will be focused on the description of an interesting cellular model, called ST38c, a mature Sertoli cell line exhibiting stable

endogenous expression of a functional AR, which presents both an androgenic induced transcriptional regulation, as well as an androgen regulated posttranslational stabilization.

Furthermore, we used this cellular model to explore new androgen-regulated genes and potential mechanisms of androgenic regulation specific to postpubertal AR expressing Sertoli cells. Using several molecular biology techniques (RT-PCR, microarray assays, fluorescence immunocytochemistry and Western blotting), I pursued the work initiated by my predecessor PhD student, Kahina Boulari, and revealed that ST38c postpubertal/mature Sertoli cells, were capable of AR posttranslational stabilization and androgenic transcriptional and posttranslational regulation. Androgens constantly enhanced AR expression by posttranscriptional mechanisms, while microarray assay and validating qPCR, identified hundreds of androgen-regulated genes in this Sertoli cell line. These results have been published in *Molecular and Cellular Endocrinology* in January 2014.

An interesting cellular marker of Sertoli cells, used for the evaluation of androgen signaling within seminiferous tubules, is the anti-Müllerian hormone (AMH), a marker of differentiation of Sertoli cells, considered to be negatively regulated by androgens at puberty and adulthood (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Rey, Musse et al. 2009; Rey and Grinspon 2011).

As this cellular model could be a useful experimental tool for studying Sertoli cell mechanisms and roles in spermatogenesis regulation, I underwent new series of experiments in order to find arguments that might answer to the question:

- Which would be the precise mechanisms explaining the androgenic negative regulation of AMH expression in Sertoli cells?

Although several *in vivo* and *in vitro* experiments have shown the negative regulation of the AMH by androgens (Young, Chanson et al. 2005; Boukari, Meduri et al. 2009), the precise molecular mechanisms responsible for the repressive effect of AR on AMH expression are still not elucidated.

In this respect, the next objective was to investigate the functional interactions between AR and AMH expression using the novel murine cellular model, the ST38c cells.

As ST38c exhibited a mature Sertoli cell phenotype, with stable AR and lack of AMH expression, I selected several experimental approaches in order to search for a direct mechanism relating AR expression to AMH suppression, such as transfection of AMH promoters and AR silencing in ST38c cell line, using a siRNA approach. However, neither AMH transfection (several transfection methods were used for ST38c, all very difficult and unsuccessful), nor successful AR silencing, brought any arguments in favor of a direct mechanism of AMH down-regulation by androgen activated AR.

As the purpose was to use a Sertoli cell line as a translational approach for better understanding of androgen signaling during human testicular development, I also explored the mechanisms of androgenic regulation in Sertoli cells during both prepubertal and postpubertal human development and in several human pathologies related to androgen impairment causing an altered spermatogenesis.

One of the human conditions related to various degrees of altered AR signaling is represented by the androgen insensitivity syndrome (AIS).

Moreover, related to the particularities of AR regulation and its implications in androgen insensitivity syndromes (AIS), the literature revealed several AR mutations in the ligand binding domain, responsible for partial (PAIS) or mild (MAIS) androgen insensitivity syndromes, that have been characterized by normal ligand binding but presenting an impaired AR N/C interaction leading to disruption of interaction with AR coregulators, such as SRC-2 (steroid receptor coregulator type 2). This implied that the mechanisms of AR regulation are more complex and involve molecular partners, such as nuclear coregulators for transcriptional control and maintenance of a normal function.

However, despite recent research advances in molecular biology, little is known at present about the regulators and the mechanisms explaining the phenomenon of stage and age-specific regulation of SCs AR expression. In this respect, identification of potential molecular partners (coactivators or corepressors) that act in conjunction with AR at different stages of testicular development would provide insight into the complex mechanisms and functional differences of Sertoli cells related to AR regulation during development and their implications in spermatogenesis.

Although in early August 2013, in the NURSA registry there were more than 350 AR coregulators, the majority have not yet been examined either in animal models or in human pathologies, therefore their real physiological relevance in normal and pathological conditions remains to be established.

In this context, a new question was raised:

- Which would be the particularities of expression and regulation of AR coregulators in the human testis?

Therefore, in the second chapter of the Results section I shall develop the study of the expression of two AR coregulators during murine and then human ontogeny and postnatal development, as well as in human pathologies associated with androgen action or AR impairment.

The two coregulators studied (SRC-2 and HBO1) were carefully selected, after an extensive review of the literature, as being relevant for testicular development and fertility.

A descriptive analysis of testicular specific expression of human SRC-2 and HBO1 from fetal life to adulthood was recently published (Vija, Meduri et al. 2013). Moreover, I also studied the expression of SRC-2 and HBO1 using transcript qPCR analysis and immunohistochemistry, in human testicular samples with androgen signaling defects (androgen insensitivity and cases of hypogonadotropic hypogonadism and 5 α -reductase type 2 deficiency), as well as in human testicular biopsies obtained from patients with obstructive or secretory azoospermia, not related to impaired androgen action, in order to clarify their potential role in testicular physiology and pathology.

In order to perform histological and molecular experimental studies on human testis, we have first established national and international collaborations with several Pathology Departments (from Bicêtre, Pitié Salpêtrière, Robert Debré and Necker Hospitals from Assistance Publique Hôpitaux de Paris, Creteil Intercommunal Hospital, France as well as Pathology Institute of Bari University, Italy and the National Institute of Endocrinology, and “Th Burghele” Pathology Department, from Bucharest, Romania) and gathered a precious tissue collection of paraffin-embedded or frozen testicular samples comprising normal fetal, neonatal, prepubertal and adult human tissue as well as postpubertal testicular tissue representative for pathologies related to impaired androgenic or AR expression/function (androgen insensitivity syndromes, 5 α -reductase type 2 deficiency, congenital hypogonadotropic hypogonadism).

Finally, I underwent in vitro experimentation using the SMAT1 Sertoli cell line, which represents an immature Sertoli cell line that was kindly provided by Dr. Jean-Yves Picard (Inserm U 782, Clamart). This Sertoli cell model allowed me to study not only the type of transcriptional regulation provided by each of the two coregulators, as well as their potential interactions, but also to evaluate the androgenic regulation of these coregulators. Transfection with hAR plasmid followed by androgen (dihydrotestosterone) treatment revealed that SRC-2 acts as an AR coactivator, while HBO1 acts as an AR corepressor in Sertoli cells.

A part of the results obtained, more precisely, the aspects related to the expression and characterization of SRC-2 and HBO1 in human testicular ontogenesis and in androgen signalling deficient patients have been published in *Molecular and Cellular Endocrinology* in August 2013.

As I had the extraordinary opportunity to study by histology and immunohistochemistry the testicular samples of two subjects (a 17 year old subject, with a 5α -R2 deficiency diagnostic considered in front of the clinical presentation, associated with a high T/DHT ratio, and confirmed by the genetic analysis, which revealed a deleterious homozygous missense mutation in the *SRD5A2* gene (c.344G > A; Gly115Asp), as well as an adult MAIS patient with a deleterious point mutation (F754S substitution) in the LBD of the AR gene who had a successful sperm retrieval by testicular extraction followed by ICSI and successful fatherhood), these two cases allowed me to explore new questions related to the roles of androgens in human reproduction:

- Is spermatogenesis impaired in the absence of dihydrotestosterone?
- Is AMH repression mainly regulated by testosterone or by its metabolite, dihydrotestosterone in human testis?

Histological and immunohistochemical analysis of AR and AMH expression in testicular paraffin-embedded samples of the postpubertal case with 5α -reductase type 2 deficiency were compared with postpubertal testicular samples obtained from a patient with complete androgen insensitivity syndrome (CAIS), and with testicular samples from an adult patient with obstructive azoospermia, but with normal histology (with complete spermatogenesis). For the patient with 5α -reductase type 2 deficiency, spermatogenesis was absent in all seminiferous tubules, while AR was immunoexpressed and AMH suppressed in 92% of the

seminiferous tubules, suggesting that intratesticular testosterone would be sufficient to repress AMH, irrespective of the conversion of testosterone into dihydrotestosterone.

The histological and immunohistochemical analysis of a testicular biopsy obtained from the adult MAIS patient highlighted that in seminiferous tubules with ongoing spermatogenesis, AMH was repressed, even though the AR mutation affected all somatic cells, contrasting with the majority of seminiferous tubules devoid of spermatogenesis, where AMH was strongly expressed. These data reinforce the importance of the indirect negative role of meiotic development of germ cells in AMH expression.

Androgenic regulation in Sertoli cells

Results

First part
Androgenic regulation in Sertoli cells

Paper 1

**Ligand-dependent stabilization of androgen receptor in a novel mouse
ST38c Sertoli cell line**

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1. Introduction

Mature SC are necessary to maintain a specific environment favorable for the development and proliferation of germ cells (Sharpe, McKinnell et al. 2003) and are involved in the hormonal regulation of spermatogenesis via AR-mediated signaling.

AR, displays distinct expression profiles in SC during testis development, tightly correlated to the stages of spermatogenesis, as previously reported (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Rey, Musse et al. 2009). AR is absent in neonatal SC while its expression becomes maximal at puberty and in adulthood, concomitantly with the progression of spermatogenesis, unlike constant AR expression in Leydig and peritubular myoid cells in both prepubertal and postpubertal testicular developmental stages.

Numerous studies previously performed in rodents, primates and humans demonstrated the importance of AR expression in SC for the regulation of spermatogenesis. For example, mouse models with AR invalidation in SC (SCARKO) exhibited variable degrees of infertility associated with spermatogenesis impairment (Wang, Yeh et al. 2009). In adult human testis, androgens initiate spermatogenesis, a complex cellular differentiation process that does not occur in fetal and newborn testis despite sustained levels of intratesticular androgen production, phenomenon known as postnatal insensitivity to testosterone due to the lack of AR in SC in neonatal testis (Rey 2003; Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009). However, the precise molecular mechanisms by which AR regulates physiological functions in SC and supports germ cell development and spermatogenesis are still poorly understood. Although the elegant studies performed on transgenic mouse models of selective invalidation of AR superficially in testicular cellular components constitute an interesting perspective to study potential androgen-regulated genes in a testicular environment, it still remains difficult to consider those candidate genes as being androgen-dependent genes in SC, due to the wide complexity of paracrine and metabolic pathways existing among the multiple cellular components of the testis.

In order to directly investigate the mechanisms of androgen regulation specific to SC, several attempts to generate immortalized mature SC lines expressing AR have been reported, with few postpubertal/mature SC models, such as MSC1 (Rao, Wayne et al. 2003), S14-1 (Boekelheide, Lee et al. 1993), 42GPA9 (Bourdon, Lablack et al. 1998) and 15P-1 cells (Paquis-Flucklinger, Michiels et al. 1993). However, in these cell lines, AR expression was very variable and was rapidly lost after several passages. Moreover, androgen signaling was

impaired in these SC models, either because androgens failed to activate endogenous AR, thus necessitating transfection of AR-encoding plasmid in order to study androgen regulation or because AR expression or regulation was rapidly lost during early passages.

Consequently, in order to provide better comprehension and study the molecular mechanisms related to physiological and developmental aspects of androgenic regulation of the AR in Sertoli cells, I continued the description of a novel immortalized murine postpubertal Sertoli cell line, named ST38c, that was generated by targeted oncogenesis, in the INSERM U693, by Kahina Boukari and her colleagues, a year before the beginning of my training as a PhD student.

Hereafter, I shall present the characterization of the abovementioned original mouse immortalized Sertoli cellular model, named ST38c. ST38c Sertoli cells harbor substantial expression of endogenous AR and present androgen-dependent transcriptional activation as well as agonist-dependent transcriptional and posttranslational regulation.

We demonstrated that androgens upregulated AR expression and stabilized AR through posttranscriptional mechanisms. In the presented paper, here attached, several androgen-regulated genes have been identified by microarray studies and some of them were validated by real-time quantitative PCR in ST38c cells.

2. Objectives

As an immortalized mature Sertoli cell line would allow better understanding of the mechanisms of androgen regulation and roles of Sertoli cells for spermatogenesis, the objectives of the first part of the project were:

- 1) To study and characterize the novel ST38c Sertoli cell line, which revealed specific interesting properties such as a stable expression of AR and androgen responsiveness;
- 2) To study the androgen-dependent mechanisms of AR regulation: at both the transcriptional and posttranslational levels;
- 3) To identify new androgen-regulated genes in the mature Sertoli cell line ST38c, using a whole genome mouse transcriptomic approach followed by validating qRT-PCR;
- 4) To explore potential molecular mechanisms responsible for the repressive effect of AR on AMH expression using two immortalized murine Sertoli cell models: prepubertal Sertoli cells-the SMAT1 cell line (Dutertre, Rey et al. 1997), kindly provided to Professor Young by Jean-Yves Picard, and the novel mature cell line ST38c, respectively.

The results obtained regarding the first three objectives are detailed in the manuscript published in *Molecular and Cellular Endocrinology*, January 2014, included in the manuscript, while the results related to the fourth objective would be detailed in the Discussion section.

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Ligand-dependent stabilization of androgen receptor in a novel mouse ST38c Sertoli cell line

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ABSTRACT

Mature Sertoli cells (SC) are critical mediators of androgen regulation of spermatogenesis, via the androgen receptor (AR) signaling. Available immortalized SC lines loose AR expression or androgen responsiveness, hampering the study of endogenous AR regulation in SC. We have established and characterized a novel clonal mouse immortalized SC line, ST38c. These cells express some SC specific genes (*sox9*, *wt1*, *tjp1*, *clu*, *abp*, *inhbb*), but not *fshr*, yet more importantly, maintain substantial expression of endogenous AR as determined by PCR, immunocytochemistry, testosterone binding assays and Western blots. Microarrays allowed identification of some (146) but not all (*rhox5*, *spinlw1*), androgen-dependent, SC expressed target genes. Quantitative Real-Time PCR validated regulation of five up-regulated and two down-regulated genes. We show that AR undergoes androgen-dependent transcriptional activation as well as agonist-dependent posttranslational stabilization in ST38c cells. This cell line constitutes a useful experimental tool for future investigations on the molecular and cellular mechanisms of androgen receptor signaling in SC function.

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1. Introduction

Sertoli cells (SCs) are essential mediators of androgen regulation of spermatogenesis, via the androgen receptor (AR)-mediated signaling. In adulthood, SCs maintain a specific environment favorable for the development and proliferation of germ cells (McKinnell and Sharpe, 1995; Sharpe et al., 2003) and are involved in the hormonal regulation of spermatogenesis. Indeed, FSH acts directly on SC through FSH receptor, while LH first activates Leydig cells and stimulates testosterone production that acts on SC through binding and activation of AR.

AR, a steroid receptor belonging to the superfamily of hormone-activated transcription factors, displays distinct expression profiles in SC during testis development, tightly correlated to the stages of spermatogenesis, as previously reported (Monet-Kuntz et al., 1984; Vornberger et al., 1994; Bremner et al., 1994; Shan et al., 1997; Suarez-Quian et al., 1999; Boukari et al., 2009; Chemes et al., 2008; Rey, 2003). Indeed, AR is absent in neonatal SC while its expression becomes maximal at puberty and in adulthood, con-

comitantly with the progression of spermatogenesis, unlike constant AR expression in Leydig and peritubular myoid cells in both prepubertal and postpubertal testis developmental stages.

Numerous studies in rodents, primates and humans have demonstrated the importance of AR expression in SC for the regulation of spermatogenesis. Several mouse models with total AR inactivation in SC exhibited variable degrees of infertility associated with spermatogenesis impairment (De Gendt et al., 2004; Wang et al., 2009; Verhoeven et al., 2010). In adult human testis, androgens initiate spermatogenesis, a complex cellular differentiation process that does not occur in fetal and newborn testis despite sustained levels of intratesticular androgen production. Thus, the lack of AR in SC in neonatal testis is responsible for a physiological postnatal insensitivity to testosterone (Rey, 2003; Chemes et al., 2008; Boukari et al., 2009).

However, the precise molecular mechanisms by which AR regulates physiological functions in SC and supports germ cell development and spermatogenesis are still poorly understood. Although the cell-selective AR deficiency animal models constitute valuable tools to study potential androgen-regulated genes in a testicular environment, it still remains difficult to identify direct androgen-dependent target genes in SC, due to the complexity and entangled paracrine and metabolic pathways existing among the multiple cellular components of the testis.

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In order to directly investigate the mechanisms of androgen regulation specific to SC, several attempts to generate immortalized mature SC lines expressing AR have been reported, resulting in very few postpubertal/mature SC models, such as MSC1 (Rao et al., 2003), S14-1 (Boekelheide et al., 1993), 42GPA9 (Bourdon et al., 1998) and 15P-1 cells (Paquis-Flucklinger et al., 1993). However, in these cell lines, AR expression was very variable and was rapidly lost after several passages. Moreover, androgen signaling was somehow impaired in these SC models, with very low levels of mRNA *Ar* expression (Rao et al., 2003), as androgens failed to activate endogenous AR, thus necessitating transfection of AR-encoding plasmid in order to study androgen regulation.

The aim of the present study was to establish a novel SC line that maintains a sustained AR expression, allowing investigation of androgen-dependent, SC specific, target genes. Herein, we generated and characterized an original mouse immortalized Sertoli cellular model, named ST38c, harboring substantial expression of endogenous AR that conserves its androgen-dependent transcriptional activation and exhibits agonist-dependent transcriptional and post-translational regulation. We demonstrated that androgens upregulated AR expression and stabilized AR through posttranscriptional mechanisms. In the present paper, hundreds of androgen-regulated genes have been identified by microarray studies and some of them were validated by real-time quantitative PCR in ST38c cells. This novel cell line thus constitutes a useful experimental system for better understanding the molecular mechanisms of androgen action in SC and may represent a valuable cell-based model for future investigations on the role of SC in spermatogenesis regulation.

2. Materials and methods

2.1. Establishment of the ST38c Sertoli cell line

The ST38c cell line was isolated from the testes of an 8-wk-old transgenic male mouse carrying a transgene in which the SV40 large T Antigen (TAG) was placed under the control of the human vimentin promoter (Schwartz et al., 1991). Mice were bred according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The animal facility was granted approval (N°C94-043-12), given by the Ministère de l'Agriculture, France. All procedures were approved by the local ethic committee CAPSud (N°2012-021). After decapsulation, the testicular tissue was incubated with collagenase (25%) (CLS type I, 146 U/mg from *Clostridium histolyticum*, Worthington Biochemical; Freehold, NJ, USA) for 15 min at 37 °C. Cellular debris were removed by sedimentation and the cell-containing supernatant was centrifuged. The cells of the formed pellet were rinsed twice with culture medium and suspended in 10 ml of DMEM/HAM's F12 (1:1) medium containing 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 20% fetal calf serum (FCS). Cells, cultured in 10 cm² Petri dishes at 37 °C in atmosphere containing 5% CO₂, were treated with 20 mM Tris-HCl (pH 7.4) hypotonic solution for 3 min, to remove any residual germ cells. After several serial passages, clonal cells morphologically similar to SC and expressing immunoreactive nuclear SV40 TAG were selected. These cells were expanded and characterized.

2.2. Cell culture

ST38c cells (passages 6–30) were seeded at 6×10^5 cells in 6 cm² Petri dishes with 5 ml of DMEM/HAM's F12 (1:1) medium containing 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 5% FCS. The medium was replaced by DMEM/HAM's F12 (1:1) with 5% Dextran-Coated

Charcoal treated serum (DCC) at least 24 h prior to study androgen signaling in steroid free medium conditions.

2.3. Hormones and drugs

Dihydrotestosterone (DHT) was purchased from Acros Organics (Noisy Le Grand, France) whereas RU486 (mifepristone), cycloheximide and actinomycin D, were purchased from Sigma (St. Louis, MO).

2.4. Quantification of specific androgen-binding sites by Scatchard analysis

Cells were grown in the DCC medium 24 h before harvesting. After being rinsed twice with cold PBS, pelleted cells were frozen in liquid nitrogen. The frozen cells were ground in a mortar under liquid nitrogen. One volume of powdered cells was homogenized in one volume of TEWG buffer (20 mM Tris-HCl, 1 mM EDTA, 20 mM sodium tungstate, 10% (vol/vol) glycerol, pH 7.4, at 20 °C) in a Teflon-glass Potter-Elvehjem (Polylabo, Strasbourg, France) apparatus. Cytosol fractions obtained after centrifugation at 4 °C at 15,000g for 20 min were incubated for 4 h at 4 °C with increasing concentrations of [³H]-testosterone (Perkin Elmer GE healthcare). Bound and unbound steroids were separated by the dextran-charcoal technique. Values of binding parameters were determined at equilibrium by Scatchard analysis using computer software Prism 5 (GraphPad Software, San Diego, CA) as previously described (Lombes et al., 1992).

2.5. Gene profiling analysis

RNA extraction was performed using TRIZOL protocol (Invitrogen) and purified with Qiagen column (Rneasy micro) from ST38c cells treated for 24 h with either vehicle (EtOH) or 10^{-7} M dihydrotestosterone. The quantity and purity of the extracted RNA was evaluated using a NanoDrop spectrophotometer and its integrity measured using Lab-on-a-chip Bioanalyser 2000 technology (Agilent Technologies, Palo Alto, CA, USA), based on the 28S/18S ribosomal RNAs ratio. Labeling of RNA samples was done according to Agilent oligo Cy5 or Cy3 probes labeling protocol using the Agilent Low Input QuickAmp labeling kit for dye swap strategy. Cy5/Cy3 cRNA mixtures were hybridized to an Agilent mouse genome GeneChip array (G4122F) allowing to independently probe 44 K transcripts from whole mouse genome. Scanning of microarrays, data normalization and quality tests were performed as previously described (Khan et al., 2012). Selection of androgen regulated genes was carried out according to fold change (FC) > 1.5, *p*-value < 10^{-5} and intensity > 50 cut offs. Among the redundant positive probes matching with the same gene, only one representative response was included in the final list of the 146 androgen-regulated genes. The raw microarray data have been submitted to Array Express database (European Bioinformatics Institute, <http://www.ebi.ac.uk/arrayexpress/>) with the accession number E-MTAB-1732.

2.6. Bioinformatics analysis

Gene ontology and functional clustering was predicted using two bioinformatics resources: the DAVID Bioinformatics Resources (<http://www.david.abcc.ncifcrf.gov>), while further information related to protein families and molecular and biological functions related to the selected genes have been confronted with PANTHER database (www.pantherdb.org).

2.7. Quantitative real time RT-PCR

Total RNA, extracted from cells was processed for RT-PCR, as previously described (Boukari et al., 2009). *Ar* and androgen-regu-

lated target gene candidates identified by microarray analysis were quantified by real-time PCR, with specific primers (primer sequences available upon request). Briefly, 1 μ g of total RNA was treated using the DNase I Amplification Grade procedure (Invitrogen), RNA was reverse-transcribed using the High Capacity cDNA RT kit from Applied Biosystems. Reverse-transcribed samples were diluted 10-fold and used for qRT-PCR using the Power SYBR[®] Green PCR Master Mix (Applied Biosystems). qPCR reactions were carried out on a StepOne Plus Detector (Applied Biosystems) as previously described (Boukari et al., 2007). Ribosomal 18S RNA was used as the internal control for data normalization.

After 24 h treatment with 10^{-7} M dihydrotestosterone, ST38c transcript expression was compared to the basal, not stimulated (ethanol at equivalent concentration) condition. The relative expression level of each gene transcript was normalized with 18S rRNA level and expressed as attomoles of gene per femtomoles of 18S. Results are means \pm SEM of at least three independent experiments.

2.8. Western blot analysis of AR expression

Total protein extracts, prepared from ST38c cell lysates (20 μ g total protein/lane) were resolved by SDS-PAGE and electrotransferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, UK Limited). Immunoblots were incubated overnight in 5% skim milk–Tris buffer saline/0.1% Tween (TBST) before incubation with rabbit anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz) at 1/200 dilution for 1 h at room temperature. After several washes, the membranes were incubated with a goat anti-rabbit peroxidase-conjugated second antibody (1:10,000) for 1 h at room temperature and proteins were visualized with the ECL⁺ Western blotting analysis system (GE Healthcare). For loading normalization, membranes were incubated with an anti α -tubulin antibody (Sigma). Relative AR protein expression was assessed after normalization with α -tubulin, using the Quantity One Analysis software (Bio-Rad, France). Results are means \pm SEM of at least three independent experiments and represent the relative fold-induction in ligand-stimulated cells compared with basal levels (arbitrarily set at 1).

2.9. Immunofluorescence studies

Cells cultured on Lab-Tek (Nunc, Roskilde, Denmark) were washed in PBS, fixed with 10% formol buffered in PBS (pH 7.3) for 10 min and washed three times in PBS before processing for immunofluorescence assays. Cells were incubated overnight with primary antibodies: rabbit polyclonal antibodies: anti-AR (sc-816, Santa Cruz Biotechnology), anti-WT1 (sc-192, Santa Cruz Biotechnology), anti-SOX9 (Chemicon international) and anti-TJP1 (Invitrogen, Life Technologies), or monoclonal antibodies: anti-SV40 T Antigen (Calbiochem/EMD Millipore) and anti- β -tubulin3 antibody (AbCys, Paris) (Meduri et al., 2002) at 1:50, 1:50, 1:100, 1:50, 1:100, 1:100 dilutions, respectively. Bound immunoglobulins were revealed by goat anti-rabbit Alexa 555 (1:1000) or goat anti-mouse Alexa488 (1:1000) (Invitrogen Cergy-Pontoise) according to the manufacturer's instructions. Negative controls were performed by substituting the primary antibodies with corresponding preimmune immunoglobulins from the same species.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad PRISM version 5 software (GraphPad Software, Inc., La Jolla, USA). Results are expressed as means \pm SEM of at least three independent experiments. Non-parametric Mann-Whitney test was used for comparison between groups.

3. Results

3.1. Morphological and molecular characterization of ST38c Sertoli cells

Sertoli cells (SC) were isolated from testes of an 8-wk-old transgenic male mouse by means of targeted oncogenesis strategy in which the expression of the SV40 large T Antigen was driven by the vimentin promoter (Schwartz et al., 1991). After clonal selection and expansion, the ST38c cells maintained stable morphological aspect during successive passages (P 6–30).

On bright-field microscopic examination, ST38c cells present a homogeneous morphology of SC, as monolayered cells with long cytoplasmic appendages and central ovoid nuclei (Fig. 1A). To assess whether ST38c were immortalized, we examined the expression of SV40 large T Antigen by immunofluorescence (Fig. 1B). Positive immunostaining of the SV40 large T Antigen was observed in the nucleus of ST38c cells, thus confirming the immortalized properties of the clonal cell line. To define the precise origin of the ST38c cells, we examined the expression of specific SC markers by immunocytochemistry and RT-PCR (Figs. 1 and 2A and B). Immunofluorescence studies in ST38c cells revealed the presence of a specific nuclear staining for both SOX9 and WT1 (Fig. 1C and D), two transcription factors expressed in both mature (post-pubertal) and immature (prepubertal) SC. We demonstrated by RT-PCR that ST38c expressed mRNA of other factors known to be involved in SC functions, such as sulphated glycoprotein-2/clusterin (*clu*), transferrin and androgen binding protein (*abp*) (Fig. 2A), consistent with the SC phenotype of ST38c cells. We also examined

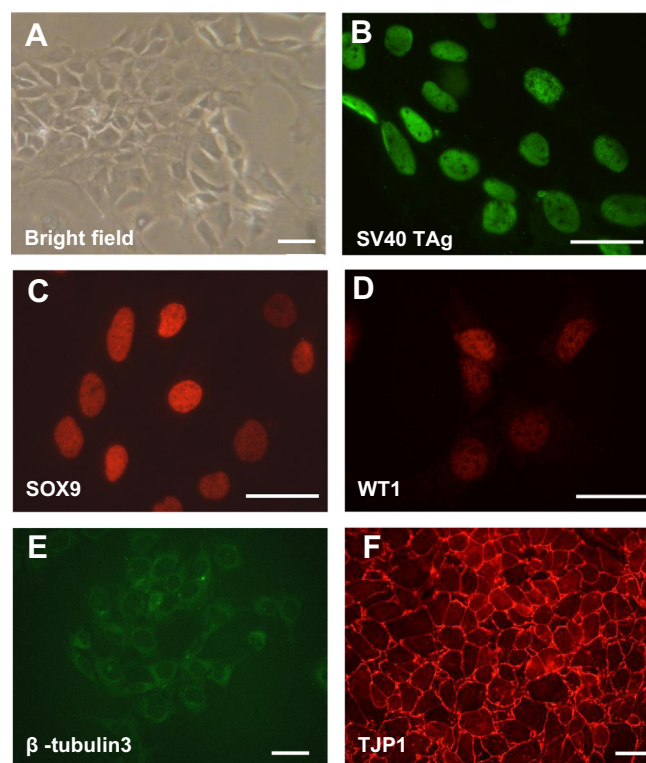


Fig. 1. Morphology and molecular markers expression in ST38c Sertoli cells. Panel A: Phase-contrast microphotograph of ST38c shows monolayered cells with long cytoplasmic appendages and central ovoid nucleus. Panels B–F: Immunofluorescence and cytochemistry studies on ST38c cells. B: Nuclear staining of the SV40 large T antigen (SV40 TAg) confirms the immortalized properties of the clonal cell line. C: Nuclear SOX9 immunofluorescence staining; D: Nuclear WT1 immunofluorescence staining; E: β tubulin 3 cytoplasmic immunofluorescence staining, F: zonula occludens 1 protein (TJP1) mostly membrane located staining. Scale bars: 50 μ m.

the expression of β -tubulin3 (*tubb3*), an androgen up-regulated protein, involved in the modulation of the SC cytoskeleton (De Gendt et al., 2011) and found that β -tubulin3 was expressed at both mRNA (Fig. 2A) and protein level as indicated by the cytoplasmic immunostaining (Fig. 1E). As expected for a mature SC line, ST38c cells also express proteins involved in the formation of tight junctions, important for the blood-testis barrier formation, such as zonula occludens1 at both protein (Fig. 1G) and mRNA levels (*tjp1*) (Fig. 2A), while anti-Mullerian Hormone (*amh*) mRNA was not detected in ST38c cells, contrasting with the high AMH expression in newborn testis (Fig. 2B). In order to determine whether ST38c cells are a clonal SC line, we confirmed the absence of Leydig specific cell markers, such as *cyp17a* and LH receptor (*lhr*) mRNA, as illustrated in Fig. 2C. Finally, we also showed that ST38c cells express detectable levels of mRNA for both α and β subunits of Inhibin B, suggesting that this new cell line might be able to produce Inhibin B (Fig. 2A and B). Altogether, these results demonstrate that ST38c cells constitute a homogenous population of mature SC.

3.2. ST38c cells express functional androgen receptors

Since our main objective was to establish a cell line expressing a functional androgen receptor (AR), as opposed to previous SC models, we used different complementary approaches to analyze AR expression in ST38c cells. RT-PCR analysis demonstrated that ST38c cells express AR mRNA (Fig. 3A). Western blot confirmed the presence of AR protein as a ~110 kDa protein, identified in ST38c cell lysates (Fig. 3B), human prostatic LNCaP cells being used as positive control. Immunofluorescence studies further demonstrated that AR is expressed in ST38c cells as a protein with both nuclear and cytoplasm distribution, more pronounced in the nucleus, under the standard experimental conditions (Fig. 3C). We next performed testosterone binding assays to characterize specific

sites and their binding parameters for AR. Cytosolic fractions of ST38c cells were incubated with increasing concentrations of [³H]-testosterone for 4 h. Scatchard plot analysis revealed that ST38c cells express approximately $5\text{--}10 \times 10^3$ specific sites/cell, with an equilibrium dissociation constant (Kd) estimated at ~0.7 nM, consistent with the high affinity of testosterone for AR (Supplemental Fig. S1). Altogether, these data indicate that ST38c cells do express high affinity, high capacity AR.

3.3. Identification of AR-regulated genes in the ST38c cells

To identify androgen-dependent and AR-regulated genes in SC, gene expression patterns were analyzed by microarray assays, comparing untreated versus DHT (10^{-7} M) treated ST38c cells. Microarray assay analysis performed as previously described (Khan et al., 2012), allowed identification of a repository of 146 differentially androgen-regulated genes including 72 up-regulated and 74 down-regulated genes, which were selected according to the following criteria: fold change FC >1.5, *p*-value < 10^{-5} and intensity >50 cutoff. A panel of genes selected upon their involvement in the biology of reproduction is presented in Table 1.

Gene ontology was predicted by using DAVID and PANTHER softwares. Among the androgen-up regulated genes in SC, there were several factors involved in testicular development (*ngfr*), androgen mediated transcriptional regulation (*nrip1-rip140*), as well as other candidates required in germ cell differentiation (*pou5f2*, *serpina3m*, *serpina3c*, *serpina3j*, *ctsc*) or genes involved in female gamete generation (*tgfb2*). Among the androgen down-regulated genes in ST38c cells there were several genes involved in the modulation of inflammatory responses (*cxcl5*, *serpina1b*, *cxcl11*),

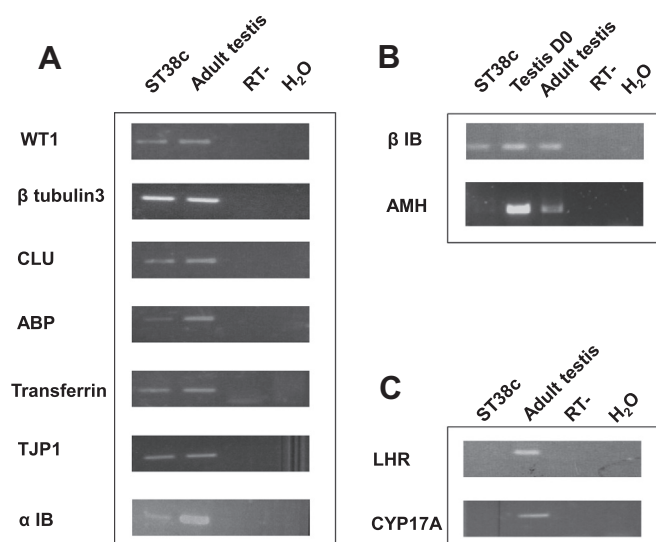


Fig. 2. Characterization of mRNA expression of specific cellular markers in ST38c cells. A: mRNA expression of specific Sertoli cell genes in ST38c cells (WT1: Wilm'sTumour; β tubulin 3; CLU: sulphated glycoproteins-2; ABP: androgen binding protein; Transferrin; TJP1: zonula occludens 1; α IB: inhibin alpha subunit) as determined by RT-PCR. B: β IB: inhibin beta subunit; AMH: anti-Mullerian hormone. Inhibin beta is expressed in murine ST38c as well as neonate and adult murine testis. AMH is not expressed in ST38c while its expression was very intense in neonate murine testis and barely detected in adult murine testis, consistent with the mature phenotype of the ST38c Sertoli cells. C: LH receptor and CYP17A genes, specifically expressed in Leydig cells are not detected in ST38c cells, consistent with the Sertoli cell origin of the ST38c cell line as well as its clonality. Testis from neonate (first day post-natally) and adult mice were included as positive controls. RT- (omission of the reverse transcriptase) and H₂O were included as negative controls.

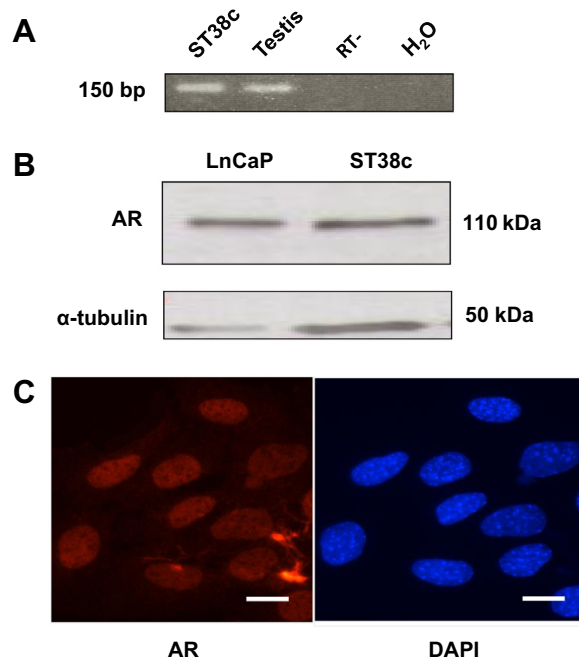


Fig. 3. ST38c cells express the androgen receptor (AR). A: RT-PCR analysis allowed the detection of a 150 bp amplicon demonstrating AR mRNA expression in the Sertoli ST38c cell line. Mouse adult testis was used as positive control. RT- (omission of the reverse transcriptase) and H₂O were negative controls. B: Western blot analysis of AR expression in ST38c and LNCaP cells, used as a positive control. 20 μ g of total protein lysates from ST38c cells and 4 μ g protein lysates from LNCaP cells were processed for immunoblotting with polyclonal anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins expression was normalized with α tubulin, blotted using an anti- α tubulin antibody. AR protein expression is revealed as a band of 110 kDa. C: Immunofluorescence studies reveal the AR mainly nuclear distribution in ST38c cells (left panel). DAPI nuclear staining was used as control (right panel). Scale bar: 50 μ m.

Table 1
Selection of androgen regulated genes in the ST38c cell line and their involvement in the biology of reproduction.

Gene ID	Gene symbol	Gene name (DAVID)	FC	p-value	Basal level	Protein class	Molecular function	Biology of reproduction	Refs.
<i>Androgen up-regulated genes in the ST38c cell line</i>									
26358	ALDH1A7	Aldehyde dehydrogenase family 1, subfamily A7	5.3	0	228	Dehydrogenase	Oxidoreductase activity	Expressed in murine testis	1
20717	SERPINA3M	Serine (or cysteine) peptidase inhibitor, clade A, member 3 M	4.1	0	601	Serine protease inhibitor	Proteolysis	Retinoid acid signaling in seminal plasma	2
16625	SERPINA3C	Serine (or cysteine) peptidase inhibitor, clade A, member 3C	3.8	0	77	Serine protease inhibitor	Proteolysis	Retinoid acid signaling in seminal plasma	2
14456	GAS6	Growth arrest specific 6	2.7	3.5E–12	24781	Extracellular matrix protein	Receptor ligand	Testicular immune response to pathogens	3
19735	RGS2	Regulator of G-protein signaling 2	2.6	1.8E–22	432	G-protein modulator	Small GTPase regulator activity		4
66695	ASPN	Asporin	2.5	8.9E–33	102	Extracellular matrix protein	Cell–cell adhesion	Extracellular matrix	
15483	HSD11B1	Hydroxysteroid 11-beta dehydrogenase 1	2.5	0	62	Dehydrogenase	Steroid metabolic process	Expressed in Sertoli and Leydig cells	5
15469	PRMT1	Protein arginine N-methyl transferase	2.3	1.3E–14	2978	Methyltransferase	Aminoacid methylation	Expressed in adult male rat testis	6
18053	NGFR	Nerve growth factor receptor	2.2	0	144	Tumor necrosis factor receptor	Tumor necrosis factor	Testis development	7
11668	ALDH1A1	Aldehyde dehydrogenase family 1, subfamilyA1	2.1	3.7E–13	3589	Dehydrogenase	Oxidoreductase activity	Expressed in murine testis	1
54635	PDGFC	Platelet-derived growth factor, C polypeptide	2.1	2.1E–24	96	Growth factor and transporter	Oxidoreductase activity	Inhibits TGF beta 1	2
20678	SOX5	SRY-box containing gene5	2.0	9.4E–19	106		Transcription factor activity	Expressed in testis	2
238395	SERPINA3J	Serine (or cysteine) peptidase inhibitor, clade A, member 3J	1.7	3.5E–14	124	Serine protease inhibitor	Proteolysis	Retinoid acid signaling in seminal plasma	2
75507	POU5F2	Sperm 1 POU Domain Transcription Factor	1.7	3.9E–06	93	Homeobox transcription factor	Transcription factor activity	Germ cell differentiation	2
268903	NRIP1	Nuclear receptor interacting protein 1	1.7	8.4E–08	97	RIP140 coregulator	Transcription factor coregulator	Up-regulated in prostate cancer	8
330119	ADAMTS3	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 3	1.7	1.0E–08	52	Metalloprotease major procollagen II N-propeptidase	Metallopeptidase activity	Expressed in human testis	2
21808	TGFB2	Transforming growth factor, beta 2	1.6	5.7E–09	1450	Growth factor	Growth factor	Expressed in postpubertal SC	9
16190	IL4RA	Interleukin 4 receptor, alpha	1.6	4.6E–10	1742	Type I cytokine receptor	Cytokine receptor activity		
56353	RYBP	RING1 and YY1 binding protein	1.6	4.4E–08	4642	Transcription cofactor	Transcription factor activity	Expressed in embryonic stem cells	10
217944	RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	1.6	2.0E–08	191	Guanyl-nucleotide exchange factor	GTP-ase regulator	Expressed in Sertoli and germ cells	2
13032	CTSC	Cathepsin C	1.6	3.7E–14	138	Cysteine protease	Cysteine peptidase	Spermatogenesis	11
68178	CGNL1	Cingulin like-1	1.5	1.1E–11	1294	G-protein modulator	Structural constituent of cytoskeleton		
<i>Androgen down-regulated genes in the ST38c cell line</i>									
242653	CLDN19	Claudin 19	–2.2	6.4E–06	134	Tight junction protein	Cell adhesion morphogenesis	Tight junctions mediated by TJP1	12
215798	GPR126	G protein-coupled receptor 126	–1.8	1.0E–15	1061	G-protein-coupled receptor	Cell–matrix interactions	Embryonic development	13
18133	NOV	Nephroblastoma overexpressed gene	–1.7	5.0E–25	5860	Matrix protein	Cell adhesion	Androgen repressed gene in prostate cancer	14
20377	SFRP1	Secreted frizzled-related protein1	–1.6	5.7E–11	1120	Wnt signaling antagonist	Wnt/βcatenin inh	Inhibits AR activity	15
21810	TGFB1	Transforming growth factor beta induced	–1.6	3.6E–09	3010	Growth factor	Cell adhesion receptor binding	Sertoli cell maturation	16
20311	CXCL5	Similar to LPS-induced CXC chemokine chemokine (C-X-C motif) ligand 5	–1.6	4.7E–07	1740	Chemokine	Chemoattractivity	SC chemoattractivity for spermatogonia	17
56066	CXCL11	Similar to small inducible cytokine B11 precursor (CXCL11)	–1.6	6.4E–18	4930	Chemokine	Immune response modulator	Expressed in human male reproductive system	18
18991	POU3F1	POU domain, class 3, transcription factor 1	–1.6	4.6E–12	215	Homeobox transcription factor	Transcription actor activity	Germ cell differentiation	2

Table 1 (continued)

Gene ID	Gene symbol	Gene name (DAVID)	FC	p-value	Basal level	Protein class	Molecular function	Biology of reproduction	Refs.
17472	GBP4	Guanylate binding protein 4	-1.5	2.8E-12	688	Heterotrimeric G-protein	Protein bounding	Androgen upregulated	19
17472	UBA7	Ubiquitin-activating enzyme E1-like	-1.5	8.7E-07	7377	Carrier protein	Protein ubiquitination	Expressed in human testis	20

FC: fold change in androgen stimulated versus basal levels of gene transcripts; AR: androgen receptor; SC: Sertoli cell; GC: germ cells; TJP1 tight junction protein 1 or zonula occludens 1. mRNA relative expression for the vehicle (EtOH) treated condition was arbitrarily set at 1.

References: 1, (Alnouti and Klaassen, 2008); 2, (www.genecards.org); 3, (Sun et al., 2010); 4, (Larminie et al., 2004); 5, (Leckie et al., 1998); 6, (Hong et al., 2012); 7, (Persson et al., 1990; Lim et al., 2009); 8, (Carascossa et al., 2006); 9, (Konrad et al., 2005); 10, (Morey et al., 2012); 11, (Mathur et al., 1997); 12, (Yamazaki et al., 2011); 13, (Waller-Evans et al., 2010); 14, (Wu et al., 2013); 15, (Kawano et al., 2009); 16, (Yin et al., 2013); 17, (Simon et al., 2010); 18, (Linge et al., 2008); 19, (Nitsche et al., 1996); 20, (www.mybiosource.com).

tight junction formation (*cldn19*, but also *cldn1* and *cldn15*) or protein ubiquitination (*uba7*). A large number of genes concerned integrin signaling (*adam11*, *itga8*, *itga11*, *adamts3*, *adamts2*, *itgb11*), and cell adhesion (*cldn19*, *cdhr1*, *itga11*, *efs*, *cldn15*, *tgfb2*, *itgb11*, *ncam1*, *vwf*, *omd*, *col14a1*, *itga8*, *tgfb1*, *cldn1*, *fbln7*, *pstpip1*, *vnn1*, *cntnap2*, *lamc2*, *vcan*, *lmln*, *spon2*, *adam12*, *dpt*). Interestingly, functional annotation clustering analysis (DAVID) revealed that the up-regulated genes preferentially influenced regulation of transcription (*fus*, *klf13*, *sox5*, *rybp*, *tle1*, *tacc1*, *purb*, *qk*, *nrip1*, *prmt1*, *dact1*, *pou5f2*, *rapgef5*, *pdgfc*) and apoptosis (*aldh1a1*, *akt1s1*, *chst11*, *sphk1*, *ngfr*, *tgfb2*). In contrast, the down-regulated genes differentially impacted cytokine (*gm2023*, *amy1*, *cxcl5*, *amy2a4*, *ifnb1*, *tgfb1*, *pf4*, *cxcl11*, *thpo*) and various extracellular signaling pathways (*wnt10a*, *vwf*, *fmod*, *col14a1*, *adamts16*, *tgfb1*, *spon2*) (for further details, see the Array Express database (European Bioinformatics Institute,

<http://www.ebi.ac.uk/arrayexpress/>), where the microarray was registered with the accession number E-MTAB-1732).

In order to confirm the androgen transcriptional regulation in ST38c cells, we next selected several genes including those previously recognized as expressed and/or androgen-regulated in mature SC or in testis. We thus studied their mRNA expression levels by qRT-PCR, comparing basal to androgen (DHT)-stimulated conditions (Fig. 4). Indeed, qRT-PCR analysis validated results of microarray studies. A well-known AR coregulator, *nrip1/rip140* mRNA, was significantly induced after androgen exposure by a 2.3 fold factor ($p = 0.0022$) (Fig. 4A). Surprisingly, *hsd11b1* mRNA encoding for the 11-beta hydroxysteroid dehydrogenase type 1, exhibited a 5-fold induction upon androgen exposure ($p < 0.0001$) (Fig. 4A). We also validated other androgen up-regulated genes such as *gas6* or *aldh1a1* (expressed in testis) (Fig. 4A), as well as androgen

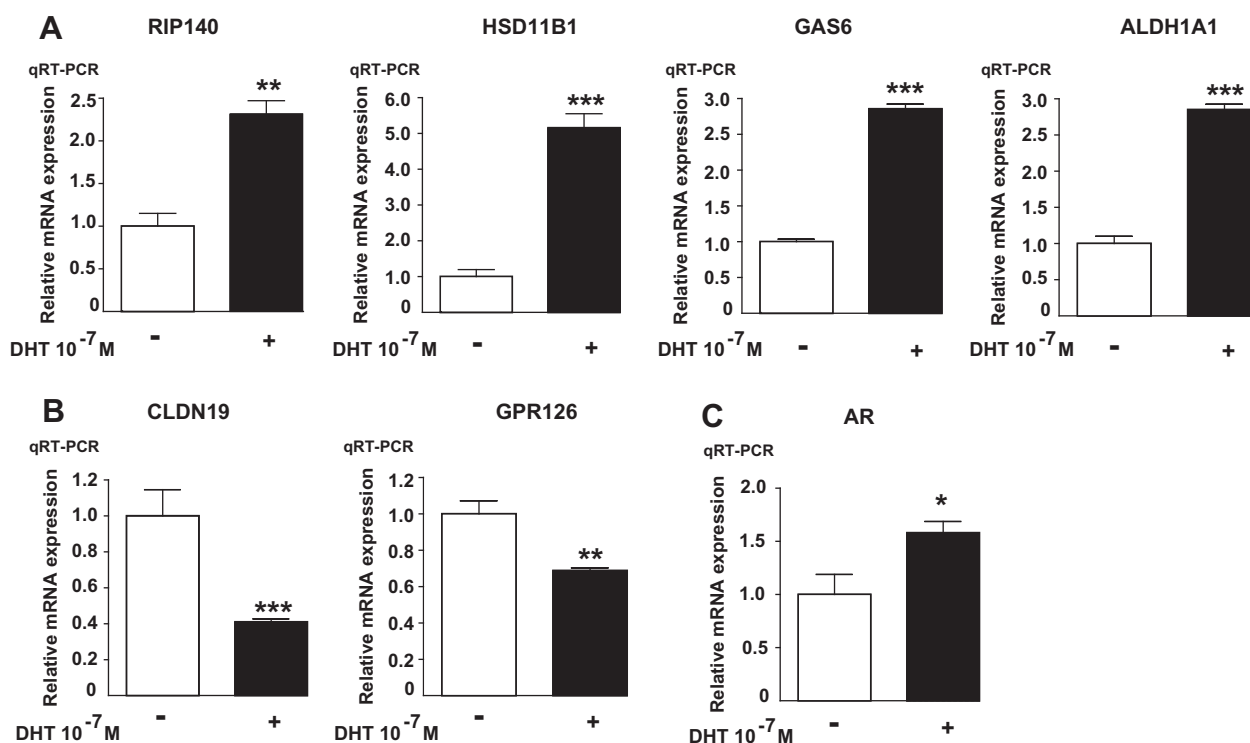


Fig. 4. Validation of some androgen-regulated genes in the ST38c cells. Relative expression of *rip140*, *hsd11b1*, *gas6*, *aldh1a1*, *cldn19* and *gpr126* and *Ar* mRNA levels was evaluated by qRT-PCR in ST38c cells treated for 24 h with either vehicle (EtOH) or dihydrotestosterone (DHT) at 10^{-7} M (primer sequences are available upon request). A: *rip140*, *hsd11b1*, *gas6*, *aldh1a1* were androgen up-regulated. B: *cldn19* and *gpr126* were androgen down-regulated genes, thus validating the gene expression profile obtained with the microarray studies. C: Androgens stimulate *Ar* mRNA levels in the ST38c cells. Relative changes in gene expression of the androgen stimulated condition, in a given sample was quantified using the $2^{-\Delta\Delta Ct}$ method, to that of another sample represented by the untreated control. Results are expressed as means \pm SEM of at least three independent experiments conducted on six reverse-transcribed samples run in duplicate (A and B), or calculated as attomoles per femtomole of 18S rRNA mRNA relative expression (C). Results were normalized to murine 18S ribosomal RNA. Relative expression in a given sample was for the vehicle (EtOH) treated condition was arbitrarily set at 1 (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ non-parametric Mann Whitney test, GraphPad Prism5).

down-regulated genes, such as *cldn19* and *gpr126* (Fig. 4B), which presented similar fold changes by RT/qPCR compared to the microarray analysis.

3.4. Androgens stabilize AR protein expression in Sertoli cells

We took advantage of the ST38c cells that do express substantial amounts of functional AR to examine the impact of androgens on AR regulation and turnover in a SC context.

To determine whether androgens could affect *Ar* gene transcription in SC, we measured *Ar* mRNA levels by RT-qPCR in ST38c cells treated or not with 10^{-7} M DHT, a saturating AR concentration. We showed that transcript levels of endogenous *Ar* were induced by androgen exposure in ST38c cells (1.6 fold induction, p -value = 0.026), indicating that androgens significantly stimulate *Ar* gene expression in SC (Fig. 4C).

Western blot analysis showed that DHT exposure also induced a strong increase in AR expression in a time- and dose-dependent manner. A two to three fold increase in AR protein expression was observed 6 h after exposure, while a 4.5 fold increase in AR protein was detected at 24 h compared to basal levels (Fig. 5A). When ST38c cells were treated with increasing concentrations of DHT (10^{-10} to 10^{-6} M), AR abundance was clearly augmented as a function of androgen concentration, already visible at a dose as low as 10^{-10} M. A maximal response was obtained with micromolar concentrations, reaching a plateau with higher concentrations (Fig. 5B).

In order to examine whether ligand-dependent AR stability directly involved AR per se, we used RU486, a weak AR antagonist (Fig. 5C). As expected, DHT at 10^{-8} M concentration induced a three-fold increase in relative AR expression. However, the combination of DHT and RU486 decreased AR expression although not

significantly, most probably due to the partial agonist activity of RU486 (p -value = 0.119) (Fig. 5C).

To further decipher the molecular mechanisms by which androgens stimulated AR abundance in SC, ST38c cells were grown in the presence or absence of actinomycin D, a transcription inhibitor, for various periods of time. As already shown, DHT (10^{-7} M) alone induced a time-dependent increase in AR protein expression, reaching two-fold after 6 h exposure (Fig. 5A and B). Actinomycin D (0.4 μ M) alone does not substantially affect AR protein levels over a 6 h period despite mRNA synthesis inhibition. Combination of DHT and Actinomycin D resulted in increased steady levels of AR expression during 6 h of exposure, indicating that AR stability is controlled by posttranscriptional events. These findings provide further evidence that androgen-dependent increase in AR abundance in SC was related not only to an enhanced AR messenger expression (Fig. 4C), but also to an increased AR protein stabilization.

To examine whether androgens modify the half-life time of AR protein, ST38c cells were treated either with DHT 10^{-7} M or cycloheximide (5 μ g/ml) or both for 1 h, 3 h or 6 h (Fig. 7). As expected, in the presence of cycloheximide, AR expression rapidly declined and was almost completely abolished after 6 h cycloheximide treatment. These findings indicate that the $t_{1/2}$ was approximately 3 h in ST38c cells, consistent with previous reports (Syms et al., 1985). On the other hand, as already shown (Figs. 5 and 6), AR protein expression was efficiently induced in the presence of 10^{-7} M DHT. However, the androgen-dependent AR stabilization was also observed in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 7), consistent with an increased half-life time of AR protein. Thus, androgens stabilized AR protein even when protein synthesis was inhibited, suggesting the involvement of direct posttranslational events on AR.

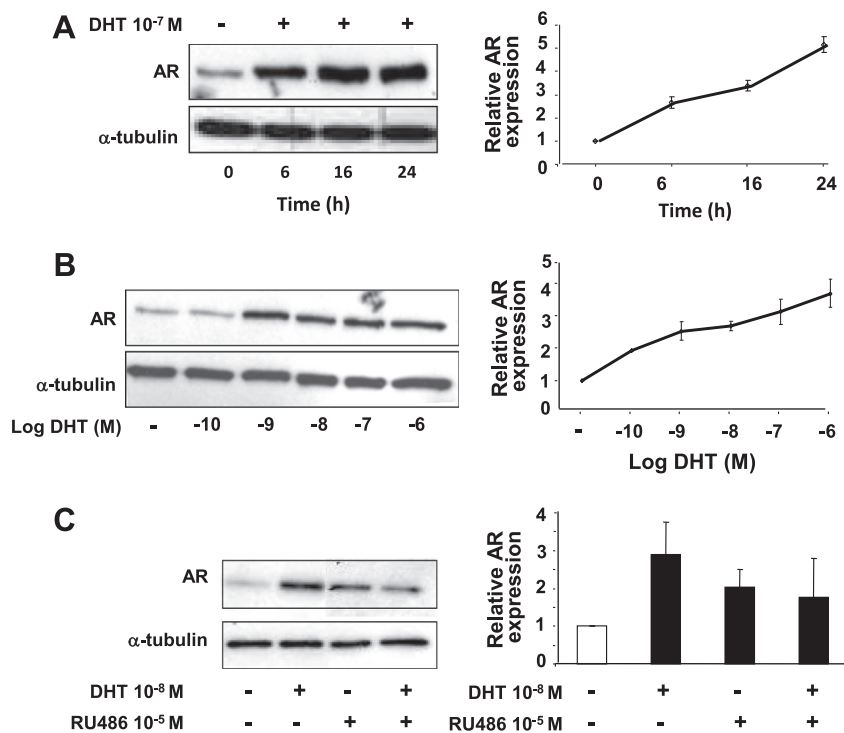


Fig. 5. Androgens up-regulate AR expression in the ST38c cells. Cell lysates were prepared from ST38c cells and AR expression was analyzed by Western immunoblots using 20 μ g protein lysate per lane. Polyclonal anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) detected an AR protein of 110 kDa, as above mentioned. Proteins were normalized by reblotting with anti α -tubulin antibody using the Bio-Rad QuantityOne software. (Bio-Rad, France). Numerized band densities corresponding to AR were normalized to α -tubulin and plotted (right panels) as fold induction relative to AR protein under unstimulated conditions arbitrarily set at 1. Data are means \pm SD of at least three independent determinations experiments. A: Time-dependent increase in androgen-bound AR protein. ST38c cells were incubated with 10^{-7} M dihydrotestosterone (DHT) for various periods of time 0 h, 6 h, 16 h, 24 h. B: Dose-dependent increase in androgen-bound AR protein. ST38c cells were stimulated with increasing concentrations of DHT (from 10^{-10} M to 10^{-6} M) for 20 h. C: ST38c cells were treated for 20 h with either vehicle, DHT at 10^{-8} M alone, with RU486 at 10^{-5} M alone or with both DHT at 10^{-8} M and RU486 at 10^{-5} M.

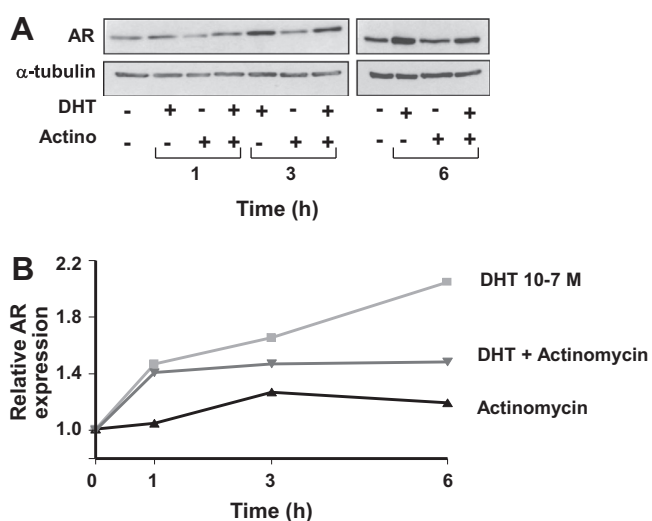


Fig. 6. Posttranscriptional events are involved in androgen-dependent AR stabilization. A: Western blotting analysis of AR expression in ST38c cells treated either with DHT 10⁻⁷ M, or with 0.4 μM actinomycin D or with a combination of both DHT and actinomycin D for various periods of time as indicated. B: Graphic representation of the relative AR expression in ST38c cells, after 1 h, 3 h or 6 h of treatment with DHT, actinomycin or their association, as previously described in the legend of Fig. 6. Relative AR protein expression is expressed as fold induction relative to basal conditions arbitrarily set at 1 except for 6 h incubation where AR expression was normalized at 6 h to unstimulated AR expression level at 6 h.

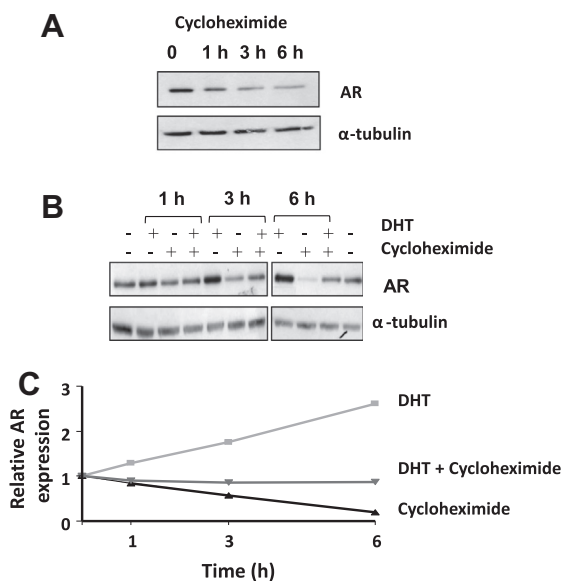


Fig. 7. Androgens induce AR upregulation by stabilizing AR protein. AR expression in ST38c cells was studied by Western blotting as described in the Figure legends above. A: ST38c cells were treated with cycloheximide (5 μg/ml) for various periods of time as indicated 1 h, 3 h and 6 h. AR expression was progressively reduced as a function of time in the presence of the protein synthesis inhibitor. B: ST38c cells were treated either with DHT 10⁻⁷ M or cycloheximide (5 μg/ml) or a combination of both DHT and cycloheximide for various periods of time as indicated. C: Graphic representation of relative normalized AR expression in ST38c cells, after treatment with DHT, cycloheximide or their association, as mentioned above. AR expression normalized to α-tubulin was expressed as fold induction relative to basal condition for untreated cells at 0 h, was arbitrarily set at 1.

4. Discussion

In the present study, we generated and described a novel immortalized mature Sertoli cell (SC) line, characterized by a substantial and stable expression of endogenous androgen

receptor (AR), exhibiting androgen-dependent transcriptional and posttranslational regulation. We established the SC origin and mature phenotype of the ST38c cells, as evidenced by the expression of several specific SC markers and other essentials factors implicated in SC functions. The presence of functional AR was unambiguously demonstrated in the ST38c cells making possible investigations on androgen-dependent AR action in a mature SC context.

SC lines have been previously derived from testis of various mammalian species (mouse, rat, sheep), or even of fish (Higaki et al., 2013), using different immortalization strategies, including the targeted oncogenesis method (Rahman and Huhtaniemi, 2004). However, these cells either contain a heterogeneous population, such as the MSC1 cell line (Peschon et al., 1992), or display an immature phenotype, such as prepubertal SC-SK11 (Sneddon et al., 2005), TM4 (Mather, 1980), or SMAT1 cells (Dutertre et al., 1997), preventing investigation on androgen regulation of SC function given the lack of AR expression in SC during the prepubertal period (Chemes et al., 2008; Boukari et al., 2009; Vija et al., 2013). Previously reported mature immortalized murine SC lines exhibited several limitations or disadvantages, such as the lack of homogeneity for the 15P-1 cell line (Paquis-Flucklinger et al., 1993), or variable degrees of FSH responsiveness owing that 42GPA9 cells expressed FSH-R (Bourdon et al., 1998) while S14-1 cells did not (Boekelheide et al., 1993). In all previously established SC lines, androgen responsiveness and stable AR expression were also an important issue given that immortalized or primary SC presented with rapid loss of AR expression after serial passages such as for SK11 (Sneddon et al., 2005) or SCIT-C8 cells (Konrad et al., 2005), or expressed very low levels of mRNA *Ar* for the MSC-1 cells (McGuinness et al., 1994).

Herein, we characterized a clonal, mature, immortalized SC line, named ST38c, expressing transcripts of specific SC markers such as *Abp*, transferrin and *clu*, as well as both mRNA and protein of SC markers of maturity (*tjp1*/TJP1). This is the first mature immortalized SC line with relatively high abundance of endogenous AR, which confers androgen-dependent transcriptional responses and exhibits androgen-mediated AR stabilization through posttranslational events. These main functional properties were maintained through several passages (P6–30).

We were interested in studying the mechanisms of AR auto-regulation in the SC context. Regulation of AR levels by androgens has been reported in almost all AR expressing cell types and was found to be both complex and tissue-specific. The androgen-dependent regulation of AR protein and gene expression has been already the subject of numerous studies, mainly in the context of prostatic tissue and prostate cancer, with contradictory results related to the different models used.

We are reporting the androgen regulation and provide possible mechanisms of androgen-mediated AR stabilization in SC. Data in the literature related to androgen-mediated AR regulation in SC are scarce. It was initially reported that androgens increase AR concentration in SC (Verhoeven and Cailleau, 1988) while it was briefly mentioned that AR protein expression was stabilized upon androgen treatment, in a primary monkey SC cultures (Chen et al., 2008). In contrast, steady state levels of *Ar* mRNA were found down-regulated after androgen exposure in several cell lines, such as the LNCaP prostatic carcinoma cells, in which Yeap et al showed that reduced mRNA transcription predominated over androgen stabilization of *Ar* mRNA, while in the MDA453 breast cancer cells, transcriptional down-regulation predominated over the reduction of the *Ar* mRNA turnover (Yeap et al., 1999). On the other hand, androgen treatment has been reported to variably up-regulate AR expression in prostate tissue. Takeda suggested a positive control of androgens on AR, given that prostate *Ar* mRNA was greatly reduced following rat castration, while normal AR transcripts were

restored upon androgen administration (Takeda et al., 1991). At the protein level, androgen treatment of prostate cells increased AR protein content, as demonstrated by both immunocytochemical and Western blot studies, such an effect suppressed by actinomycin D and cycloheximide, consistent with the involvement of both transcriptional activation and protein neosynthesis (Blanchere et al., 1998). Taken together, owing to the important variation of AR regulation as a function of cell environment, it was crucial to generate a specific cell model, which could recapitulate AR regulation in a Sertoli cell environment.

Thus, our results significantly differ from those reported in other androgen-responsive tissues, since androgen exposure of ST38c cells resulted in both an increased *Ar* mRNA expression as well as a AR protein stabilization. This androgen-stimulated *Ar* transcription associated with an enhanced AR stability was observed in ST38c cells in a time- and dose-dependent manner. In addition, we showed that AR abundance in the presence of DHT was not completely suppressed by actinomycin D, while AR protein remained stable after combined exposure of DHT and cycloheximide, indicating that both androgen transcriptional up-regulation and androgen posttranslational stabilization contribute equally to AR expression in ST38c cells.

Recently, Hazra et al. (2013), developed a unique transgenic mouse model, (TgSCAR) in which human AR was specifically over-expressed in SC using the rat androgen-binding protein (*Abpa*) gene promoter. As expected, it was found a strong and premature postnatal expression of AR in SC as early as D2, leading to reduction of SC number and testis weight and associated with upregulation of *rhox5* but also of tight junction transcripts (*cldn11* and *tjp1*). Interestingly, AMH expression rapidly declined to normal levels, however only in postpubertal testis, indicating that differential age-related down-regulation of *amh* might be in part independent of AR expression.

Transcriptomic analysis followed by qPCR validation identified a large series of androgen-regulated genes, some already reported as expressed in testis or androgen regulated. Previous microarrays were mostly performed in AR knockout mouse models, comparing whole testis gene expression of knockout and wild-type animals. Even with sophisticated engineered mouse models of selective ablation of AR in SC, such as SCARKO (Denolet et al., 2006; Abel et al., 2008; Willems et al., 2010) or S-AR^{-ly} (Zhang et al., 2012), it is always challenging to unambiguously appreciate whether identified genes are directly or indirectly androgen-dependent and SC specific.

Claudins form a large family of transmembrane proteins involved in tight junction formation with paracellular sealing function (Krause et al., 2008), which have been reported to be androgen regulated during developmental stages in several mouse models. For instance, it was shown that DHT treatment stimulated claudin 11 expression in rat Sertoli cells (Kaitu'u-Lino et al., 2007), as well as mRNA expression and the protein redistribution of claudin 11 in hpg mice testes (McCabe et al., 2012). On the other hand, claudins 3, 11 as well as *tjp1* mRNA and protein were decreased in SCARKO models, associated with defective barrier formation (Willems et al., 2010). In the ST38c cells, we observed a different pattern of claudin expression, as claudins 19, 15 and 1 were all androgen-downregulated targets, while claudin 3 did not seem to be androgen-regulated and claudins 2, 5 and 11 were barely expressed. These findings are not quite surprising, given that classical and non-classical claudin expression, their regulation and tightness properties, as well as their association to TJP1 seems to be largely dependent upon cell context.

We also identified *rip140* as an androgen up-regulated gene in ST38c cells. *Nrip/rip140* was originally reported as an AR coregulator expressed in epididymal epithelial cells, prostate and testis (Steel et al., 2005). It was shown that *rip140* mRNA steady-state levels were increased by androgens in prostate cancer cells (Caras-

cosa et al., 2006); however, the precise role of *rip140* in SC needs to be further evaluated. We also found that 11 β -hydroxysteroid dehydrogenase type 1 (*hsd11b1*) expression was up-regulated by androgens in ST38c cells. This steroid metabolic enzyme may exert a dual reductase/dehydrogenase enzymatic activity (Chapman et al., 2013; Gathercole et al., 2013) according to the tissue distribution, but most likely regenerating active cortisol or corticosterone in rodents from inactive 11 keto-metabolites (cortisone and 11-dehydrocorticosterone), thus enhancing local glucocorticoid action. *Hsd11b1* was previously shown to be expressed in testis, most notably in Leydig cells (Philips et al., 1989; Monder et al., 1994; Gao et al., 1997; Leckie et al., 1998; Ferguson et al., 1999; Gomez-Sanchez et al., 2008; Guo et al., 2012). The role of this glucocorticoid-converting enzyme in SC, the reductase or dehydrogenase activity and its androgen-regulated expression remain to be investigated.

Finally, few androgen-regulated genes previously described as expressed in SCs, such as *rhox5* (Maclean et al., 2005; Hu et al., 2010) presented neither a significant expression, nor androgen regulation in the ST38c cells. Indeed, *rhox5* was absent or very weakly expressed in several other immature or mature Sertoli cell lines (such as 15P1, TM4, MSC1) (Bhardwaj et al., 2008). It has been recently reported that GATA factors (mainly GATA4 and GATA6) are essential for AR regulated *rhox5* transcriptional activation (Bhardwaj et al., 2008). As other Sertoli cell lines (15P1, TM4), our Sertoli cell line, lacked expression of GATA factors, including, GATA4 and GATA6, explaining the lack of *rhox5* transcription.

In sum, our ST38c SC model could certainly complement *in vivo* results obtained with animal models, even though, as most cell-based systems, the ST38c cell line has its advantages as well as its own limitations. For instance, we were unable to efficiently transfect these cells without high cellular mortality, even by means of electroporation. FSH responsiveness was also an important issue. We failed to detect *fshr* mRNA in the ST38c cells under standard experimental conditions. However, in addition to the lack of GATA factors important for *fshr* transcription, it is known that FSH-R expression highly depends upon cultured conditions, notably timing and duration of gonadotropin exposure. Further studies are needed to reevaluate FSH signaling in SC context.

Anyhow, the ST38c cell model should facilitate not only better understanding of the androgen regulation in SC but also might constitute a suitable system to decipher the molecular mechanisms accounting for the negative correlation between AR and AMH expression in postpubertal Sertoli cells.

5. Conclusion

To our knowledge, ST38c cells represent the first mature, clonal, immortalized mouse Sertoli cell line, expressing a functional AR. Detailed analysis revealed not only that ST38c express *Ar* RNA messenger and protein, but also that androgens stabilize AR protein turnover in Sertoli cells *via* post-transcriptional mechanisms. We also investigated androgen induced AR mRNA transcriptional regulation as well as some of the transcriptional androgen-regulated genes, showing that this new cell line might be a valuable model for the study of Sertoli-cell specific gene expression and function.

Reviewers might temporary account to access the microarray data stored in Array Express database (http://www.ebi.ac.uk/arrayexpress/help/how_to_search.html#Login). Username: Reviewer_E-MTAB-1732; Password: iggwutoa.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mce.2014.01.008>.

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Fig. S1

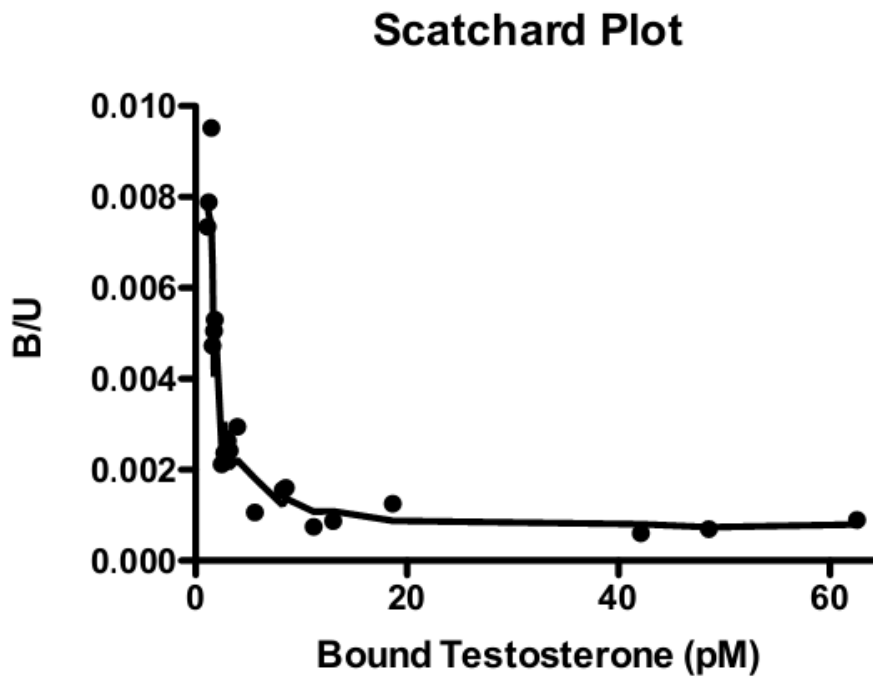


Fig. S1. ST38c cells express the androgen receptor (AR) with high affinity for ligand (testosterone). Cytosolic fractions of ST38c cells, previously maintained in dextran charcoal-coated FBS for 24h, were incubated with increasing concentrations of [³H]- testosterone (Perkin Elmer) for 4 h at 4 C. Bound (B) and unbound (U) hormone were separated by the charcoal-dextran technique. U was calculated as the difference between total and bound hormone concentration. The curve was simulated from the best model of interaction. The calculated values of binding parameters were determined at equilibrium. The dissociation constant K_d was estimated at 0.7 nM while the maximum number of specific binding sites was calculated at $5-10 \times 10^3$ sites/cell.

3. Discussion

In the first part of the project, we generated and described a novel immortalized mature Sertoli cell (SC) line, characterized by a substantial and stable expression of endogenous androgen receptor (AR), exhibiting androgen-dependent transcriptional and posttranslational regulation. We established the SC origin and mature phenotype of the ST38c cells, as evidenced by the expression of some specific SC markers and other essential factors implicated in SC functions. The presence of functional AR was unambiguously demonstrated in the ST38c cells making possible investigations on androgen-dependent AR action in a mature SC context.

SC lines have been previously derived from the testis of various mammalian species (mouse, rat, sheep), or even in fish (Higaki, Koyama et al. 2013), using different immortalization strategies, including the targeted oncogenesis method (Rahman and Huhtaniemi 2004), as detailed in Table 6. However, these cell lines either contain a heterogeneous population, such as the MSC1 cell line (Peschon, Behringer et al. 1992), or display an immature phenotype (prepubertal SC-SK11 cells (Sneddon, Walther et al. 2005), TM4 (Mather, 1980), or SMAT1 cells (Dutertre, Rey et al. 1997), preventing investigation on androgen regulation of SC function given the lack of AR expression in SC during the prepubertal period (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Rey, Musse et al. 2009). Previously reported mature immortalized murine SC lines were either contaminated by germ cells, 15P-1 (Paquis-Flucklinger, Michiels et al. 1993), or exhibited variable degrees of FSH responsiveness with 42GPA9 cells (Bourdon, Lablack et al. 1998) expressing FSH-R, while S14-1 cells did not (Boekelheide, Lee et al. 1993).

Previous data related to androgen responsiveness and AR expression in SC lines concerns few immature and mature SC lines (Table 6). It was reported that AR expression was rapidly lost after serial passages in several immortalized SC lines, such as the SK11 cells, or the rat SCIT-C8 cells (Konrad, Munir Keilani et al. 2005). On the other hand, Other SC lines, such as MSC-1, expressed very low levels of endogenous AR, necessitating AR cDNA transfection to evaluate androgen regulation of SC-expressed genes, such as the Rhox genes (McGuinness, Linder et al. 1994).

We characterized a clonal, mature, immortalized SC line, named ST38c, expressing transcripts of specific SC markers such as ABP, transferrin and SGP-2/CLU, as well as both mRNA and protein of SC markers of maturity (ZO-1/TJP1). This is the first mature

immortalized SC line with relatively high abundance of endogenous AR, which is characterized by both androgen-dependent transcriptional responses and androgen-mediated AR stabilization through posttranslational events. These two functional properties were maintained through several passages (P6-30).

We were interested in studying the mechanisms of AR autoregulation in the SC context. Regulation of AR levels by androgens has been reported in almost all AR expressing cell types and was found to be both complex and tissue-specific. The androgen-dependent regulation of AR protein and gene expression has been already the subject of numerous studies, mainly in the context of prostatic tissue and prostate cancer, with contradictory results related to the different models used. Steady state levels of *Ar* mRNA were found down-regulated after androgen exposure in several cell lines, such as the LNCaP prostatic carcinoma cells, in which Yeap et al showed that reduced mRNA transcription predominated over androgen stabilization of *Ar* mRNA, while in the MDA453 breast cancer cells, transcriptional down-regulation predominated over the reduction of the *Ar* mRNA turnover (Yeap, Krueger et al. 1999). On the other hand, androgen treatment has been reported to variably up-regulate AR expression in prostate tissue. Takeda suggested a positive control of androgens on AR, given that prostate *Ar* mRNA was greatly reduced following rat castration, while normal AR transcripts were restored upon androgen administration (Takeda, Nakamoto et al. 1991). At the protein level, androgen treatment of prostate cells increased AR protein content, as demonstrated by both immunocytochemical and Western blot studies, such an effect suppressed by actinomycin D and cycloheximide, consistent with the involvement of both transcriptional activation and protein neosynthesis (Blanchere, Berthaut et al. 1998). Taken together, owing to the important variation of AR regulation as a function of cell environment, it was crucial to generate a specific cell model, which could recapitulate AR regulation in a Sertoli cell environment.

Thus, our results significantly differ from those reported in other androgen-responsive tissues, since androgen exposure of ST38c cells resulted in both an increased *Ar* mRNA expression as well as AR protein stabilization. This androgen-stimulated *Ar* transcription associated with an enhanced AR stability was observed in ST38c cells in a time- and dose-dependent manner. In addition, we showed that AR abundance in the presence of DHT was completely suppressed neither by actinomycin D, nor by cycloheximide exposure, indicating that both androgen transcriptional up-regulation and androgen posttranslational stabilization contribute equally to AR expression in ST38c cells.

Transcriptomic analysis followed by qPCR validation identified a large series of androgen-regulated genes, some already reported as expressed in testis or androgen regulated. Previous microarrays were mostly performed in AR knockout mouse models, comparing whole testis gene expression of knockout and wild type animals. Even with sophisticated engineered mouse models of selective ablation of AR in SC, such as SCARKO (Tan, De Gendt et al. 2005; Abel, Baker et al. 2008) or S-AR^{-y} (Zhang, Zhang et al. 2012), it is always challenging to unambiguously appreciate whether identified genes are directly or indirectly androgen-dependent and SC specific.

Recently, De Gendt et al (De Gendt, Verhoeven et al. 2014) used RiboTag mice containing a *Rpl22* gene with a duplicate exon 4 tagged with a hemagglutinin epitope that was floxed; the incorporation of this mutant *Rpl22* into the ribosomes allowed isolation of ribosomes with their bound mRNAs; these mice were crossed with mice expressing CRE specifically in SC; by isolating the ribosome bound RNA fraction, authors identified a large panel of genes expressed in SC. They compared gene expression between these mice (called SCRIBO) with RiboTag mice lacking a functional AR gene in SCs. The analysis allowed identification of new sets of androgen regulated genes in Sertoli cells after comparison of gene expression made between SCRIBO/versus SCARIBO mice at postnatal day 10, associated with an analysis of mRNAs predominantly translated in SC between postnatal day 10 and adult SCRIBO testis. However, in this work AR-regulated genes identification was realised at postnatal day 10 and not in adult mice.

In our approach of identification of androgen regulated genes, in an adult SC line we found some new AR regulated genes that were in concordance with De Gendt's findings using the RiboTag approach, such as the *TGFB2*, that we found AR up-regulated, shown to have roles in SC barrier formation and maintenance (De Gendt, Verhoeven et al. 2014).

Claudins form a large family of transmembrane proteins involved in tight junction formation with paracellular sealing function (Krause, Winkler et al. 2008), which have been reported to be androgen regulated during developmental stages in several mouse models. For instance, it was shown that DHT treatment stimulated claudin 11 expression in rat sertoli cells (Kaitu'u-Lino, Sluka et al. 2007) as well as mRNA expression and the protein redistribution of claudin 11 in hpg mice testes (McCabe, Allan et al. 2012).

Table 6. Main characteristics of previously reported Sertoli cell lines. Sp species;h Telomere RT-human telomere reverse transcriptase

References:1,(Dutertre, Rey et al. 1997); 2, (Mather 1980); 3, (Sneddon, Walther et al. 2005); 4, (Hofmann, Narisawa et al. 1992); 5,(Peschon, Behringer et al. 1992); 6, (Boekelheide, Lee et al. 1993); 7,(Bourdon, Lablack et al. 1998), 8 (Paquis-Flucklinger, Michiels et al. 1993); 9, (Sato, Yoshida et al. 2013); 10, (Chuang, Lee et al. 2007); 11,(Konrad, Munir Keilani et al. 2005); 12, (Higaki, Koyama et al. 2013).

Immature Sertoli cell lines														
Name	Species	Immort. method	Sertoli cell markers									FSH Response	Ref.	
			WT1	transferrin	SGP-2	ABP	ZO-1	inhibin	AMH	FSH-R	AR			
SMAT1	mouse	SV40-hAMH	+	-	+	+			$\alpha+\beta$ B	++	-	-	+	1
TM4	mouse			+							+	+	+	2
SK11	mouse	SV40			+									3
Other	mouse	SV40									+			4
Mature Sertoli cell lines														
MSC1	mouse	SV40-hAMH		+	+	+			β B		-		+	5
S14-1	mouse	SV40-tsA255		+	+								-	6
42GPA9	mouse	PyLT		+	+		+				+		+	7
15P-1	mouse	PyLT												8
Other	mouse	starvation	+	+			+		α	+	+	+	+	9
B6SC-2,3	mouse	h telomere RT					+			+	+	+	+	10
SCIT-C8	rat	pSV 40-neo		+							-	-	-	11
Other	fish											+		12

On the other hand, claudins 3, 11 as well as ZO-1 mRNA and protein were decreased in SCARKO (McCabe, Allan et al. 2012) associated with defective barrier formation (Willems, Batlouni et al. 2010). In the ST38c cells, we observed a different pattern of claudin expression, as claudins 19, 15 and 1 were all androgen-downregulated targets, while claudin 3 did not seem to be androgen-regulated and claudins 2, 5 and 11 were barely expressed. These findings are not quite surprising given that classical and non-classical claudin expression, their regulation and tightness properties, as well as their association to ZO-1/TJP1 seems to be largely dependent upon cell context.

We also identified *rip140* as an androgen up-regulated gene in ST38c Sertoli cells (SC). *Nrip/rip140* was originally reported as an AR coregulator expressed in epididymal epithelial cells, prostate and testis (Steel, White et al. 2005). It was shown that *rip140* mRNA steady-state levels were increased by androgens in prostate cancer cells (Carascossa, Gobinet et al. 2006); however, the precise role of *rip140* in SC needs to be further evaluated. We also found that 11 β -hydroxysteroid dehydrogenase type 1(*hsd11b1*) expression was up-regulated by androgens in ST38c cells. This enzyme that regenerates 11-keto glucocorticoids and controls glucocorticoid receptor signaling was already reported to be expressed in testis notably in Leydig cells (Guo, Yuan et al. 2012). The role of this glucocorticoid-converting enzyme in Sertoli cells and its androgen-regulated expression remain to be investigated.

Finally, few androgen-regulated genes previously described in SCs, such as *Rhox5* (Maclean, Chen et al. 2005; Hu, Dandekar et al. 2010) presented a weak expression, and not significant evidence of androgen regulation in the ST38c cells (Fig.21).

Rhox5 gene is crucial for spermatogenesis maintenance, as targeted disruption of *Rhox5* leads to fertility impairment due to increased germ cell apoptosis and decreased sperm mobility (Rao, Wayne et al. 2003; Maclean, Chen et al. 2005).

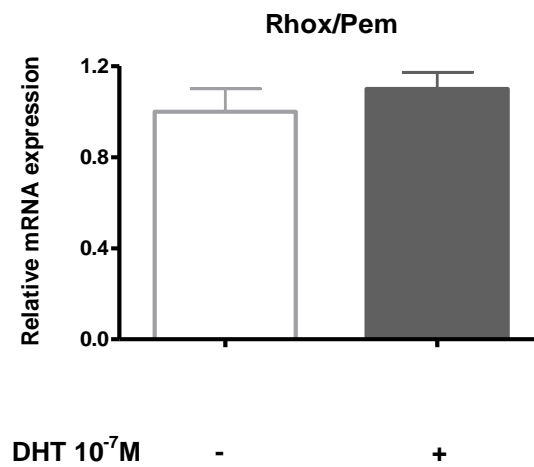


Fig. 21. Androgen stimulated ST38c cells express low levels of *Rhox5* mRNA with no androgenic regulation.

Our ST38c SC model could certainly complement *in vivo* results obtained with animal models, even though, as most cell-based systems, the ST38c cell line has its advantages as well as its own limitations. For instance, we were unable to efficiently transfect these cells without high cellular mortality, even by means of electroporation. FSH responsiveness was also an important issue. We failed to detect FSH-R mRNA in the ST38c cells under standard experimental conditions. However, it is known that FSH-R expression highly depends upon cultured conditions, notably timing and duration of gonadotrophin exposure. Further studies are needed to reevaluate FSH signalling in SC context.

Anyhow, the ST38c cell model should facilitate not only better understanding of the androgen regulation in SC but also might constitute a suitable system to decipher the molecular mechanisms accounting for the negative correlation between AR and AMH expression in postpubertal Sertoli cells.

4. Limits, further perspectives and conclusions

To our knowledge, ST38c cells represent the first mature, clonal, immortalized mouse Sertoli cell line, expressing a functional AR. Detailed analysis revealed not only that ST38c express *Ar* RNA messenger and protein, but also that androgens stabilize AR protein turnover in Sertoli cells via post-transcriptional mechanisms. We also investigated androgen induced AR mRNA transcriptional regulation as well as some of the transcriptional androgen regulated genes, showing that this new cell line might be a valuable model for the study of Sertoli-cell specific gene expression and function.

Moreover, we used this novel cellular model in order to explore the potential AR/AMH regulation explaining the inverse relationship between serum AMH and testosterone levels at puberty and in adulthood.

4.1. AR induced- AMH repression: hypotheses, results and perspectives

4.1.1. Introduction

It has already been reported that AMH production and expression declines with the increase in intratesticular androgen concentration during puberty and adulthood (Rey, Mebarki et al. 1994; Rajpert-De Meyts, Jorgensen et al. 1999; Rey 2003; Rey, Lukas-Croisier et al. 2003; Rey, Musse et al. 2009; Rey and Grinspon 2011). This negative correlation is reinforced by clinical evidence in boys with central or gonadotropin-independent early puberty (Rey, Lukas-Croisier et al. 2003; Young, Rey et al. 2003), while in patients with deficient testosterone production or androgen insensitivity syndromes, lack of functional testosterone intratesticular levels result in abnormal high AMH production (Rey, Mebarki et al. 1994; Rey, Lukas-Croisier et al. 2003). Indeed, Prof Young and his collaborators have previously demonstrated that the hCG-driven increase in intratesticular T in 10 CHH patients was associated with a marked reduction in the serum AMH level (Young, Chanson et al. 2005). Experimental evidence for the negative regulation of androgens on AMH expression has also been observed in androgen insensitive *Tfm* mice, which continued to express high levels of AMH until the meiotic entry of seminiferous tubules (Al-Attar, Noel et al. 1997).

We and others have also previously demonstrated that during fetal life and in the neonatal period (1 to 4 months postnatally), there is a transient coexistence of high concentrations of androgens and AMH, related to a physiological androgen insensitivity, explained (both by in

vivo results but also by immunohistochemical analysis) by the lack of functional AR expression in SC during this developmental periods (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Vija, Meduri et al. 2013).

Moreover, the histological and immunohistochemical testicular description of a testicular sample obtained from a patient with minimal androgen insensitivity syndrome (MAIS), allowed me to confirm that in this MAIS male subject, presenting a partially inactive AR mutation and azoospermia, in the rare seminiferous tubules presenting spermatogenesis on testicular biopsy, AMH was repressed, while AR was expressed, contrasting with the majority of seminiferous tubules devoid of spermatogenesis, where AMH was strongly expressed (Massin, Bry et al. 2012).

In accordance with these *in vivo* findings, my colleague, and predecessor, Kahina Boukari, obtained the first *in vitro* direct evidence that AMH repression by androgens occurs when AR is expressed in Sertoli cells, using the immortalized murine cell line, SMAT1. The immature SMAT1 Sertoli cells (Dutertre, Rey et al. 1997), express AMH, which is increased after exposure to forskolin through a protein kinase A-dependent mechanism (Lukas-Croisier, Lasala et al. 2003), despite the absence of FSH-R. SMAT1 do not express endogenous AR. Whether dihydrotestosterone treatment did not interfere with the forskolin-induced AMH expression, when SMAT1 cells were transiently transfected with a hAR plasmid/expression vector, forskolin-stimulated AMH mRNA levels returned to basal values after dihydrotestosterone exposure, arguing that functional AR is crucial for full AMH repression by androgens in SC (Boukari, Meduri et al. 2009).

However, the precise molecular mechanisms responsible for the repressive effect of AR on AMH expression are still not elucidated.

4.1.2. Objectives

As ST38c exhibit a mature Sertoli cell phenotype, with stable AR expression and lack of AMH expression, we used this novel cellular model in order to explore whether it exists a direct effect of AR on AMH expression and its potential molecular mechanisms.

4.1.3. Results

In this respect, I have realized several transfection assays of AMH promoter constructs, kindly provided by Pr Jean-Yves Picard (UMR782, Clamart). However, despite having performed repeated experiences, for a period during more than 10 months, and using different transfection methods of AMH promoter constructs, such as lipofectamines (with different transfection kits-Lipofectamine 2000, GeneJuice, Fugene6), calcium phosphate, electroporation, direct as well as reverse transfection, and even viral transduction, all these assays have proven to be unsuccessful, as this Sertoli line was not easily transfectable.

In order to evaluate whether an AMH transcriptional induction, occurs when AR transcriptional expression is silenced, which would have demonstrated its direct negative regulation by AR, I used a small interfering RNA (siRNA) approach. Therefore, we designed two different siRNA targeting the eighth exon of AR (siRNA sequences are available in the Additional Tables).

Preliminary results, obtained 48h after siRNA transfection were encouraging, as AR mRNA silencing was associated with barely increased AMH expression. However, despite both mRNA and protein AR silencing, in repeated experiments, mRNA AMH expression didn't register any important modifications maintaining the same steady levels. Moreover, even 3h stimulation with 10^{-6} M forskolin, didn't succeed in stimulating any AMH increase (Fig. 22 and 23).

4.1.4. Discussion

As the hormonal profile in children and adolescents with AIS and precocious puberty revealed an inverse relationship between serum AMH level and testosterone levels (Rey, Mebarki et al. 1994; Al-Attar, Noel et al. 1997; Rey, Lukas-Croisier et al. 2003), and as Boukari (Boukari, Meduri et al. 2009), already demonstrated that the decrease in AMH secretion is mediated directly by androgens via the AR present on SCs, we were motivated to search for a cellular mechanism for a direct AR/AMH regulation.

However, previous studies in XXSxr^b mice, model characterized by normal testicular maturation and AR expression, presenting germ cell failure before meiotic entry,

demonstrated that the AMH inhibition at puberty is related to a synergistic effect of androgens and meiotic entry (Al-Attar, Noel et al. 1997).

Moreover, this aspect is reinforced by immunohistochemical data related to heterogeneous AMH expression in relation with the entry in meiosis of germ cells in testicular biopsies of patients with AIS or azoospermia (Steger, Rey et al. 1996; Rajpert-De Meyts, Jorgensen et al. 1999).

Recent reports related to AR/AMH relations in murine testis ontogeny, bring evidence that potential AR/AMH interactions might be more complex than expected.

Recently, Hazra et al developed a SC-specific transgenic mouse model overexpressing AR in postnatal Sertoli cells. In this model, human AR was specifically overexpressed in SC using the rat androgen-binding protein (Abpa) gene promoter. As expected, it was found a strong and premature postnatal expression of AR in SC as early as day 2, leading to reduction of SC number and testis weight and with early maturation of the seminiferous tubules and the entry of germ cells into meiosis and, for testosterone rates similar to WT (Hazra, Corcoran et al. 2013). Strong and persistent AR postnatal expression was associated not only with up-regulation of several AR-regulated genes, such as *Rhox5* and *Spinlw1* but also of transcripts crucial for SC tight-junction formation (*Cldn11* and *Tjp1*).

Postnatal *Amh* expression was initially elevated and then declined to normal levels with the evolution of developmental stages, in both pubertal TgSCAR and WT, indicating that differential age-related down-regulation of *Amh* might be at least in part independent of AR expression. Interestingly, AMH transcripts were more expressed in prepubertal TgSCAR (5 to 10 days of postnatal life), than WT, whereas, just before puberty and adulthood, AMH levels decreased in the same way for both TgSCAR and WT.

The authors attempted to confirm the hypothesis that GATA-1, which could be directly regulated by AR, represses AMH in Sertoli cells (as the GATA-1 promoter exhibits several ARE (androgen response element), in opposition to the AMH promoter, which has a single ARE, with low potential).

The particularity in Hazra's work was that in this TgSCAR mouse model, the inverse relationship GATA1 / AMH was found only during the first 5 days of postnatal life and not at

puberty or later on, in the adult, which presented a very important expression of AR, before a collapse of AMH. Concerning the testicular phenotype, in developing and mature TgSCAR mouse testes total SC numbers and testicular volume were reduced, despite normal or higher *Fshr* mRNA and circulating FSH levels.

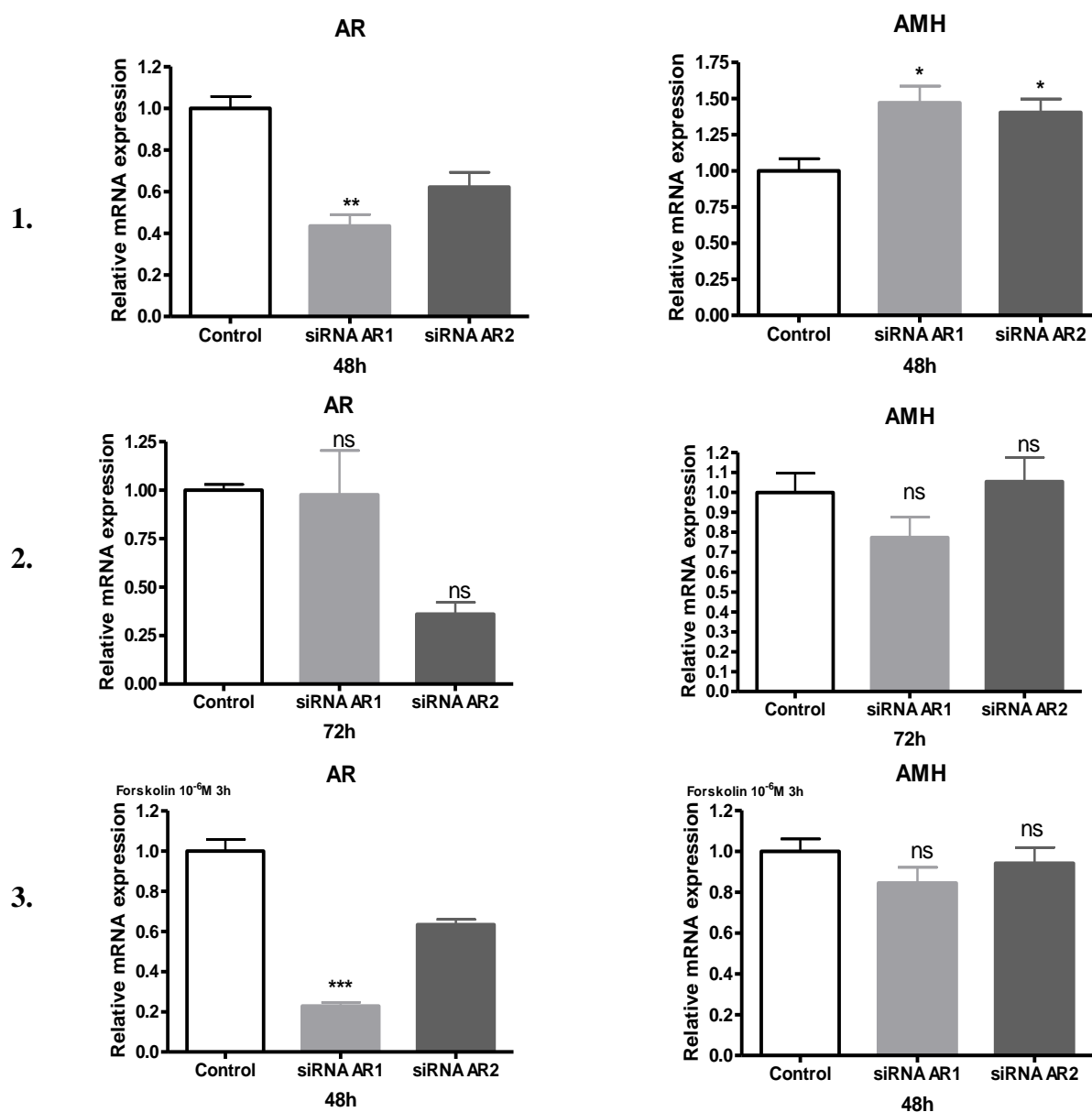


Fig.22. In ST38c cells, *Amh* mRNA expression is not upregulated upon mRNA and protein AR silencing, using a siRNA approach. Line 1, *Ar* and *Amh* mRNA expression 48h after siRNA AR transfection (two siRNA AR couples have been tested-siRNA AR1 and siRNA

AR2). Line 2, Ar and Amh mRNA expression 72h after siRNA AR transfection. Line 3, Ar and Amh mRNA expression 48h after siRNA AR transfection and 3h of treatment with forskolin $10^{-6}M$.

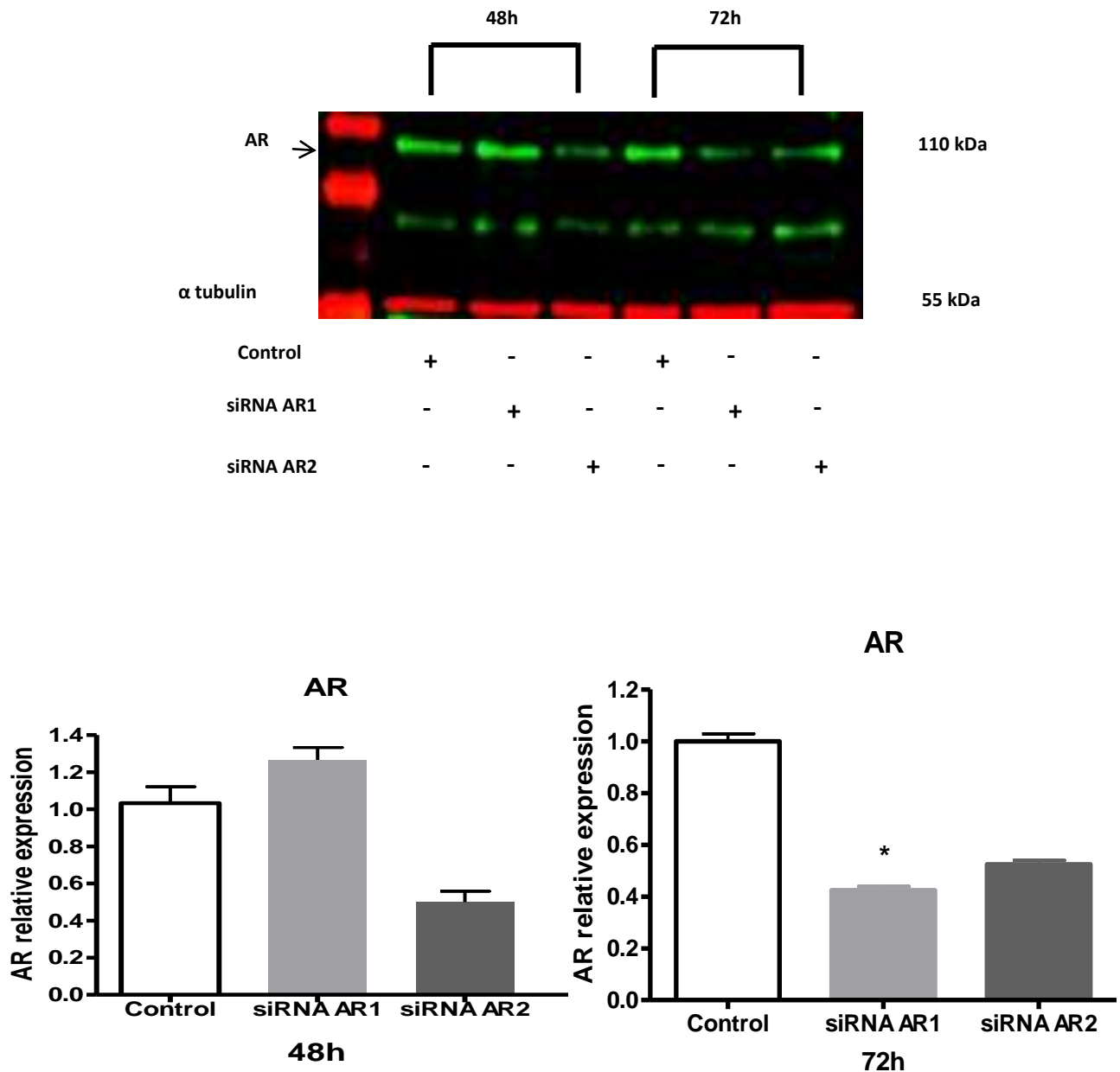


Fig.23. AR protein expression in ST38c SC after 48 and 72 h of transfection with siRNA AR. The control samples were arbitrarily set as 1.

5. Conclusion

In conclusion, the precise molecular mechanisms responsible for the repressive effect of AR on AMH expression remain to be elucidated. It was hypothesized that AR might directly repress AMH transcription although no androgen response elements are present in the regulatory regions of the AMH gene. Alternatively, AR might compromise FSHR-mediated AMH stimulation by altering activator protein 2 and nuclear factor κ B signaling (Lukas-Croisier, Lasala et al. 2003). Further investigations on the functional interaction between AR and FSHR activation cascade on AMH expression using a SC model, either expressing endogenously the FSHR or being easily transfectable. Although presenting some pitfalls (such as lack of FSHR expression, alike the majority of SC lines obtained by SV40 targeted oncogenesis, as listed in Table 6) and transfection difficulties, the ST38c Sertoli cell line, could still represent a useful tool for studying androgenic regulation in Sertoli cells, spermatogenesis regulation or the potential effects and mechanisms of action of endocrine disruptors on testicular functions.

Supplemental table 1. Mus musculus primer sequences for real time PCR.

Name	Forward primer	Reverse primer
m18S	CCCTGCCCTTTGTACACACC	CGATCCGAGGGCCTCACTA
mAR	TCCCAGTCCCAATTGTGTTCAA	CCACAGATCAGCCAGGTCTTC
m HBO1	GACAGGGCTACGGGAAGATG	TGTGCAGGTATC GGA GCAGTA
m SRC-2	GGC TGG GAAGATCTGGTAAGAA	GCCATC AGACAAAGAAAAACGAT
m CLDN 19	CTACGAAGGGCTGTGGATGTC	GGGCTCGTGCTGACTGGATA
m GPR126	GAAGAAGCTCCCACTGTATCTATGA	GCAGTCGCTCCACAGAATTTT
m NOV	GCACCAAGAAATCCCTGAAA	GAGGGCAGTTGGAGTAGCAG
m GAS6	GAGCTCGTCCAGCCGATTAA	TTGCCTTGACTGTCTCCTGGAT
m ALDH1A1	GCTGCAGGGAAAAGCAATCT	TCGGCATCTGCAAACACAAT
m PRMT1	GGCCAAGCAGAAAGTAGTGAGAA	AAAGTGGGCATAGGAGTCAAAGTAG
m hsd11b1	TCCCTGTTTGATGGCAGTTATG	CCTGGAGCATTTCTGGTCTGA
m CXCL11	GACATCCTGGGAACGTCTGACT	CCTGGTAATACGTGCCTGCAT
m WT1	CACGGCACAGGGTATGAGAGT	TTCAGATGCTGACCGGACAAG
m β TUB3	CCAAGAACATGATGGCTGCCTGT	ACCTTGACGTTGTTGGGGATCC
m SGP-2	GACAATGAGCTCCAGAAATG	CAGGCATCCTGTGGAGTTGTG
m Inhibin B α	GCAATGGATGGGGAAGGTGG	GGTGGCTGCGTATGTGTTGG
m Inhibin B β	CCGAGATCATCAGCTTTGCA	CCTTCATTAGAGACGAAGAAGTACA
m AMH	CACACAGAACCTCTGCCCTACTC	AAGGCTTGCAGCTGATCGAT
m LHR	GAATTTTGCCGAAGAAAGAACA	GAATTTTGCCGAAGAAAGAACA

Abbreviations of the genes and the 5' and 3' nucleotide sequences of their sense and antisense primers

Supplemental table 2. Oligonucleotide 5'-3' primer sequences of murine couples of siRNA for AR.

siRNA AR	Sequence (5'-3')
siRNA AR1	CUGC ⁺ UAAUCAAGUCCCAUA UAUGGGACUUGAUUAGCAG
siRNA AR2	CCAAGA ⁺ UCCUUCUGGGAA U ⁺ UCCAGAAAGGAUCUUGG

Second part

Androgen receptor coregulators (SRC-2 and HBO1) expression and characterization during testicular development

Paper 2

**Expression and characterization of androgen receptor coregulators, SRC-2
and HBO1, during human testis ontogenesis and in androgen signaling
deficient patients.**

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Mol Cell Endocrinol. 2013 Aug 15;375(1-2):140-8

1. Introduction

As previously discussed in the introduction part, little is known at present about the phenomenon of stage and age-specific regulation of AR expression in Sertoli cells. In this respect, identification of potential co-activators or co-repressors that act in conjunction with AR at different stages of testicular development would provide insight into the complex mechanisms and functional differences of Sertoli cells related to AR regulation during different stages of spermatogenesis. Among the several hundreds of AR coregulators, a great part have not yet been examined neither in animal models nor in human pathologies, therefore their biological relevance in normal and pathological conditions remains to be established.

In this context, another established objective was to study the differential expression of AR coregulators during murine and human normal ontogeny and postnatal development, as well as in pathologies associated with androgen or AR action impairment (androgen insensitivity syndromes- a human model of AR invalidation, congenital hypogonadotropic hypogonadism- a situation of reduced testicular androgenic exposure, 5-alpha reductase type 2 deficiency- a situation of reduced/absent testicular exposure to dihydrotestosterone).

After studying previous data related to animal models of AR coregulator invalidation, or clinical data related to AR coregulator dysfunctions (as synthesized in the Introduction part), as well as their relevance for testicular development and fertility, we selected two potentially interesting AR coregulators: SRC-2 and HBO1.

Indeed, there are limited data on the role of androgen receptor coregulators in male reproductive events, notably almost no information in humans. Some reports exist on the identification in the human testis by means of Northern blot analysis, real time RT-PCR and immunohistochemistry, of some AR coregulators, such as SRC-1, ARA54, ARA55, ARA70 (Lan, Hseh et al. 2008) and PIAS1 (Tan, Hall et al. 2000).

Herein, I studied for the first time the expression profile of two androgen receptor coregulators, SRC-2 and HBO1 during both murine and human testicular development, from fetal life to adulthood. In human testicular samples, I specifically localized by double immunolabeling studies, that both coregulators are expressed in Sertoli cells, suggesting that they both interact with and modulate AR signaling.

Moreover, after localizing these two coregulators, I performed preliminary experiments, in order to understand the mechanisms of regulation of HBO1 on AR expression.

The results related to the studies of the expression profiling of SRC-2 and HBO1 in human testis, as well as in diseases associated with AR signalling alteration leading to infertility caused by reduced or absence of spermatogenesis have been recently published in the “Molecular and Cellular Endocrinology” journal. We revealed that SRC-2 was present in Sertoli cells at all developmental stages. HBO1 was barely or focally detected in the fetal testis yet its expression, in Sertoli and germ cells, drastically increased postnatally from early infancy to adulthood. In transient co-transfection studies we showed that SRC-2 induced, while HBO1 inhibited AR-mediated transactivation of reporter constructs in murine Sertoli SMAT1 cells. HBO1, but not SRC-2, expression was reduced in testes of patients with AIS compared to normal testes.

In this chapter I shall present the rationale for the selection of each of the two acetyl transferases exerting AR coregulatory properties, as well as additional data related to further coregulator profiling studies performed in murine primary as well as immortalized Sertoli cells and spermatogonia and murine testis during ontogenesis and data related to clinical and genetic profile of the CAIS and CHH patients for whom I analysed the testicular expression of SRC-2 and HBO1.

1.1. Rationale for SRC-2 selection

The steroid receptor coactivator 2 (SRC-2) formerly named TIF2, is a class II coactivator that modifies chromatin structure through the histone acetyltransferase or ATP-dependent chromatin remodelling activity. It interacts with both N-terminal activation function (AF-1) and C-terminal region (AF-2) of AR (Heinlein and Chang 2002; van de Wijngaart, Dubbink et al. 2012).

Studies performed on SRC-2 mutant phenotypes allowing in vivo studies of androgen induced AR activity, brought to evidence that SRC-2 is a preferential coactivator for AR in the murine testis (Ye, Han et al. 2005). Moreover, several authors reported that SRC-2 is not only an AR coactivator, but also a coactivator of GR (Awasthi and Simons 2012), PR (Hofman, Swinnen et al. 2002) and ER (Warnmark, Treuter et al. 2002), all these steroid receptors being also expressed in human testis during ontogenesis, with normal or impaired spermatogenesis (Shah, Modi et al. 2005; Boukari, Ciampi et al. 2007; Cavaco, Laurentino et al. 2009). However, there were no data related to SRC-2 expression and localization in human testis.

It was previously reported that SRC-2 was expressed in murine adult testes and its complete inactivation leads to a hypofertile phenotype. Null SRC-2^{-/-} female mice present a poor development of the chorioallantoic placenta leading to embryonic growth retardation and lethality. SRC-2 null male mice become hypofertile as early as three months of life and exhibit testicular alterations in cell adhesion between Sertoli and germ cells, explaining the decreased fertility. These data imply that SRC-2 expression, exclusively confined to Sertoli cells, would be essential for spermatogenesis in SRC-2^{-/-} male mice (Gehin, Mark et al. 2002). Moreover, there are also several reported patients with either partial AIS or idiopathic azoospermia, presenting mutations in the ligand-binding domain of AR, characterized by normal ligand binding, but defective transactivation, due to altered interaction with coactivators, such as SRC-2 (Wang, Ghadessy et al. 1998; Ghadessy, Lim et al. 1999; Adachi, Takayanagi et al. 2000; Quigley, Tan et al. 2004).

Despite the interesting role of SRC-2 in murine reproduction, there are limited available data regarding the presence and the potential role of SRC-2 in human reproduction. Immunohistochemical studies demonstrated that SRC-2 protein is expressed in a subset of steroid-responsive tissues in human, such as the epithelial cells of human prostate (which is a well-known cellular target for androgen receptor action)(Culig, Klocker et al. 2002; Ye, Han et al. 2005), the stromal and epithelial compartments of human endometrium (Mukherjee,

Amato et al. 2007) and in a subgroup of epithelial cells within normal human breast (Mukherjee, Amato et al. 2007).

However there is very limited data implying a potential role for SRC-2 in human testis.

In the article recently published in *Molecular and Cellular Endocrinology* (Vija, Meduri et al. 2013), we evaluated the transactivation properties of SRC-2 on the human AR expression vector, in a Sertoli cell context (namely in the immortalized SC line, SMAT1, transfected with a hAR expression vector) and confirmed that SRC-2, characterized as an AR coactivator in murine testis (Gehin, Mark et al. 2002; Ye, Han et al. 2005) acts as a potent human AR coactivator.

1.2. Rationale for HBO1 selection

Histone acetyltransferase binding to origin recognition complex (HBO1), also named MYST2 or KAT7 in rodents and humans, is a putative histone acetyltransferase (HAT), which is unique among HAT enzymes in that it serves as a positive regulator of DNA replication (Iizuka, Takahashi et al. 2009; Avvakumov, Lalonde et al. 2012).

Indeed, HATs are enzymes that acetylate internal lysine residues of histone N-terminal domains, which is associated with transcriptional activation, DNA replication, DNA repair and exert their enzymatic actions not only on the transcription factors (Kuo and Allis 1998) (on their ligand-bound or non-ligand bound states) and their complexes, but also on nucleosomes or even on other histones .

HBO1 carries a functional histone acetyltransferase (HAT) domain and is a component of a large protein complex that contains histone acetyltransferase activity; a fraction of HBO1 protein associates with ORC1 in human cell extracts. The origin recognition complex (ORC) is an initiator protein for DNA replication, but also affects transcriptional silencing (Iizuka and Stillman 1999). Localization analysis on the human genome indicates that HBO1 complexes are enriched throughout the coding regions of genes, supporting a role in transcription elongation (Saksouk, Avvakumov et al. 2009).

Moreover, HBO1 acts as a modulator of transcriptional regulation of several nuclear receptors.

Previous reports showed that HBO1 acts as an AR corepressor (Sharma, Zarnegar et al. 2000). Indeed, HBO1 associates with the LBD of AR and inhibits transcriptional coactivation by the ARA70 cofactor in mammalian cell. Using a GAL4 DNA-binding domain assay,

Sharma et al (Sharma, Zarnegar et al. 2000), mapped a transcriptional repression domain within the N-terminal region of HBO1. Co-transfection experiments revealed that HBO1 specifically repressed AR-mediated transcription in both CV-1 (kidney monkey cell line) and PC-3 (human prostate cancer cell line) cells, testifying for the co-repressor effect.

HBO1 was also described as a coactivator of ER α , its expression being induced by oestradiol, via an ERK1/2 signaling pathway (Wang, Liu et al. 2010). HBO1 expression is induced by oestradiol in breast cancer cell lines, as well as in ER α positive human breast cancer tumours (Wang, Liu et al. 2010). Not only 17 β -estradiol (E2) upregulated HBO1 protein expression, but also ER α siRNA inhibited HBO1 protein expression in both T47D and MCF7 cell lines (Wang, Liu et al. 2010).

Hugues Loosfelt and his PhD student, Maria Georgiakaki, showed that HBO1 also acts as a coactivator of PRB by recruiting SRC-1, as well as a coactivator of GR and MR (Georgiakaki, Chabbert-Buffet et al. 2006).

However, HBO1 invalidation in animal models leads to embryonic lethality, with HBO1-deficient embryos being arrested at the 10-somite stage, due to blood vessels, mesenchyme and somite disorganization by extensive cell death and DNA fragmentation. However, in HBO1 mutant embryos neither cell proliferation, nor DNA replication are affected, suggesting that, HBO1 acts as a transcriptional activator for essential genes regulating embryonic development (Kueh, Dixon et al. 2011).

We were interested in HBO1's localization, expression pattern during ontogeny and mechanisms of regulation of male reproduction. Especially as previous experiments, presented by Sharma et al, identified by Northern blot analysis the abundance of HBO1 in human testis (Iizuka and Stillman 1999; Sharma, Zarnegar et al. 2000). Moreover, immunohistochemical detection of HBO1 in several primary human tumor categories revealed an important expression of HBO1 in testicular carcinomas (seminomas), with a less important expression in ovary, breast, stomach, bladder and liver (Iizuka, Takahashi et al. 2009).

As we and others have previously demonstrated that AR is differentially expressed in a cell-specific and developmental manner in human testis (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009), we questioned on the precise ontogeny and histological localization of HBO1 in both normal murine and human testis, as well as in human pathologies related to impaired androgen signalling and defective spermatogenesis.



Expression and characterization of androgen receptor coregulators, SRC-2 and HBO1, during human testis ontogenesis and in androgen signaling deficient patients



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ABSTRACT

Androgen receptor (AR) is essential for testicular physiology and spermatogenesis. SRC-2 and HBO1 are two AR coregulators yet their expression and roles in human testis are unknown. For the first time, we studied by immunohistochemistry and RT-PCR, the expression and distribution of these two coregulators during human testicular ontogenesis, in patients with altered AR signaling (Androgen insensitivity syndrome, AIS) and evaluated the functional impact of SRC-2 and HBO1 on AR signaling in a Sertoli cell context. SRC-2 was present in Sertoli cells at all developmental stages. HBO1 was barely or focally detected in the fetal testis yet its expression, in Sertoli and germ cells, drastically increased postnatally from early infancy to adulthood. In transient co-transfection studies we showed that SRC-2 induced, while HBO1 inhibited AR-mediated transactivation of reporter constructs in murine Sertoli SMAT1 cells. HBO1, but not SRC-2, expression was reduced in testes of patients with AIS compared to normal testes.

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1. Introduction

Androgen receptor (AR) functions as a transcription factor that regulates expression of genes involved in a large array of physiological processes, most notably male sexual differentiation and maturation, and onset and maintenance of spermatogenesis in adulthood (Rey et al., 2009; Brinkmann, 2011). In situations of severe testicular testosterone deficiency (Young, 2012) or in AR loss of function mutations, spermatogenesis is impaired (Hiort et al., 2000, 1996; Yong et al., 2003; Ferlin et al., 2006; Massin et al., 2012). The transcriptional activity of AR is regulated by several

molecular partners, such as coregulators that drastically modulate functional properties of AR, including ligand selectivity, DNA binding and recruitment of the basal transcriptional machinery (Brinkmann, 2011; Heinlein and Chang, 2002; Heemers and Tindall, 2007; Lonard and O'Malley, 2012; van de Wijngaert et al., 2012; Gottlieb et al., 2005).

In contrast to the well-established and unequivocal roles of AR in male reproductive physiology, there are limited data on the role of its coregulators in male reproductive events, notably almost no information in humans (Lan et al., 2008; van de Wijngaert et al., 2012; Tan et al., 2000; Sharma et al., 2000). Among AR coregulators, we focused our attention on two steroid receptor coregulators that could play a role in human testicular pathophysiology. Indeed, steroid receptor coactivator 2 (SRC-2) formerly named TIF2, is a class II coactivator that modifies chromatin structure through the histone acetyltransferase or ATP-dependent chromatin remodeling activity. It interacts with both N-terminal activation function

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(AF-1) and C-terminal region (AF-2) of AR (van de Wijngaert et al., 2012; Heinlein and Chang, 2002). In addition, SRC-2 is expressed in murine adult testes and its inactivation leads to a hypofertile phenotype with impaired spermatogenesis related to testicular alterations in cell adhesion between Sertoli cells and germ cells (Gehin et al., 2002; Ye et al., 2005). On the other hand, histone acetyltransferase binding to origin recognition complex (HBO1), also named MYST2 or KAT7 in rodents and humans respectively, is a putative histone acetyltransferase (HAT) essential for DNA replication, that has been shown to act as an AR corepressor (Sharma et al., 2000).

We and others have previously demonstrated that AR is differentially expressed in a cell-specific and developmental manner in human testis (Boukari et al., 2009; Chemes et al., 2008; Rey et al., 2009), questioning on the precise ontogeny and histological localization of these two AR partners in human testis.

The aim of our present study was to map the specific expression of human SRC-2 and HBO1 in the testis from fetal life to adulthood and in patients with androgen signaling defects (androgen insensitivity syndrome, AIS) in order to clarify their potential role in testicular pathophysiology.

2. Materials and methods

2.1. Tissue collections

All selected samples had a 46 XY karyotype and all subjects or their parents/tutors gave their informed written consent to allow the study of testicular samples. This study used licenced collections from Bicêtre, Pitié Salpêtrière and Necker Hospitals from Assistance Publique-Hôpitaux de Paris, Creteil Intercommunal Hospital, France and Pathology Institute of Bari University, Italy and the National Institute of Endocrinology, Bucharest, Romania. This study was approved by the corresponding local ethics committees and was in accordance with the French Bioethics law No. 2004-800.

2.1.1. Paraffin-embedded tissue samples

Archival paraffin-embedded human testicular samples were collected at different stages of development and at different ages from fetal life to adulthood. Tissue samples were fixed in 10% neutral-buffered formalin for less than 24 h. Fetal testis samples ($n = 13$) collected at 14–35 weeks of gestation were selected as reported (Boukari et al., 2009). Infant (age: 1 and 11 month old, $n = 2$), prepubertal (age: 2–7 yr old, $n = 5$) and pubertal (age: 12–14 yr old, $n = 3$) as well as adult (age: 19–59 yr old, $n = 28$) paraffin embedded human testis samples were also obtained. Newborn, infant and prepubertal testis samples were obtained from cases of sudden infant death or testicular biopsies for germ cell preservation before any gonadotoxic treatment. Testicular tissue samples used as controls originated from adults undergoing surgery for non-secreting benign tumours (such as teratomas, paratesticular epididymal cysts, adenomatoid tumours) (immunohistochemical studies were performed on the normal tissue adjacent to the tumour) and from young adults undergoing surgical investigation for obstructive azoospermia but with normal testicular histological structures and spermatogenesis on histological analysis.

In addition, human testicular samples of patients with androgen insensitivity syndrome (AIS) ($n = 12$, 7 patients with complete AIS (CAIS) and 5 patients with partial AIS (PAIS)) were obtained from the licenced collections as indicated above. All AIS patients were diagnosed on the basis of clinical, hormonal and genetic presentations (for adult CAIS, female phenotype contrasting with XY karyotype, high plasma testosterone levels and/or identified AR mutations; for PAIS undervirilized phenotype and gynecomastia

contrasting with high plasma testosterone levels and/or identified AR mutations) (Hiort et al., 1996; Massin et al., 2012).

2.1.2. Frozen-tissue samples

Snap-frozen testis specimens from human fetuses of 20 to 37 weeks ($n = 5$) were obtained from the fetopathology department of Robert Debré Hospital, Paris (Prof Anne-Lise Delezoide). These fetal samples were obtained from fetuses that had died in utero and had been rapidly delivered and autopsied within 6–36 h post-mortem. None of the pregnancy terminations were performed for fetal abnormalities.

Frozen testis samples from adult men with obstructive azoospermia, but with normal spermatogenesis on histological analysis ($n = 4$) were also obtained from the licensed tissue collection of the Urology Department from Bicêtre Hospital (French Bioethics law No. 2004-800).

2.2. Immunohistochemistry

Briefly, as reported (Boukari et al., 2007, 2009) 5 μm -thick tissue sections were deparaffinized and rehydrated in successive baths of Roti-histol (Carl Roth, Germany) and graded alcohol solutions. Slides were then subjected to microwave antigen retrieval for 15 min either in Tris-EDTA buffer pH 9 (for SRC-2, HBO1, PCNA) or in citrate buffer pH 6 (for AR, vimentin). Slides were incubated overnight at 4 °C with the following primary antibodies: anti-SRC-2 (Bethyl Laboratories Inc, Montgomery, USA), anti-HBO1 and anti-AR (sc-25379 and sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-vimentin (clone V9, DAKO Cytomation, France) anti-PCNA (clone PC10, DAKO Cytomation) at 1:200, 1:50, 1:50, 1:200 and 1:100 dilution, respectively. Bound Ig were revealed with peroxidase-aminoethylcarbazole kit (ImmPRESS reagent kit, VECTOR, CA and LSAB: Dako Cytomation), or for immunofluorescence, with fluorescent secondary antibodies (Cy3-donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, Suffolk, UK), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Eugene, Oregon, USA)). Negative controls were performed by substituting the primary antibodies with corresponding preimmune immunoglobulins from the same species. These sections were all immunonegative (Supplemental data, Fig. S4). In addition, positive SRC-2, HBO1 and Sertoli cell AR, adult testicular samples were always run in parallel for all immunohistochemical experiments in order to ease comparison of coregulator expression in different developmental stages.

2.2.1. Quantification of SRC-2 and HBO1

To quantify the immunohistochemical detection of SRC-2 and HBO1 in the human testis samples, immunopositive nuclei were counted on examination of series of ten consecutive seminiferous tubules sections of at least ten randomly selected microscopic fields, on $\times 20$ or $\times 40$ magnification (Nistal et al., 1982; Regadera, 1999). The total number of positive nuclei for SRC-2 or HBO1 was divided by the number of seminiferous tubules sections counted (see Fig. 2A, B and Supplemental data Fig. S2).

2.3. Real-time RT-PCR

SRC-2 and HBO1 gene expression was quantified by real-time RT-PCR (for primers, see Supplemental Table 1). Total RNA, extracted from frozen tissues using the mRNA-easy extraction kit (Qiagen) as recommended by the manufacturer, was reverse-transcribed and amplified on a Step One Plus real-time q PCR system (Applied Biosystems, Foster City, CA) as previously described (Boukari et al., 2007, 2009). Ribosomal 18S RNA was used as an internal control for data normalization. Results were expressed as means of at least two independent analyses of

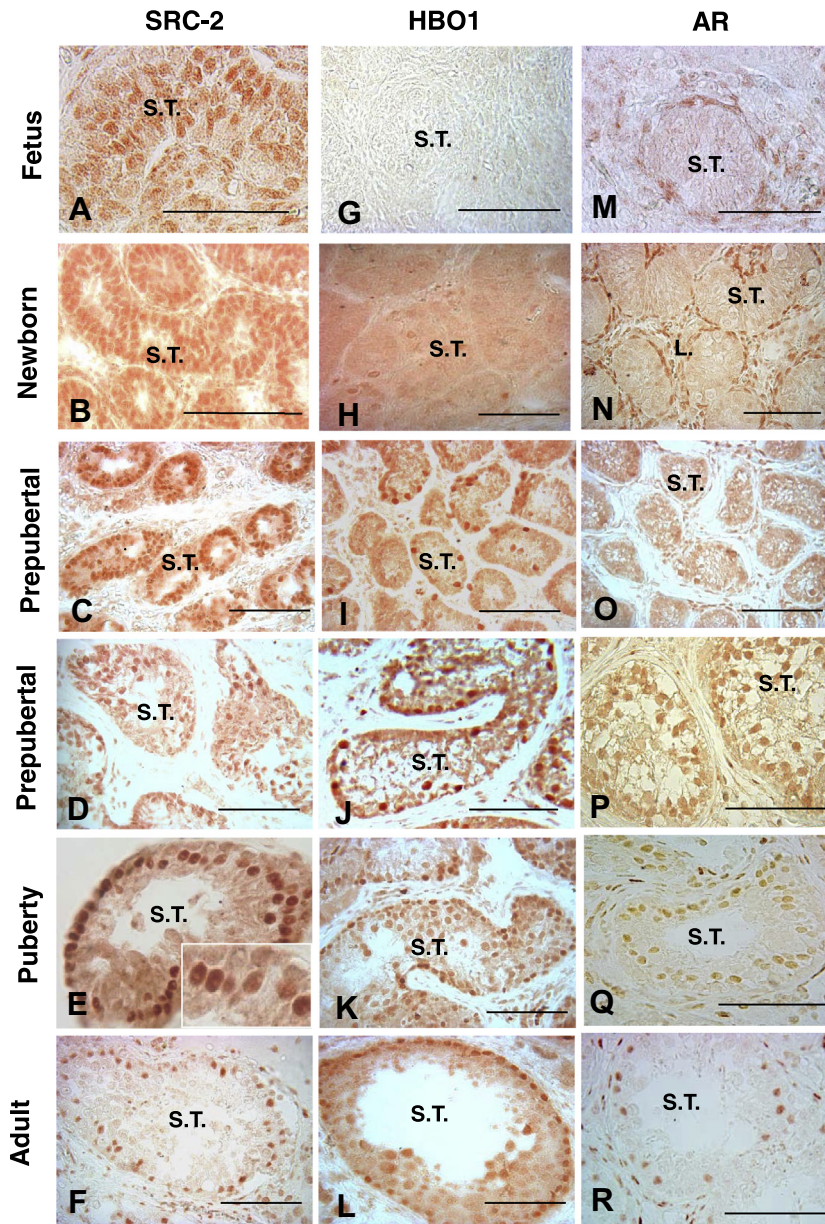


Fig. 1. Immunoreexpression of the coregulators SRC-2 and HBO1 comparatively to the androgen receptor (AR) in human testis: representative illustrations of immunoreactivity during development. Panels A, G, M – fetus (at 20 gestational weeks). Panels B, H, N – 1 month old newborn. Panels C, I, O – 2 year old boy. Panels D, J, P – 7 year old boy. Panels E, K, Q – pubertal boy (14 years old). Panels F, L, R – 29 year old man. SRC-2 is localized in the nuclei of seminiferous tubules from foetal life to adulthood (A–F). HBO1 expression is restricted to seminiferous tubules nuclei. HBO1 is not expressed in the fetal testis (Panel G); its expression increases in infant (Panels H, I) and prepubertal testis (J), reaching a maximum in postpubertal testis (K, L). Comparatively, gradual increase in AR expression in human testis during development, is shown in panels M–R. Scale Bars: 100 μ m. Magnification $\times 40$. S.T. seminiferous tubules; L. Leydig cells.

reverse-transcribed samples run in duplicate. The relative expression level of each gene was expressed relative to 18S rRNA (attomoles of specific gene per femtomoles of 18S).

2.4. Cell culture and transfection

SMAT1 cells, an immortalized murine immature Sertoli cell line, lacking AR (Dutertre et al., 1997), were cultivated in DMEM (Invitrogen, Life Technologies, Les Mureaux, France) supplemented with 10% fetal calf serum, 1x amino acid mix (Eurobio, Les Ulis, France), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Eurobio). Cells plated at 2×10^5 cells/well in six-well plates, while at 70% confluency, were transiently co-transfected as previously reported (Boukari et al., 2009), with human AR pcDNA3 expression vector (165 ng), human HBO1 pSG expression vector coding for the 611

amino acids isoform (165 ng), or SRC-2 pcDNA3 (165 ng) (or their corresponding empty vector plasmids-pcDNA3 or pSG), together with luciferase reporter plasmid (GRE2-luc) (330 ng) and β -galactosidase plasmid (80 ng) used as control for transfection efficiency. For evaluation of endogenous HBO1 transcripts regulation by hAR and DHT, cells plated at 2×10^6 cells/well in 60 mm Petri dishes, while at 70% confluency, were transiently transfected as previously reported (Boukari et al., 2009), with either pcDNA3 empty vector or human AR pcDNA3 expression vector.

Transfection was carried out in OPTIMEM culture medium (Invitrogen, Life Technologies) by using the FuGENE 6 Transfection Reagent kit (Roche, Promega Corporation, Madison, USA) according to the manufacturer's instructions. Transfected cells were incubated for 24 h with 10^{-7} M dihydrotestosterone or vehicle (ethanol) in DMEM supplemented with dextran-coated charcoal (DCC)

treated serum. Luciferase activity was determined by photometry using either a Victor luminometer (Perkin–Elmer Victor 3 1420 Multilabel Counter) or TriStar LB 941 (Berthold France SAS) and normalized for β -galactosidase activity estimated by spectrophotometry at 455 nm. Each experiment has been performed under the same experimental conditions for at least six times.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad PRISM version 4 software (GraphPad Software, Inc., La Jolla, USA). Results are expressed as means \pm SEM. Nonparametric Mann–Whitney test has been used for analysis between two groups. Non-parametric Kruskal–Wallis one way-ANOVA test followed by Dunn post-test for multiple comparisons between groups was used for transcriptional regulation of SRC-2 and HBO1 and coregulators immunostaining quantification.

3. Results

3.1. SRC-2 and HBO1 protein expression during human testicular development

We first examined the ontogenesis of the two coregulators (SRC-2 and HBO1) expression during human testicular

development in comparison with that of AR by immunohistochemistry.

In fetal, newborn, prepubertal as well as postpubertal human testis, the SRC-2 protein was strongly expressed as revealed by a positive nuclear staining and was almost exclusively detected within seminiferous tubules, mainly in Sertoli cells (Fig. 1, panels A–F and see below). In contrast, HBO1 was barely detected in fetal testis (Fig. 1, panel G), but a positive nuclear staining became readily detectable in newborn seminiferous tubules while its expression gradually increased with age, reaching a maximum of expression level in adult testis (Fig. 1, panels H–L). We also examined whether the evolution profile of these coregulators followed that of AR in the same samples. As previously reported (Boukari et al., 2009; Chemes et al., 2008), we found that AR was expressed in human seminiferous tubules with an increased expression in Sertoli cells with age (Fig. 1, panels M–R). This contrasts with AR expression in the peritubular myoid cells and Leydig cells which is detected irrespective to the developmental stage. Thus, HBO1 expression in the seminiferous tubules seems to be expressed in parallel to that of AR.

In order to get a more precise evaluation of the evolution of SRC-2 and HBO1 immunoexpression during testicular development, we quantified the number of immunopositive nuclei per seminiferous tubule section as detailed in Section 2. As presented in Fig. 2, we confirmed that SRC-2 was constantly expressed at

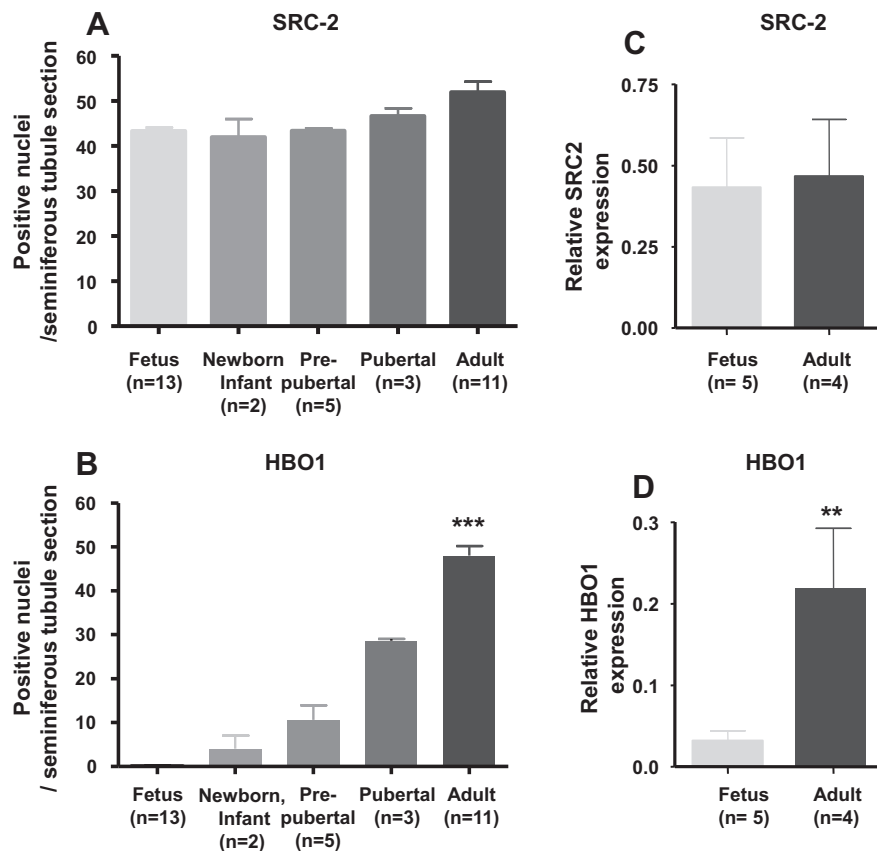


Fig. 2. Relative quantification of SRC-2 and HBO1 protein immunoexpression and mRNA levels in fetal and adult human testis. SRC-2 (A) and HBO1 (B) immunoexpression in human testis were quantified on immunostained paraffin-embedded slides of normal testicular biopsies obtained during human ontogenesis from 46 XY fetuses ($n = 13$), newborn and infant (<1 year) ($n = 2$), prepubertal boys (2–7 yrs-old) ($n = 5$), pubertal boys ($n = 3$) and adults with preserved complete spermatogenesis ($n = 11$). Scoring was performed by counting all positive nuclei found in at least three blindly selected microscopic fields, on $\times 20$ or $\times 40$ magnification (Section 2). Results, expressed as means of ratios between number of positively immunostained nuclei per seminiferous tubules section, were compared to the mean value of the fetal testes. (***) $p < 0.001$; non-parametric Kruskal–Wallis one way-ANOVA test with Tukey post hoc test for comparison between groups; GraphPad PRISM4). Relative expression of SRC-2 (C) and HBO1 (D) mRNA levels were evaluated by RT-PCR in 5 fetal human testes obtained at 21 ($n = 1$), 25 ($n = 1$), 34 ($n = 1$), 35 ($n = 1$), 37 ($n = 1$) gestational weeks, in comparison with 4 adults (aged between 30 and 50 years). (See Supplemental Table 1 for primers.). Results were normalized to human 18S ribosomal RNA. Relative expression in a given sample was calculated as attomoles per femtomole of 18S RNA. Each experiment (run in duplicate) was performed twice. Results are expressed as means \pm SEM of reverse-transcribed samples run in duplicate. (** $p < 0.01$; non-parametric Mann–Whitney test; Graph Pad PRISM 4).

all stages of testicular development (Fig. 2A) while HBO1 displayed an ongoing increasing expression, reaching a maximum in adult human testis (Fig. 2B).

3.2. SRC-2 and HBO1 mRNA analysis in fetus versus adult human testis

To further validate quantitative changes in SRC-2 and HBO1 gene expression during testicular ontogenesis, mRNA levels of these 2 genes were determined by real-time quantitative PCR in 5 fetal testes obtained respectively at 21, 25, 34, 35, 37 gestational weeks, in comparison with 4 adults (aged from 30 to 50 years). As anticipated by the stable SRC-2 expression levels detected by immunohistochemical studies (Fig. 1) and quantification (Fig. 2A), there was no significant difference between SRC-2 mRNA expression in fetal versus adult testicular tissues ($p = 0.73$) (Fig. 2C). On the contrary, the relative expression level of HBO1 mRNA in fetal testes was <15% of that measured in adult testes ($p < 0.01$) (Fig. 2D), confirming that HBO1 expression significantly

increases during human testis development thus following the evolution profile of the protein immunodetection (Fig. 2B).

3.3. Cellular localization for SRC-2 and HBO1 in human adult seminiferous tubules

To specify the cellular localization of the two coregulators, SRC-2 and HBO1 within the human testis, double immunolabeling experiments with either vimentin (chosen as marker of Sertoli cell (Rogatsch et al., 1996)) or PCNA (proliferating cell nuclear antigen) (chosen as a marker of proliferating germ cells) (Bar-Shira Maymon et al., 2003; Kelnar et al., 2002; Steger et al., 1998; Takagi et al., 2001) were performed on sections of adult normal human testis.

All SRC-2 positive nuclei were delineated by vimentin positive cytoplasm, suggesting that SRC-2 is expressed almost exclusively in Sertoli cells (Fig. 3, line 1–thin arrow). This Sertoli-specific cellular localization was further confirmed by the fact that PCNA positively immunolabeled nuclei were negative for SRC-2

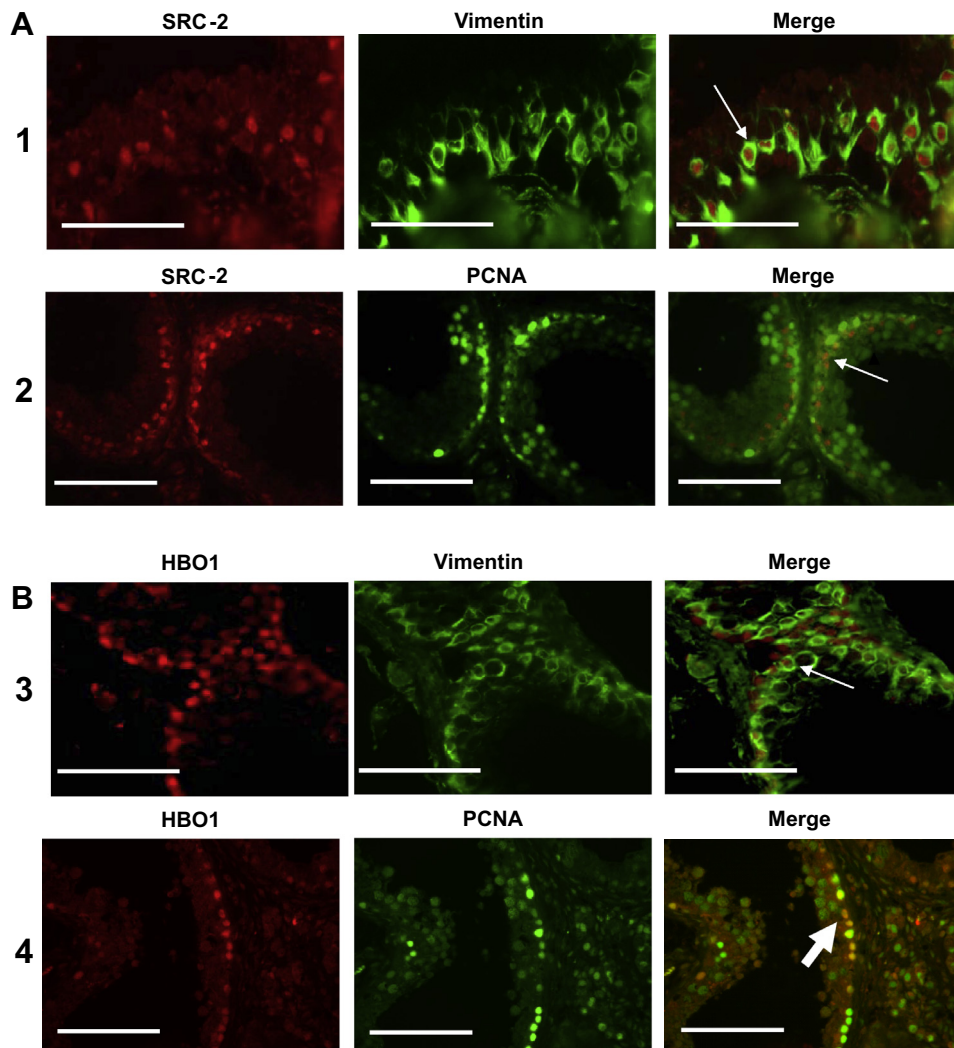


Fig. 3. Cellular localization of SRC-2 and HBO1 in human adult testis by double immunostaining (A). Line 1: left panel shows nuclear SRC-2 immunostaining; middle panel shows cytoplasmic vimentin immunostaining of Sertoli cells and right panel, shows the merge of vimentin and SRC-2, indicating that SRC-2 is expressed in Sertoli cells (adapted from magnification $\times 40$). Line 2: left panel shows nuclear SRC-2 immunostaining; middle panel shows nuclear PCNA immunostaining of testicular proliferating cells and right panel, shows the merge of the two previous panels, indicating that SRC-2 is not expressed in proliferating (germ) cells (magnification $\times 20$). (B) Line 3: left panel shows nuclear HBO1 immunostaining; middle panel shows cytoplasmic vimentin immunostaining of Sertoli cells and right panel, shows the merge of the two previous panels, indicating that HBO1 is expressed in some but not all Sertoli cells (arrow), (adapted from magnification $\times 40$) Line 4: left panel shows nuclear HBO1 immunostaining; middle panel shows nuclear PCNA immunostaining of testicular proliferating cells and right panel, shows the merge of the two previous panels, indicating that HBO1 is also expressed in part in proliferating (germ) cells (full arrows). These observations -Lines 3 and 4-indicate that HBO1 is expressed in both Sertoli and germ cells (magnification $\times 20$). Scale Bars: 100 μm .

immunostaining (Fig. 3, line 2–thin arrow), indicating that SRC-2 was not expressed in proliferating cells, presumably germ cells, within the adult human seminiferous tubules.

Similarly, the colocalization of HBO1 and vimentin readily suggests that HBO1 is also expressed in Sertoli cell nuclei (Fig. 3, line 3–thin arrow). However, some nuclei within the seminiferous tubules were positively stained for both HBO1 and PCNA, consistent with the expression of HBO1 in cells, other than Sertoli cells, most likely in proliferating germ cells in human adult testis (Fig. 3, line 4–thick arrow). This hypothesis was reinforced by the fact that some nuclei within the seminiferous tubules were positively stained for HBO1 but not for vimentin (Fig. 3, line 3).

3.4. SRC-2 acts as an AR coactivator, while HBO1 acts as an AR corepressor in Sertoli cells

To examine the possible functional consequences of SRC-2 and HBO1 coexpression with AR in human Sertoli cells, we studied their regulatory impact on AR transcriptional activity in a Sertoli cell context. In absence of available human Sertoli cell line, transient co-transfection assays using expression plasmids of human AR, SRC-2 or HBO1 together with reporter GRE2-luciferase plasmid (Boukari et al., 2009; Georgiakaki et al., 2006) and with a β -galactosidase plasmid were performed in the murine Sertoli cell line, SMAT1 (Dutertre et al., 1997) and dihydrotestosterone (DHT)-stimulated transactivations were examined (Fig. 4).

As expected, a strong induction of AR-mediated transcriptional activity was observed after 100 nM DHT exposure in the absence of transfected SRC-2. However, ligand-dependent AR activation was further stimulated by a 3-fold factor after a 1:2 AR/SRC-2 cotransfection ratio, consistent with the coactivating property of SRC-2 (Fig. 4A). In contrast, under similar experimental conditions with a 1:2 AR/HBO1 cotransfection ratio, a 60% reduction in DHT-stimulated AR transactivation was observed when HBO1 was coexpressed, indicating that it behaves as a potent AR co-repressor in a Sertoli cell context (Fig. 4B). Finally, to assess the HBO1 effects on SRC-2 stimulation of DHT-induced AR transactivation we similarly measured ligand-dependent AR activation in SRC-2 and HBO1 co-transfected SMAT1 cells. As shown in Fig. 4C a significant decrease in luciferase activity induced by SRC-2 was observed in presence of HBO1 suggesting a repression of the SRC-2 coactivating activity by HBO1.

To confirm that SRC-2 and HBO1 exert direct effects on AR transactivation of their target promoters, we showed by western blot that AR was expressed at equivalent levels in all experimental conditions tested, excluding potential posttranslational regulation of AR expression by its molecular partners (see Supplemental data, Fig. S1).

3.5. SRC-2 and HBO1 expression in human testicular pathologies with impaired androgen signaling

Given that SRC-2 and HBO1 are physiologically expressed in human testis following differential evolution profile during ontogenesis, we next evaluated the expression of these two coregulators in testicular samples of patients presenting with impaired testicular androgen signaling by immunohistochemistry and quantification (as specified in Section 2).

First, in subjects with androgen insensitivity syndromes (AIS) ($n = 12$), SRC-2 was expressed in seminiferous tubules, similarly to the normal testes (Fig. 5, panels A–C and Supplemental data Fig. S2, panel A). On the contrary, HBO1 was not expressed in seminiferous tubules of patients with complete AIS (CAIS) ($n = 7$) (Fig. 5, panel E and Supplemental Figs. S2 and S3, panel B), while the immunostaining of HBO1 in partial AIS (PAIS) ($n = 5$) was barely detected (Fig. 5, panel F and Supplemental data Fig. S2,

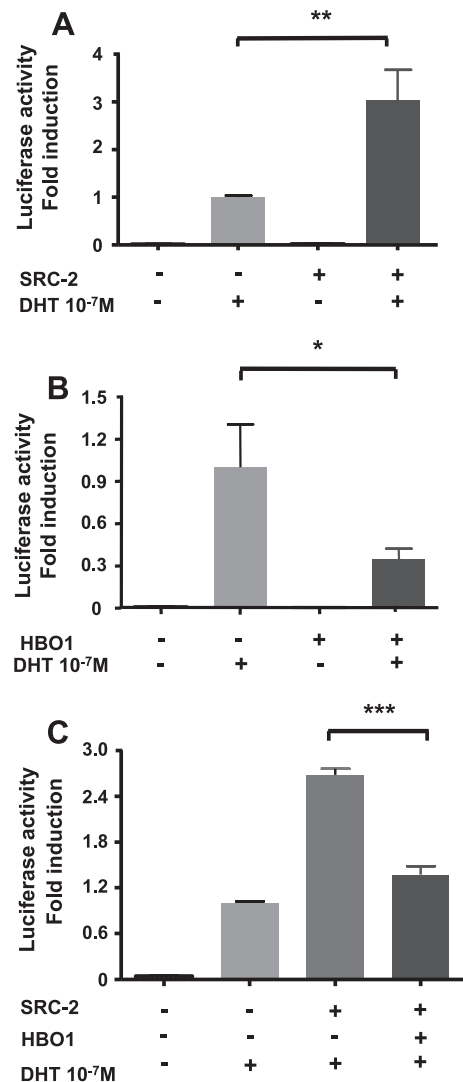


Fig. 4. Coregulatory function of SRC-2 and HBO1 on AR transcriptional activity in SMAT1 Sertoli cells. SMAT1 cells transiently transfected with pcDNA3-hAR plasmid, GRE2luc reporter vector and β -galactosidase plasmid, have been co-transfected with either SRC-2 expression vector (A), HBO1 expression vector (B), SRC-2 and HBO1 (C) or their corresponding empty plasmids. Cells were treated with 10⁻⁷ M dihydrotestosterone (DHT) or with vehicle (ethanol) for 24 h. Luciferase activities normalized by β -galactosidase activities were measured. Results are means \pm SEM of at least six independent determinations and represent the relative fold induction compared with basal levels in ligand stimulated cells in the absence of cotransfected coregulators (arbitrarily set at 1). (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$ nonparametric Kruskal Wallis one way-ANOVA, GraphPad PRISM4 followed by Dunn post-test for multiple comparison between groups).

panel B). Given that HBO1 was never detected in any of the 7 CAIS testicular samples analyzed, we wondered whether the lack of HBO1 expression was correlated with the presence of AR protein. As shown in Supplemental Fig. S3, we found that the absence of HBO1 immunodetection was independent of the AR protein status.

3.6. Androgens induce HBO1 gene expression in SMAT1 SC in the presence of AR

In order to evaluate the possibility that HBO1 could be an AR target gene we assessed the HBO1 expression by qPCR in human AR transfected SMAT1 cells. As shown in Fig. 6, we show that DHT significantly induces HBO1 expression.

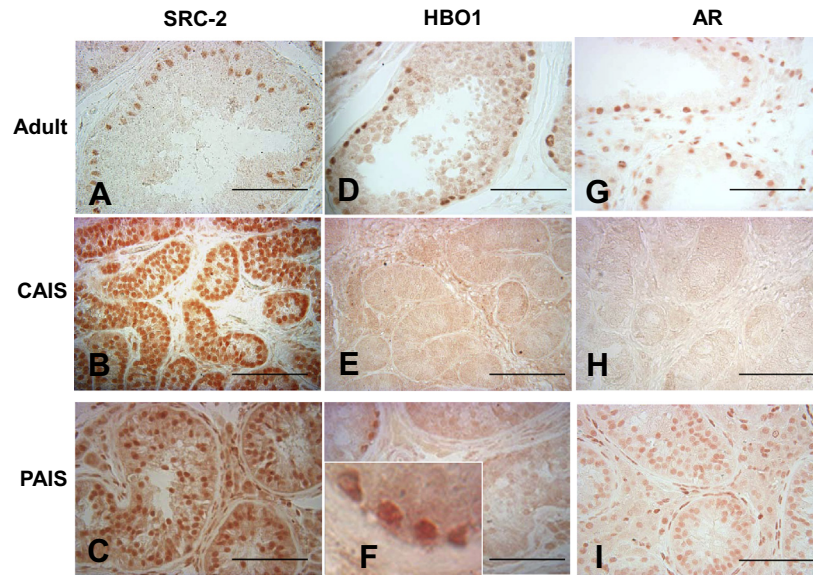


Fig. 5. SRC-2 and HBO1 immunostaining comparatively to AR in normal and pathological adult human testis. Representative illustrations of immunoreactivity in normal young adult testicular biopsy and in pathologies related to dysregulation in androgen signaling such as: complete androgen insensitivity syndrome (CAIS) and partial androgen insensitivity syndrome (PAIS). Panels A, D, G – 19 year young adult. Panels B, E, H – 18 year old CAIS teenager. Panels C, F, I – 15 year old PAIS boy. SRC-2 is constantly localized in Sertoli cell nuclei in all testes analyzed. HBO1 expression is restricted to seminiferous tubules nuclei. It is absent in CAIS testes; it is immunodetected in some nuclei within seminiferous tubules from a PAIS 46 XY male testis (Fig. 5F, magnified insert); HBO1 is expressed in seminiferous tubules nuclei in normal adult human testis. Magnification $\times 40$. S.T. seminiferous tubules. Scale Bars: 100 μm .

4. Discussion

The aim of the present paper was to evaluate the expression of two AR molecular partners, SRC-2 and HBO1, in human testis during development and in diseases associated with AR signaling alteration leading to infertility caused by reduced or absence of spermatogenesis.

To the best of our knowledge, we show for the first time the expression profiling of two AR coregulators SRC-2 and HBO1 in human testis. We have demonstrated their precise testicular localization and provided evidence that both SRC-2 and HBO1 are exclusively expressed in the seminiferous tubules yet absent in the interstitium and in Leydig cells. However, the expression of these two AR coregulators is not identical in the seminiferous tubules. Indeed, coimmunolocalization studies indicate that SRC-2

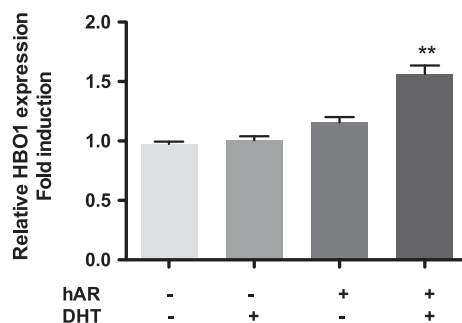


Fig. 6. Quantification of endogenous HBO1 mRNA levels in SMAT1 Sertoli cell line. SMAT1 cells were transiently transfected with pcDNA3 empty vector or human pcDNA3-hAR plasmid. Empty vector or human pcDNA3-hAR transfected cells were treated with vehicle (ethanol) or with 10^{-7} M dihydrotestosterone (DHT) for 24 h. Results were normalized to murine 18S ribosomal RNA. Relative expression in a given sample was calculated as attomoles per femtomole of 18S RNA. Each condition was performed in quadruplicate. Each sample was reverse-transcribed and then run in duplicate for qPCR ($n = 8$). (** $p < 0.01$; non-parametric Mann-Whitney test; Graph Pad PRISM 4). Results are means \pm SEM and represent the relative fold induction compared with basal levels (arbitrarily set as 1) in cells in the absence of hAR and DHT.

is exclusively expressed in Sertoli cells while HBO1 is localized in both Sertoli and germ cells most notably those adjacent to the basal membrane.

Of particular interest was the testicular ontogenesis of the two AR partners during development keeping in mind the differential AR expression profiling already reported along human testis maturation (Boukari et al., 2009; Chemes et al., 2008). Herein, we show that SRC-2 expression was first immuno-detected in Sertoli cells during the early phases of testis development as early as 14 gestational weeks even though AR was not expressed at this developmental stage. The expression of this coregulator remains stable post-natally and throughout adulthood. This stable SRC-2 expression contrasts with the progressive evolution profile of the AR signaling and suggests that Sertoli SRC-2 expression was independent of the androgen signaling. The lack of coexpression of AR and its molecular partner at this fetal/neonatal period of development obviously questions on the exact role of SRC-2 in the testicular physiology during embryonic life. It is very likely that SRC-2 interacts with several other nuclear receptors during fetal testis development. Indeed, it is well established that SRC-2 is not only an AR coactivator, but also a coactivator of glucocorticoid receptor (Awasthi and Simons, 2012), Progesterone receptor (Hofman et al., 2002) and estrogen receptor (Warnmark et al., 2002), all these steroid receptors being also expressed in human testis during ontogenesis (Cavaco et al., 2009; Shah et al., 2005). Along this line, as opposed to AR, we have previously demonstrated that estrogen receptor β (ER β) but not ER α was expressed in the Sertoli cells of human testis between 14 and 22 weeks of foetal life (Boukari et al., 2007). As a result, SRC-2 might exert important coactivating control of ER β transcriptional activities given the physical interaction, already reported, between ER β and SRC-2 (Warnmark et al., 2001).

On the other hand, we demonstrate in the present paper a strict parallel maturation profile for HBO1 and AR expression during human testis development. This temporal and positively correlated expression pattern of both AR and HBO1 suggests that HBO1 expression is linked to or required a functional AR signaling in

the Sertoli cell. This hypothesis is further validated by the lack or drastic reduction of testis HBO1 expression in pathological conditions in which androgenic signaling was impaired such as in AIS patients. As previously mentioned, HBO1 is a nuclear protein not only expressed in Sertoli cells, but also in spermatogonia. Thus the physiological role of HBO1 in germ cells is very likely distinct from that exerted in Sertoli cells given that it is well established that AR is not expressed in spermatogonia in humans (Boukari et al., 2009; Chemes et al., 2008; Suarez-Quian et al., 1999). We propose that HBO1 might affect other steroid receptor signaling such as PR or ER although their expression in human germ cells remains still controversial (Luetjens et al., 2006; Han et al., 2009; Pais et al., 2003).

Alternatively, given that HBO1 plays an important role on DNA replication (Han et al., 2009; Iizuka et al., 2006; Luetjens et al., 2006; Pais et al., 2003) and is potentially involved in the control of cellular proliferation (Avvakumov et al., 2012) it could thereby be involved in the initiation and maintenance of spermatogenesis. HBO1 expression could also serve as a suitable index of germ cell proliferation.

In order to clarify the potential impact of SRC-2 and HBO1 on testicular function and AR signaling pathway, notably within the Sertoli cell where all molecular partners are expressed in adult testis, their functional properties were studied by cotransfection assays in murine Sertoli SMAT1 cells. Transactivation experiments showed that SRC-2 behaves as a potent AR coactivator in a Sertoli cell context while cotransfection of HBO1 with AR markedly reduced DHT-stimulated AR transactivation, consistent with its corepressor property. These results are reminiscent of previous work that reported AR mutations responsible for impaired SRC-2 interaction, associated with an altered spermatogenesis (Ghadessy et al., 1999; Lim et al., 2000; Quigley et al., 2004). Collectively, these findings suggest that the coactivating activity of SRC-2 on AR function in the Sertoli cell could play a pivotal role for the sustained production of spermatozooids in human adults. This is also true in other species given the hypofertility of SRC-2 knock-out male mice (Gehin et al., 2002; Ye et al., 2005). On the other hand, the exact role of the corepressor HBO1 in Sertoli cells remains to be established. A possibility would be that HBO1 as a AR corepressor could attenuate a maximal AR activation in Sertoli cells induced by very high intra-testicular testosterone concentrations (Coviello et al., 2005). Alternately, HBO1 expression in adult Sertoli cells could induce a partial testicular resistance to testosterone and thus explain the necessity of very high intratesticular testosterone levels needed to induce spermatogenesis in men (Coviello et al., 2005; Jarow and Zirkin, 2005; Schaison et al., 1993).

The drastic alteration of HBO1 expression in severe/complete androgen insensitivity syndrome, irrespective of deleterious natures of AR mutations i.e. the presence or absence of detectable AR protein, suggest that the integrity of AR signaling within the Sertoli cell is germane for HBO1 expression in both Sertoli and germ cells. This hypothesis was further supported by the increase of Hbo1/Myst2 transcripts levels in AR transfected SMAT1 cells treated by the nonaromatizable androgen DHT.

If and how HBO1 impacts androgenic regulation of spermatogenesis remains to be further explored.

In conclusion, even if other AR coregulators may be expressed and play a role in human testis physiology, we provide first evidence that the expression of SRC-2 and HBO1, two important AR coregulators, follows distinct evolution profile during human testis development. Our results also suggest that modulation of SRC-2 and HBO1 abundance within the Sertoli cells might constitute molecular markers for altered spermatogenesis. Given the opposite coregulatory properties of SRC-2 and HBO1 on AR signaling, it would be of interest to examine whether infertile patients might

harbor either loss of function or gain of function deleterious mutations of these two coregulators.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mce.2013.05.004>.

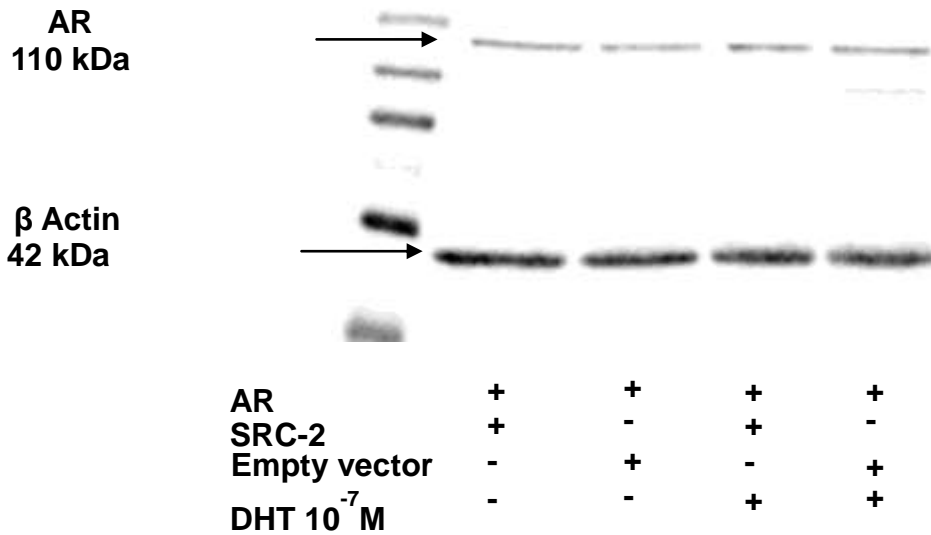
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Fig. S2

A



B

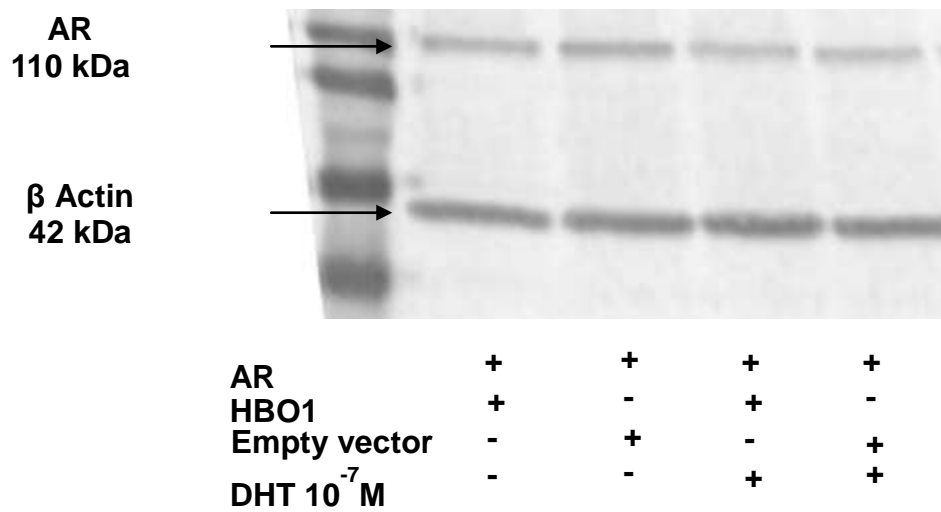


Fig. S2. Expression of transfected AR in the presence of SRC-2 (A), HBO1 (B) or their empty corresponding plasmids. SMAT1 cells have been transfected as mentioned in the Materials and Methods section. Western blot was performed on equal amounts of proteins using the anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β actin was used as an internal control. Note that AR expression was comparable in all experimental conditions.

Fig. S3

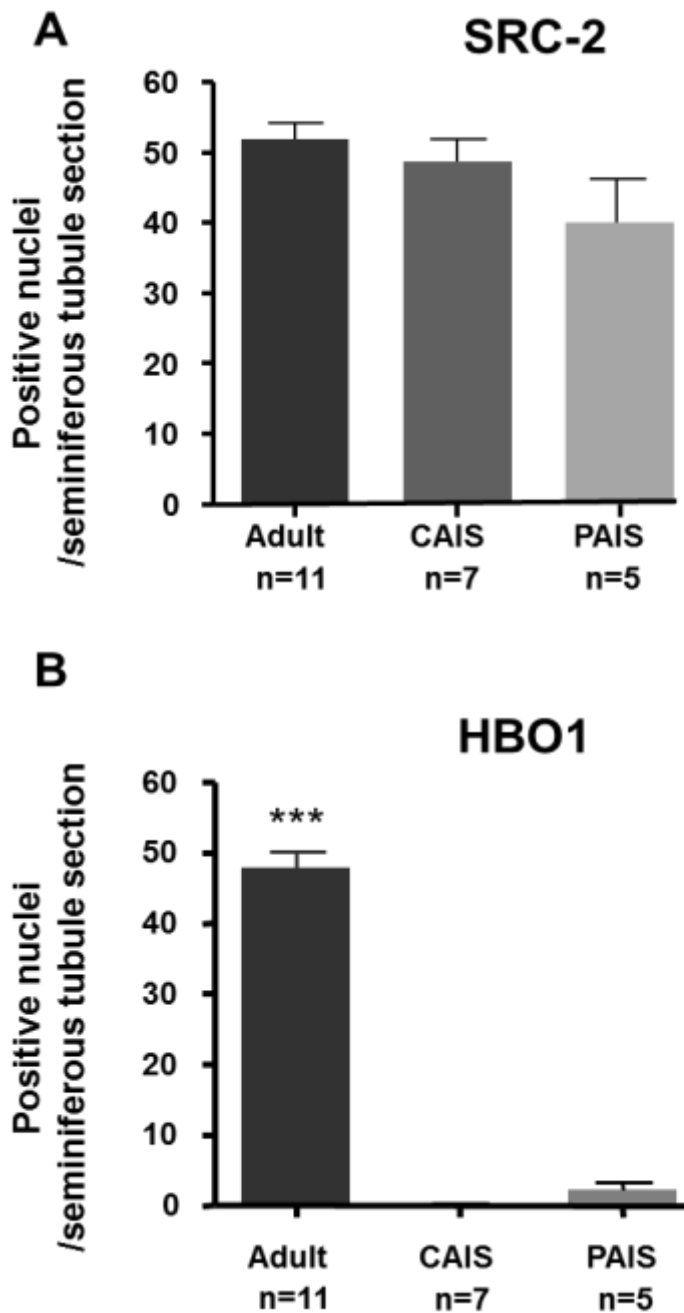


Fig. S2. SRC2 and HBO1 nuclear immunostaining quantification in testicular samples obtained from normal adults and patients with androgen insensitivity syndromes.

*Results are presented as means \pm SEM of ratio between number of positively immunostained nuclei per seminiferous tubule sections. For details, see Materials and Methods section (***, $P = 0,0002$). CAIS: complete androgen insensitivity syndrome; PAIS: partial androgen insensitivity syndrome; n: number of patients.*

2. Murine and human testicular samples and patient profiles

2.1. Murine testicular samples

During the second year of the PhD project, I obtained a testicular collection from wild type Balb/C57 male mice aged between day 1 and 4 months. Tissue samples were either cryopreserved or fixed in 10% neutral-buffered formalin for less than 24h. Paired testis samples were obtained from newborn mice (n=6), prepubertal mice, with ages ranging from day 1 (n=6), day 7 (n=6), day 14 (n=10), day 21 (n=10), and more than 8-week-old adults (n=10); for each animal one testis was cryopreserved for mRNA extraction, while the other one was formalin-fixed.

2.2. Human testicular samples

Due to an established multicentric national and international collaborations with Pathology Departments from Bicêtre, Pitié Salpêtrière and Necker Hospitals from Assistance Publique-Hôpitaux de Paris, Creteil Intercommunal Hospital, France, as well as Pathology Institute of Bari University, Italy, the National Institute of Endocrinology, and the “Th Burghele” Hospital from Bucharest, Romania. During the first trimester of my thesis, we gathered during the first 18 months of the project, an exhaustive collection of testicular paraffin-embedded or frozen human samples. All selected samples had a 46 XY karyotype and all subjects or their parents/tutors gave their informed written consent to allow the study of testicular samples.

Paraffin-embedded human testicular samples were collected at different stages of development and at different ages from fetal life to adulthood. Tissue samples were fixed in 10% neutral-buffered formalin for less than 24h. Fetal testis samples (n=13) collected at 14 to 35 weeks of gestation were selected as reported (Boukari et al., 2009). Infant (age: 1 and 11 month old, n=2), prepubertal (age: 2 to 7 yr old, n=5) and pubertal (age: 12 to 14 yr old, n=3) as well as adult (age: 19 to 59 yr old, n=28) paraffin embedded human testis samples were also obtained. Newborn, infant and prepubertal testis samples were obtained from cases of sudden infant death or testicular biopsies for germ cell preservation before any gonadotoxic treatment. Testicular tissue samples used as controls originated from adults undergoing surgery for non-secreting benign tumours (such as teratomas, paratesticular epididymal cysts, adenomatoid tumours)(immunohistochemical studies were performed on the normal tissue adjacent to the tumour) and from young adults undergoing surgical investigation for

obstructive azoospermia but with normal testicular histological structures and spermatogenesis on histological analysis.

In addition, human testicular samples of patients with androgen insensitivity syndrome (AIS) (n=12, 7 patients with complete AIS (CAIS) and 5 patients with partial AIS (PAIS)) were obtained from the licensed collections as indicated above. All AIS patients were diagnosed on the basis of clinical, hormonal and genetic presentations (female phenotype contrasting with XY karyotype, high plasma testosterone levels and/or identified AR mutations were diagnostic features of CAIS; undervirilized phenotype and gynecomastia contrasting with high plasma testosterone levels and/or identified AR mutations were diagnostic features for PAIS(Hiort and Holterhus 2003; Massin, Bry et al. 2012). Moreover, in order to study the particularities of SRC-2 and HBO1 immunoexpression in more medical conditions associated or not with androgenic impairment I also performed immunohistochemical studies on paraffin-embedded testicular samples obtained from an adult patient with congenital hypogonadotropic hypogonadism and a postpubertal patient with 5- α reductase type 2 deficiency. Details related to their clinical and genetic characteristics are presented in Tables 7 and 8.

Moreover, in order to verify whether the changes in SRC-2 and HBO1 expression in pathologies related to androgen impairment and spermatogenesis alterations are related to disturbed androgen signalling and not to other factors, we also compared the immunohistochemical expression of SRC-2 and HBO1 in AIS patients to testicular samples obtained from patients with idiopathic azoospermia (n=5), without any other mutations or alterations in androgen signaling.

For details related to the RT-PCR and immunohistochemistry protocols, as well as further details related to the molecular biology techniques, please see the published manuscript (Vija, Meduri et al. 2013).

2.3. Data related to the genetic profile of the studied patients

Concerning the seven patients with CAIS, we also documented the presence and the severity of the AR dysfunctions. Of these, two siblings (two sisters) (CAIS patients P3 and P4) had the nonsense deleterious mutation c.1659C>G (formerly c.1656C<G) (Y552X), located in the exon 2, which deleted both the DNA binding and hormone binding domains.

However, this mutation has not yet been registered in the AR mutation database (www.androgendb.mcgill.ca).

Another CAIS patient (P6), had the frameshift deleterious mutation c.310-311insA (p.Pro104Hisfs*9) in exon 1, mutation responsible for a deletion of part of the amino-terminal domain, and for the complete deletion of both DNA and ligand binding domains.

Two additional siblings (P1 and P5) carried the AR missense c.2086G>A (D695N) mutation (which nucleotide sequence was formerly called c.2083G>A). This mutation was functionally evaluated by Ris et al. (Ris-Stalpers, Trifiro et al. 1991). These authors demonstrated that the deleterious effect of this missense mutation which had only 28% of the wild type transcriptional activity.

The P2 CAIS patient had the recurrent mutation (already reported in CAIS patients), c.2197G>A in exon 5 of the LBD, with the protein nomenclature, p.Asp733Asn (previously known as D732N). This mutation induced a functionally less active protein with only 5% of the wild-type transcriptional activity when treatment was performed with 1mM mibolerone (Hannema, Scott et al. 2004). Moreover, this mutation seems to be deleterious for the interactions with HBO1/MYST2, severely altering the interaction AR-MYST2/HBO1 (AlaMut; PolyPhen).

The P7 presented a synonymus substitution due to a transition of C in T in exon 9 (AlaMut); the cDNA nomenclature of this new mutation is c.2697C>T, whereas the protein nomenclature is p.Ile899Ile. However, it seems that this mutation would not explain the phenotype of CAIS.

An adult azoospermic patient with a phenotype of MAIS, and a substitution in the exon 5 of the LBD, F755S (formerly called F754S), who reached double paternity by in vitro assisted reproduction techniques, shall be presented in a new section of the results (Massin, Bry et al., 2012).

The adult presenting with congenital hypogonadotropic hypogonadism and infertility had a mutation in the TACR3 gene, c.738-1G>A, leading to codon stop and a truncated protein (Young, Bouligand et al. 2010).

The 17 year old patient with 5- α reductase type 2 deficiency presented with primary amenorrhea, failure of breast development, virilisation, clitoromegaly and bilateral cryptorchidism (with testes located in the inguinal canals), related to a homozygous missense deleterious mutation in the exon 2 of the *SRD5A2* gene (c.344G>A; Gly115Asp) (Sarfati,

Trabado et al. 2011). Data related to some clinical features and genetical profiles of the patients whose testicular samples were studied are briefly presented in Tables 7 and 8.

Patient Number	Initials	Age at gonadectomy (years)	Sex	Pathology	Cryptorchidism Yes/No	Testicular Volume (ml)
1	SE	19	F	CAIS	Yes	16
2	MA	18	F	CAIS	NA	15
3	JA	16	F	CAIS	Yes	24
4	JL	14	F	CAIS	NA	NA
5	GL	17	F	CAIS	Yes	28
6	GE	16	F	CAIS	Yes	26
7	KA	21	F	CAIS	Yes	16
8	DW	15	M	PAIS	Yes	14
9	SA	39	M	PAIS	NA	12
10	DC	25	F	PAIS	NA	14
11	HM	17	F	PAIS	NA	NA
12	GMA	25	F	PAIS	NA	9
13	H	29	M	MAIS	No	NA
14	MM	35	M	CHH	No	NA
15	HN	17	F	5ARD	Yes	11

Table 7. Main clinical features of patients included in the study. CAIS, complete androgen insensitivity syndrome; PAIS, partial androgen insensitivity syndrome; MAIS, minimal androgen insensitivity syndrome; CHH, congenital hypogonadotropic hypogonadism; 5ARD, 5- α reductase type 2 deficiency. Testicular volumes were calculated using the equation of ellipsoid [$V = (4/3) \pi ab^2$], based on the measures for testicular length (2a) and width (2b).

Pt No	Initials	Age (years)	Sex	Phenotype	AR mutation Nucleotidic/Protidic nomenclature	Exon	Other mutation	Funct. Mut. Charact.
1	SE	19	F	CAIS	c.2194G>A D732N	5	-	1
2	MA	18	F	CAIS		1	-	
3	JA	16	F	CAIS	c.2083G>A	4	-	1,2
4	JL	14	F	CAIS	c.2083G>A D695N	4	-	1,2
5	GL	17	F	CAIS	c.1696C<G Y552X	2	-	NA
6	GE	16	F	CAIS	c.1696C<G Y552X	2	-	NA
7	KA	21	F	CAIS			-	
8	DW	15	M	PAIS	-		-	
9	SA	39	M	PAIS	A765S	5	-	NA
10	DC	25	F	PAIS	-		-	
11	HM	17	F	PAIS	Q60X	1	-	NA
12	GMA	25	F	PAIS			-	
13	H	29	M	MAIS	F755S	5	-	3
14	MM	35	M	CHH	no	-	TACR3 c.738-1G>A	4
15	HN	17	F	5ARD	no	-	SRD5A2 c.344G>A D115N	4

Table 8. Presentation of the genetic profile (mutations in AR, TACR3 and SRD5A2 genes) for the patients whose testicular samples were included in the present study. AR mutations responsible for androgen insensitivity syndromes (CAIS, n=7, PAIS n=5, MAIS, n=1) as well as one case of congenital hypogonadotropic hypogonadism (CHH)(n=1) and one case of 5- α reductase type 2 deficiency. CAIS, complete androgen insensitivity syndrome; PAIS, partial androgen insensitivity syndrome; MAIS, minimal androgen insensitivity syndrome; CHH, congenital hypogonadotropic hypogonadism; 5ARD, 5- α reductase type 2 deficiency; NA, not available.

References:1, (Hannema, Scott et al. 2004);2, (Ris-Stalpers, Trifiro et al. 1991);3, (Massin, Bry et al. 2012); 4, (Francou, Bouligand et al. 2011) ;5, (Sarfati, Trabado et al. 2011)

Supplemental table 3. Oligonucleotide primer sequences of human and murine primers for real-time quantitative reverse transcriptase- PCR.

Gene	Forward (F) and reverse (R) primers (5'-3')
Human 18S	F: GTGCATGGCCGTTCTTAGTTG R: CATGCCAGAGTCTCGTTCGTT
Human AR(C-terminal)	F (3442): TGC ACA AGT CCC GGA TGT AC R (3591): TTT GAT TTT TCA GCC CAT CCA
Human SRC-2	F: GAACAGCCCCTCACAAAGCA R: ACTGACTGGGTGGGATTCGA
Human HBO1	F: CTCAGCCAGGCTAAGCCAGA R: TGTCAGTGGGTAGGCTGCT

Abbreviations of the genes and the 5' to 3' nucleotide sequences of their sense and antisense primers

3. Additional results

3.1. SRC-2 and HBO1 mRNA expression during murine testicular postnatal development

Murine models of SRC-2 invalidation were characterized by various degrees of both female and male fertility impairment. Moreover, in wild type adult testis, *Src-2* transcripts and immunofluorescence labeled protein were detected in the seminiferous tubules epithelium, exclusively in Sertoli cells nuclei (Gehin, Mark et al. 2002).

Therefore, we questioned on the mRNA relative expression of SRC-2 during postnatal testicular development. In a similar manner to the postpubertal surge in *Ar* expression, *Src-2* expression in murine testis increases during testicular development, reaching a maximum in young postpubertal mice, corresponding to Sertoli cell proliferation (Sharpe, McKinnell et al. 2003) (Fig.24, panels A and C). *Hbo1*, which was found to exhibit an abundant expression in murine testis, presented a very weak expression postnatally, with increasing levels thereafter, reaching maximum levels in postpubertal testis (Fig.24, panel B).

The parallel expression of *Src-2*, *Hbo1* and *Ar* suggests that both coregulators might interfere with *Ar* transcription regulation.

3.2. SRC-2 and HBO1 cellular localization within the adult murine seminiferous tubules

In order to specifically localize SRC-2 and HBO1 expression within the murine testis I performed immunohistochemistry assays on paraffin-embedded murine testicular biopsies. Unfortunately, despite several attempts of immunohistochemical detection of SRC-2 and HBO1 using referenced antibodies purchased from Betyl Laboratories (SRC-2) and Santa Cruz (HBO1), no experiment was successful.

In this context, I evaluated mRNA expression for *Ar*, *Src-2* and *Hbo1*, in primary murine cultures of Sertoli cells and spermatogonia, obtained from adult Balb/c wild type mice.

Reverse transcribed samples of primary murine Sertoli cells and spermatogonia, were kindly provided by Dr Isabelle Allemand, researcher in the “Laboratoire de Radiobiologie des

Cellules Germinales”, The Commission for Atomic Energy (Commission d’Energie Atomique), Fontenay aux Roses.

As expected (Sharpe, McKinnell et al. 2003), *Ar* was not expressed in spermatogonia, whereas abundant in mature Sertoli cells (Fig.25, panel A). *Src-2* transcripts were weakly expressed in spermatogonia, while well detectable in Sertoli cells (Fig.25, panel B). Conversely, *Hbo1* was two-fold more expressed in adult testicular murine spermatogonia than in Sertoli cells, suggesting that *Hbo1* might present complex and different roles in each cellular system (Fig.25, panel C).

3.3. SRC-2 and HBO1 expression during human postnatal testicular development

As reported in the published article, immunohistochemical detection and quantification of SRC-2 and HBO1 expression within testicular samples obtained from neonate to adult, confirmed that SRC-2 was constantly expressed at all stages of testicular development (Fig. 1 and 2A of the published manuscript) while HBO1 displayed an ongoing increasing expression, reaching a maximum in adult human testis (Fig. 1 and 2B of the published manuscript).

3.4. Cellular localization of SRC-2 and HBO1 in human adult seminiferous tubules

To specify the cellular localization of the two coregulators, SRC-2 and HBO1, within the human testis, double immunolabeling experiments with either vimentin (chosen as marker of Sertoli cells (Rogatsch, Jezek et al. 1996) or PCNA (proliferating cell nuclear antigen) (chosen as a marker of proliferating germ cells) (Rogatsch, Jezek et al. 1996; Steger, Aleithe et al. 1998; Takagi, Itoh et al. 2001; Kelnar, McKinnell et al. 2002; Bar-Shira Maymon, Yogev et al. 2003) were performed on sections of adult normal human testis.

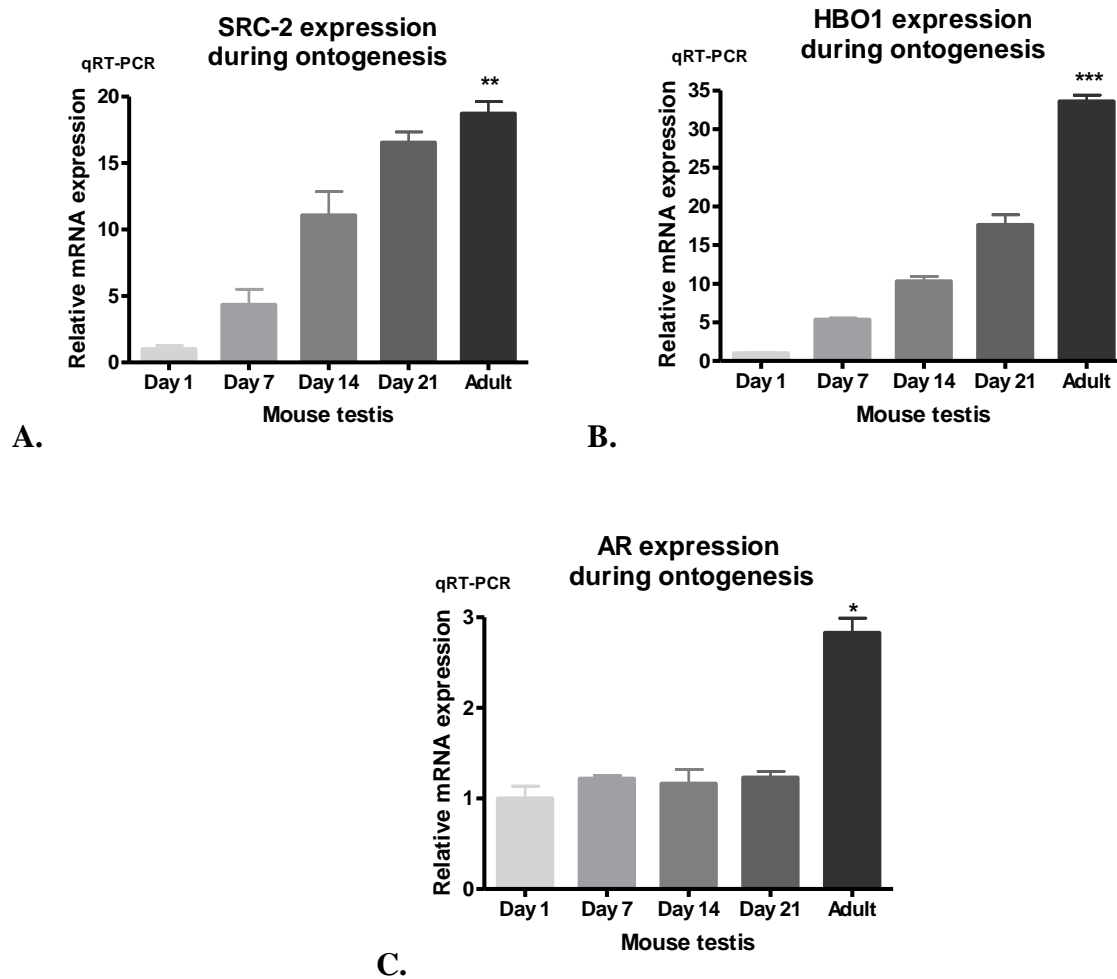


Fig.24. Relative *Src-2* (A), *Hbo1* (B) and *Ar* (C) mRNA expression in murine postnatal testis at various ages, such as neonate (Day 1), prepubertal (Days 7, 14 and 21) and postpubertal (adult aged of more than 5 weeks and less than 4 months). Relative expression of mRNA levels were evaluated by RT-PCR. Results were normalized to murine 18S ribosomal RNA. Relative expression in a given sample was calculated as attomoles per femtomole of 18S rRNA. mRNA relative expression for testicular samples of newborn mice (day 1) was arbitrarily set at 1. Results are expressed as means \pm SEM of at least two independent experiments conducted on six reverse-transcribed samples run in duplicate (*, $P < 0.05$, **, $P < 0.01$, *, $P < 0.001$, non parametric Mann Whitney test).**

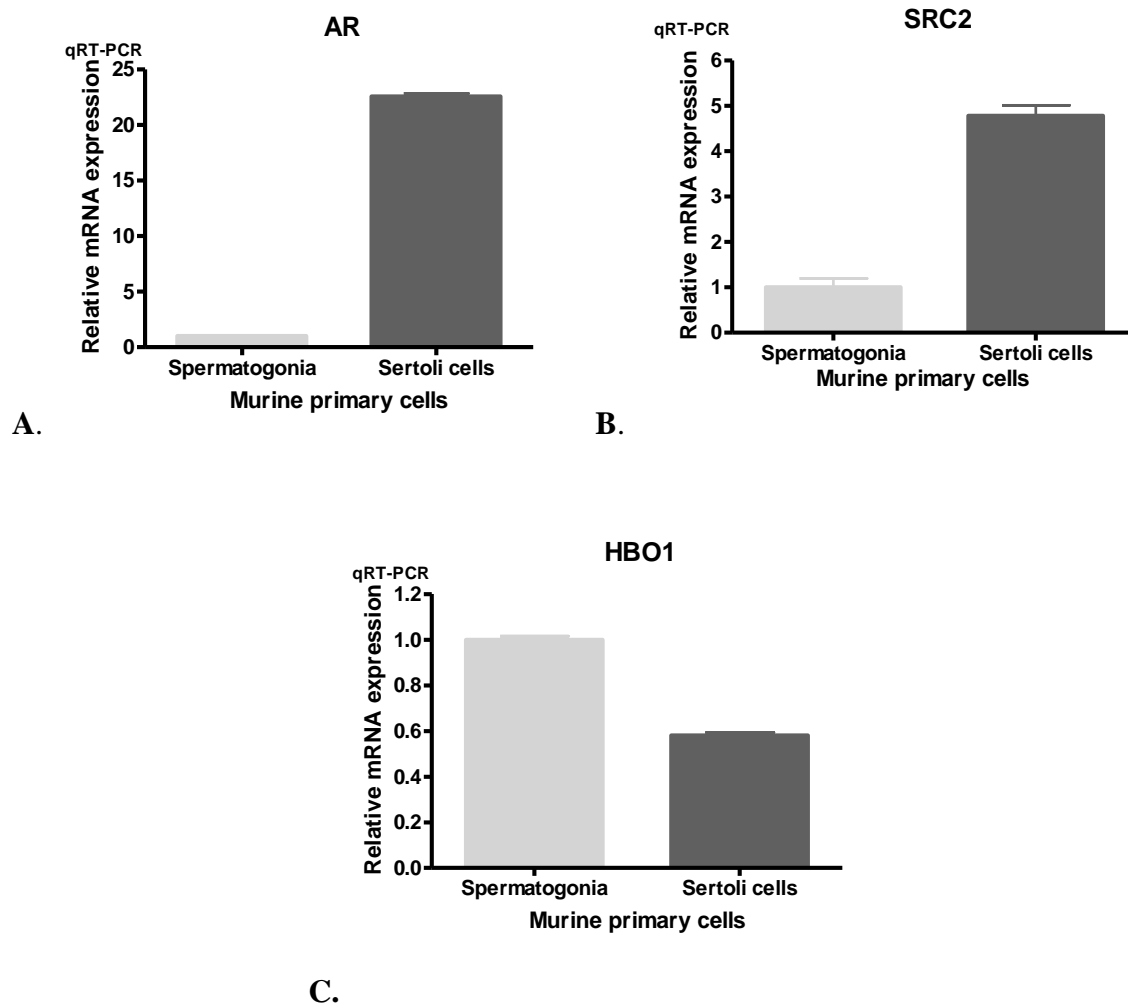


Fig.25. Relative Ar (A) Src-2 (B), Hbo1 (C) and mRNA expression in murine adult primary cultures of spermatogonia and Sertoli cells. Relative expression of mRNA levels were evaluated by RT-PCR. Results were normalized to murine 18S ribosomal RNA. Relative expression in a given sample was calculated as attomoles per femtomole of 18S rRNA. mRNA relative expression for spermatogonia was arbitrarily set at 1. The experiment was performed only once, using two primary cell RNA extracts for both spermatogonia and Sertoli cells; each sample was run in duplicate.

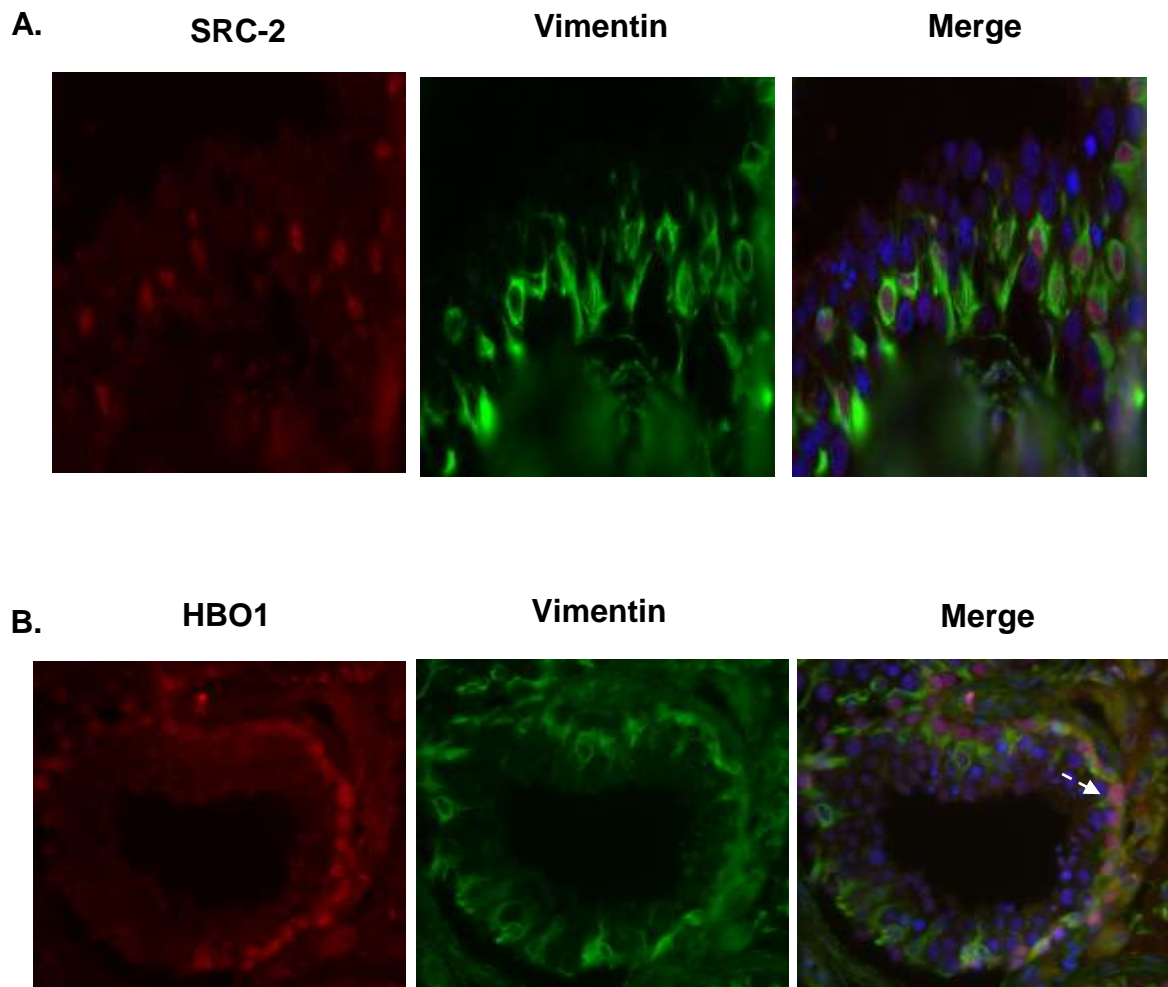


Fig.26. Cellular localization of SRC-2 and HBO1 in human adult testis by double immunostaining **A.** Panels show nuclear SRC-2 immunostaining, vimentin cytoplasmic immunostaining and the merge of the markers (SRC-2 and Vimentin), indicating that SRC-2 is expressed in Sertoli cells, as all SRC-2 positive nuclei are surrounded by cytoplasm positive for vimentin (magnification X 100). **B.** Panels show nuclear HBO1 immunostaining, vimentin positive cytoplasmic immunostaining, and their merge (HBO1 and Vimentin), indicating that HBO1 is expressed not only in Sertoli cells, but also in germ cells (white arrow). Magnification x 20. Blue- DAPI immunostaining.

All SRC-2 positive nuclei were delineated by vimentin positive cytoplasm, suggesting that SRC-2 is expressed almost exclusively in Sertoli cells (Fig. 26.A). This Sertoli-specific cellular localization was further confirmed by the fact that PCNA positively immunolabeled nuclei were negative for SRC-2 immunostaining (see published manuscript and Fig. 26), indicating that SRC-2 was exclusively expressed in Sertoli cells, similarly to the localisation within adult murine seminiferous tubules.

Similarly, the co-localization of HBO1 and vimentin suggests that HBO1 is also expressed in Sertoli cell nuclei (Fig. 26. B). However, some nuclei within the seminiferous tubules were positively stained for HBO1 but not for vimentin (Fig. 26. B).

3.5. SRC-2 and HBO1 expression in human testicular pathologies with impaired androgen signaling

As SRC-2 and HBO1 are physiologically expressed in human testis following a differential evolution profile during ontogenesis, we next evaluated, by means of immunohistochemistry and quantification, the expression of these two coregulators, in testicular samples of patients presenting impaired testicular androgen signaling.

First, in subjects with androgen insensitivity syndromes (AIS) (n=12), SRC-2 was expressed in seminiferous tubules, similarly to the normal testes (see published manuscript and fig. 27). On the contrary, HBO1 was not expressed in seminiferous tubules of patients with complete AIS (CAIS) (n=7) (see published manuscript and Fig. 27), while the immunostaining of HBO1 in partial AIS (PAIS) (n=5) was barely detected (see published manuscript and Fig. 27). Given that HBO1 was never detected in any of the 7 CAIS testicular samples analyzed, we wondered whether the lack of HBO1 expression was correlated with the presence of AR protein. As shown in Fig 5 or the Supplemental Fig. 3 presented in the published manuscript, we found that the absence of HBO1 immunodetection was independent of the AR protein status.

In a patient with congenital hypogonadotropic hypogonadism (CHH), SRC-2 and HBO1 were both present in seminiferous tubules, although the expression of HBO1 seemed to be slightly reduced (Fig. 4). In a postpubertal patient with 5- α reductase type 2 deficiency (5ARD), SRC-2 expression was preserved, while HBO1 was absent (Fig. 27).

Given that in all the 7 CAIS samples as well as in the patient with 5- α reductase type 2 deficiency (5ARD), spermatogenesis was severely impaired (Fig. 28), with complete absence of germ cells in the majority of seminiferous tubules, we also questioned on the possibility that in part, the lack of expression of HBO1 was related to the absence of spermatogonia but also, in part, HBO1 expression was related to an intact AR signaling.

In this context, we next evaluated the immunoexpression of SRC-2 and HBO1 in situations of idiopathic azoospermia, in testicular samples obtained from adult subjects presenting with a normal biological, hormonal and genetic profile (Fig. 29). As already observed, SRC-2 was immunoexpressed in all seminiferous tubules for all the testicular biopsies analysed. In all situations, AR was immunoexpressed within seminiferous tubules in Sertoli cells nuclei and in peritubular myoid cells, as well as within the interstitial space in Leydig cells, as already reported (Rey 2003; Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Rey, Musse et al. 2009). In seminiferous tubules from patients with complete androgen insensitivity and absence of spermatogenesis, HBO1 was never detected. In a single patient presenting with partial androgen insensitivity syndrome, few nuclei were positively immunostained for HBO1 (Fig. 27 and Fig. 29). These nuclei were situated at the basal level, round-shaped and well delineated, being suggestive for spermatogonia. Moreover, in patients with idiopathic azoospermia and no androgen signalling disturbance, HBO1 was immunodetected within few round nuclei situated near the basal membrane, similarly to the PAIS testicular samples, in nuclei with a typical aspect for germ cells.

These data bring evidence of HBO1 protein immunodetection within germ cells and are in accordance with the previous analysis of *Hbo1* transcripts in murine primary cultures of spermatogonia (Fig. 25, C).

However, HBO1 was never immunoexpressed in neither of the 7 CAIS patients, all presenting various deleterious mutations and severe alteration in AR signalling, while HBO1 was weakly expressed in partial androgen insensitivity, or idiopathic azoospermia, suggesting that HBO1 expression in both Sertoli cells and spermatogonia is conditioned by an intact and functional AR present in Sertoli cells.

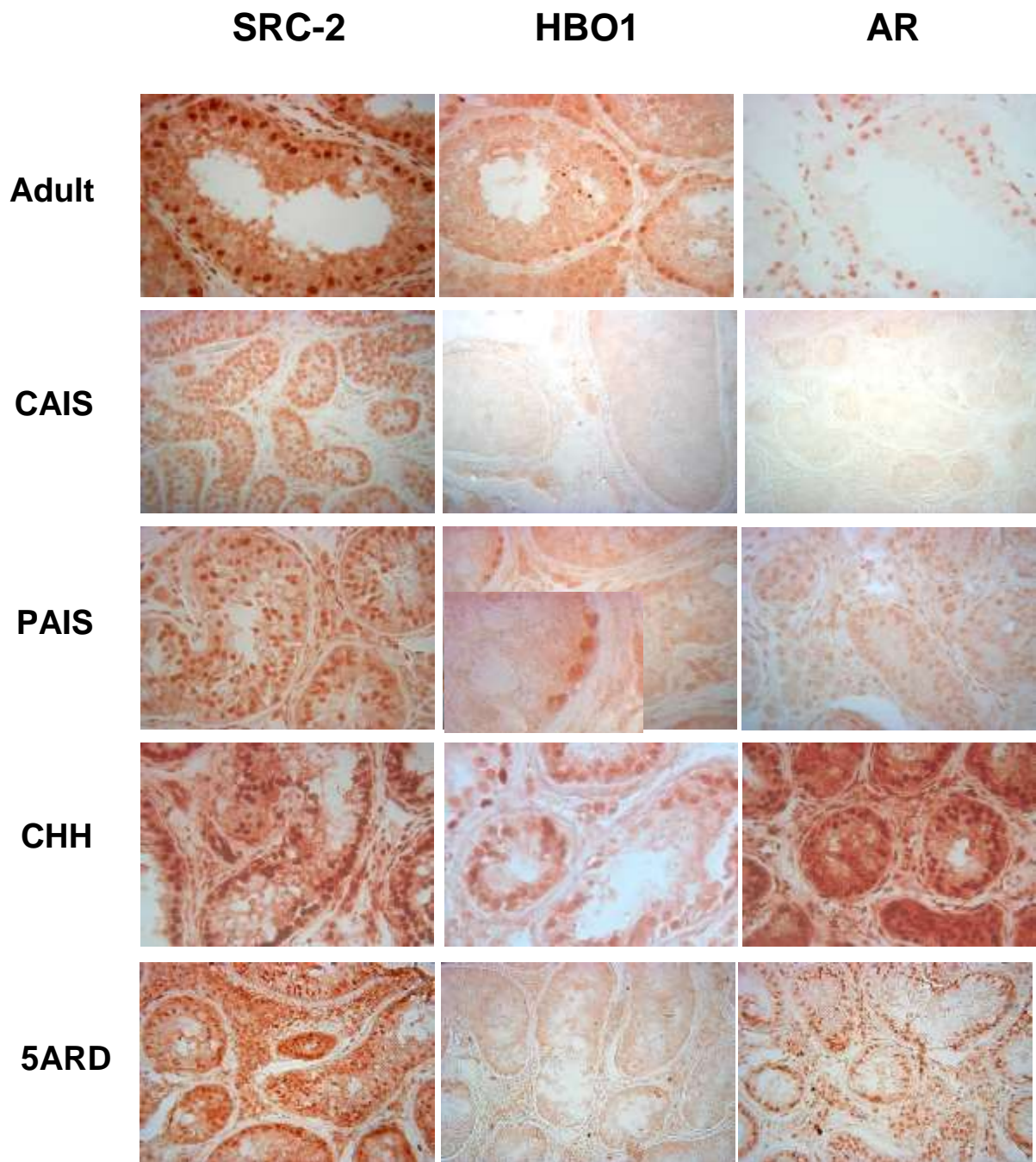


Fig. 27. SRC-2 (right-sided panels), HBO1 (panels on the central column) and AR (left-sided panels) immunoexpression, in paraffin-embedded testicular samples obtained from 46, XY subjects such as: an adult with preserved spermatogenesis, from a posptubertal patient with complete androgen insensitivity syndrome (CAIS), a patient with partial androgen insensitivity syndrome (PAIS), a patient with congenital hypogonadotropic hypogonadism (CHH) and a patient with 5- α reductase type 2 deficiency (5ARD). Magnification: x40.

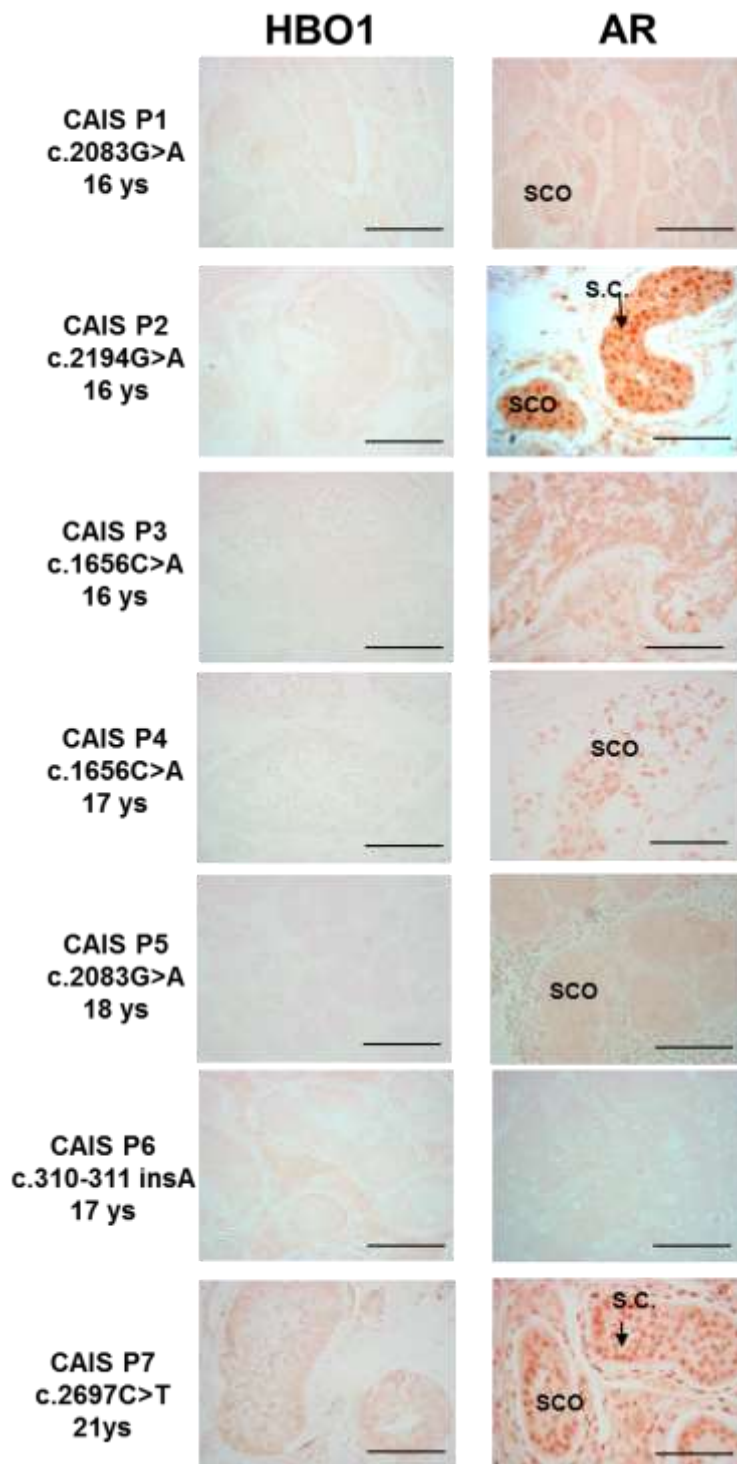


Fig. 28. HBO1 and AR immunostaining in 7 complete androgen insensitivity (CAIS) patients, presenting various AR mutations. S.C.-Sertoli cells, SCO-Sertoli cell only seminiferous tubules. HBO1 was not expressed in CAIS patients irrespective of the presence of detectable AR protein. Magnification: x40. Scale Bars 100µm.

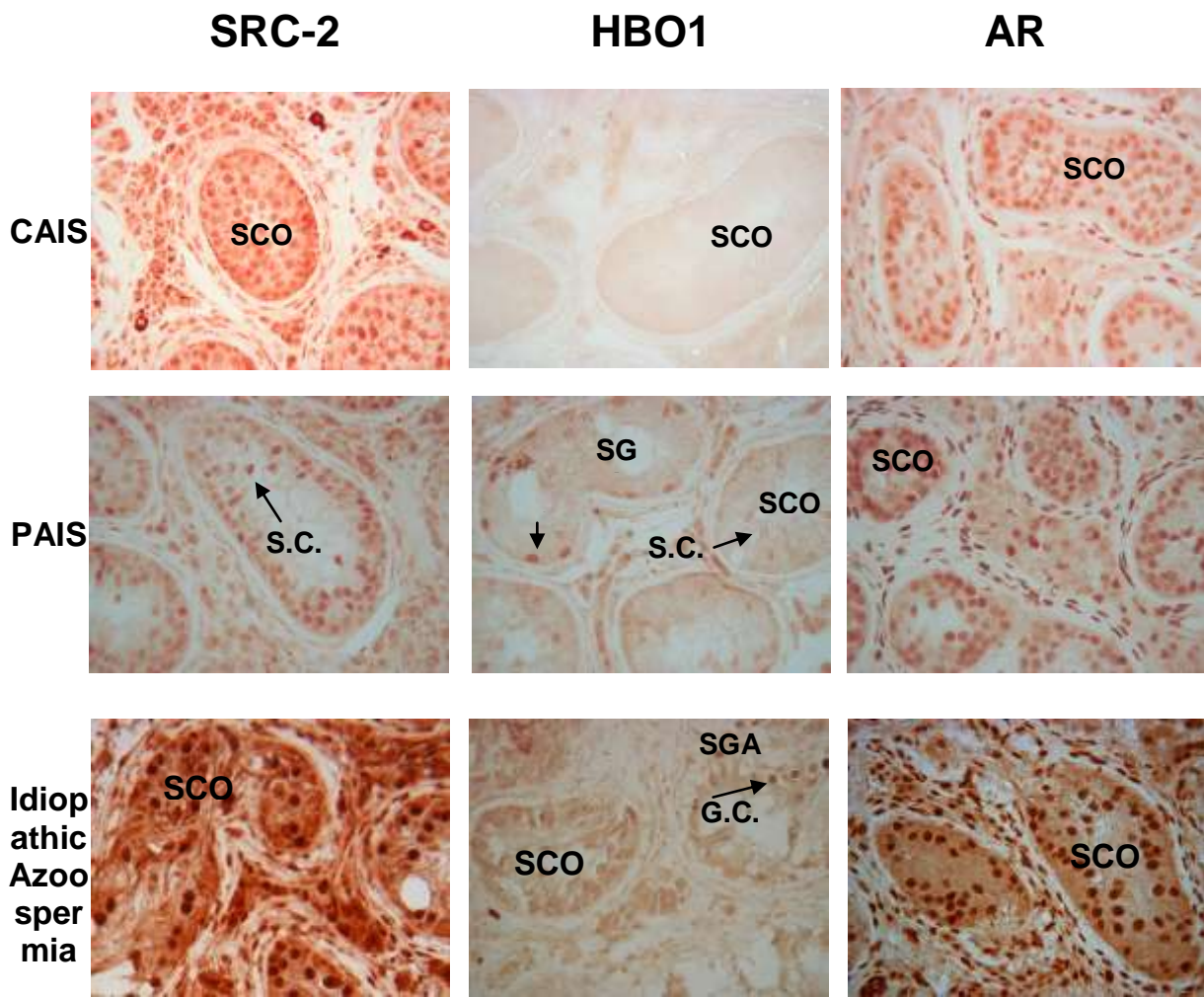


Fig. 29. SRC-2 and HBO1 immunostaining in seminiferous tubules with various degrees of spermatogenesis impairment. SCO, Sertoli cell only tubules; SGA, arrest at the level of spermatogonia. G.C., germ cells, S.C., Sertoli cells. Magnification x40.

3.6. Androgens induce HBO1 gene expression in SMAT1 Sertoli cells in the presence of AR.

As one of the objectives was to study the regulatory impact of SRC-2 and HBO1 on AR transcriptional activity, I performed transient co-transfection assays using expression plasmids of human AR, SRC-2 or HBO1 together with reporter GRE2-luciferase plasmid (Georgiakaki, Chabbert-Buffet et al. 2006; Boukari, Meduri et al. 2009) and with a β -galactosidase plasmid. Due to the lack of an immortalized human Sertoli cell line, I used as a cellular model, the murine Sertoli cell line, SMAT1, whose characteristics have been previously presented (Table 2 of the First Chapter of the Results) (Dutertre, Rey et al. 1997). Vehicle (EtOH) and dihydrotestosterone (DHT)-stimulated transactivations were examined. We showed that in SMAT1 Sertoli cells SRC-2 acts as an AR coactivator, while HBO1 acts as an AR corepressor (see published manuscript, Fig.4).

Moreover, in order to evaluate the possibility that HBO1 could be an AR target gene we assessed the HBO1 expression by qPCR in SMAT1 human AR transfected cells. As shown in the Fig. 6 of the published article, DHT significantly induces HBO1 expression, suggesting not only that HBO1 expression is induced by androgens, but also that HBO1's expression and coregulatory functions are conditioned by a functional and transcriptionally active, ligand-stimulated-AR.

4. Discussion, limits and perspectives

The objective of this part of the thesis, was to evaluate the expression of two AR molecular partners, SRC-2 and HBO1, in murine and human testis during development and in human diseases associated with AR signaling alteration leading to infertility caused by reduced or absence of spermatogenesis.

We are the first to reveal the expression profiling of SRC-2 and HBO1 in murine and human testis during postnatal development. We have demonstrated their precise testicular localization and provided evidence that both SRC-2 and HBO1 are exclusively expressed in the seminiferous tubules yet absent in the interstitium and in Leydig cells. However, the expression of these two AR coregulators is not identical in the seminiferous tubules. Indeed, coimmunolocalization studies indicate that SRC-2 is exclusively expressed in the Sertoli cells while HBO1 is localized in both Sertoli cells and germ cells most notably those adjacent to the basal membrane.

Of particular interest was the testicular ontogenesis of the two AR partners during development keeping in mind the differential AR expression profiling already reported along human testis maturation (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009).

We have previously found that SRC-2 presented a stable expression during human ontogenesis. This coregulator is expressed early in prenatal life (detected on 14 week old fetal testis), when AR is still not expressed in Sertoli cells. Its constant presence in Sertoli cells during human ontogenesis in healthy subjects as well as in human testes of patients with defective androgen signaling (androgen resistance) or in congenital hypogonadotropic hypogonadism (CHH), a condition associated with low testicular testosterone indicates that SRC-2 expression is not exclusively dependent on the androgen status despite its important functional role as an AR coactivator. It is very likely that SRC-2 coactivates transcriptional activities of several nuclear receptor expressed in human testis, other than the AR.

The stable SRC-2 expression contrasts with the progressive evolution profile of the AR signaling and suggests that in humans SRC-2 expression in Sertoli cells was independent of the androgen signaling. The lack of coexpression of AR and its molecular partner at these fetal and neonatal periods of development obviously questions on the exact role of SRC-2 in the testicular physiology during embryonic life. It is very likely that SRC-2 interacts with several other nuclear receptors during fetal testis development, as previous works presented SRC-2 not only as an AR coactivator, but also as a coactivator of GR (Awasthi and Simons 2012), PR (Hofman, Swinnen et al. 2002) and ER (Warnmark, Treuter et al. 2002), all these

steroid receptors being also expressed in human testis during ontogenesis(Shah, Modi et al. 2005; Cavaco, Laurentino et al. 2009).

On the other hand, we presented a strict parallel maturation profile for HBO1 and AR expression during both murine and human testis developments. This temporal and positively correlated expression pattern of both AR and HBO1 suggests that HBO1 expression is linked to or requires a functional AR signaling in the Sertoli cells. This hypothesis is further validated by the lack or drastic reduction of testis HBO1 expression in pathological conditions in which androgenic signaling was impaired such as in AIS patients and 5- α reductase type 2 deficiency. As previously mentioned, HBO1 is a nuclear protein not only expressed in Sertoli cells, but also in spermatogonia. Thus the physiological role of HBO1 in germ cells is very likely distinct from that exerted in Sertoli cells given that it is well established that AR is not expressed in spermatogonia neither in mice nor in humans (Suarez-Quian, Martinez-Garcia et al. 1999; Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009) We propose that HBO1 might affect other steroid receptor signaling such as PR or ER although their expression in human germ cells remains still controversial (Pais, Leav et al. 2003; Luetjens, Didolkar et al. 2006; Han, Feng et al. 2009).

Alternatively, given that HBO1 plays an important role on DNA replication (Pais, Leav et al. 2003; Iizuka, Matsui et al. 2006; Luetjens, Didolkar et al. 2006; Han, Feng et al. 2009)and is potentially involved in the control of cellular proliferation (Avvakumov, Lalonde et al. 2012) it could thereby be involved in the initiation and maintenance of spermatogenesis. In this respect, HBO1 expression could also serve as a suitable index of germ cell proliferation.

In order to clarify the potential impact of SRC-2 and HBO1 on testicular function and AR signaling pathway, notably within the Sertoli cell where all molecular partners are expressed in adult testis, their functional properties were studied by cotransfection assays in murine Sertoli SMAT1 cells. Transactivation experiments showed that SRC-2 behaves as a potent AR coactivator in a Sertoli cell like context while cotransfection of HBO1 with AR markedly reduced DHT-stimulated AR transactivation, consistent with its corepressor property. These results are consistent with previous works that reported AR mutations responsible for impaired SRC-2 interaction, associated with an altered spermatogenesis (Ghadessy, Lim et al. 1999; Lim, Ghadessy et al. 2000; Quigley, Tan et al. 2004) Collectively, these findings suggest that the coactivating properties of SRC-2 on AR function in the Sertoli cell could play a pivotal role for the sustained production of spermatozooids in human adults. This is also true in other species given the hypofertility of SRC-2 knock-out male mice (Gehin, Mark et al.

2002; Ye, Han et al. 2005). On the other hand, the exact role of the corepressor HBO1 in Sertoli cells remains to be established. A possibility would be that HBO1 as a AR corepressor could attenuate a maximal AR activation in Sertoli cells induced by very high intra-testicular testosterone concentrations (Coviello, Matsumoto et al. 2005). Alternately, HBO1 expression in adult Sertoli cells could induce a partial testicular resistance to testosterone and thus explain the necessity of very high intratesticular testosterone levels needed to induce spermatogenesis in men (Schaison, Young et al. 1993; Coviello, Matsumoto et al. 2005; Jarow and Zirkin 2005).

The drastic alteration of HBO1 expression in severe/complete androgen insensitivity, irrespective of deleterious natures of AR mutations *i.e* the presence or absence of detectable AR protein, suggest that the integrity of AR signaling within the Sertoli cell is germane for HBO1 expression in both Sertoli and germ cells. The presence of AR immunostaining despite a deleterious mutation of AR is not unusual. Previous reports (Avila, Wilson et al. 2002) have described preserved immunoreactivity of AR in patients with AR substitution/missense mutations within the ligand binding domain, as the case of the presented CAIS patient. In our CAIS patient, AR was detected by an anti-AR antibody, frequently used for AR immunohistochemical detection, which recognizes the first 50 amino acids of the NH₂-terminal region of human AR (AR (N-20): sc-816; NCBI: P10275 <http://www.ncbi.nlm.nih.gov/protein/P10275>), AR component which was not altered by the distal mutation affecting the ligand-binding domain.

If and how HBO1 impacts androgenic regulation of spermatogenesis remains to be further explored.

In conclusion, even if other AR coregulators may be expressed and play a role in human testis physiology, we provide first evidence that the expression of SRC-2 and HBO1, two important AR coregulators, follows distinct evolution profile during human testis development. Our results also indicate that modulation of SRC-2 and HBO1 abundance within the Sertoli cells might constitute molecular markers for altered spermatogenesis. Given the opposite coregulatory properties of SRC-2 and HBO1 on AR signaling, it would be of interest to examine whether infertile patients might harbor either loss of function or gain of function deleterious mutations of these two coregulators.

Third part
Androgen regulation in Sertoli cells
Additional papers

Paper 3

Testicular histological and immunohistochemical aspects in a post-pubertal patient with 5 alpha-Reductase type 2 Deficiency. Case report and review of the literature in a perspective of evaluation of potential fertility of these patients.

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1. Introduction and Objectives

As androgens are important for the regulation of Sertoli cells we wished to assess the consequences of testosterone and dihydrotestosterone (DHT) on spermatogenesis, AR expression and AMH repression in mature, postpubertal Sertoli cells and the consequences of the absence of DHT for human reproduction.

As I had the opportunity of studying a postpubertal subject carrying a homozygous missense deleterious mutation in the exon 2 of the *SRD5A2* gene (c.344G>A; Gly115Asp), leading to complete absence of 5 α reductase type 2 activity, I took advantage of this particular situation of absence of intratesticular testosterone conversion into dihydrotestosterone in order to search for answers to the following questions:

- Is spermatogenesis impaired in the absence of testicular dihydrotestosterone?
- Is AMH still repressed in the absence of testicular dihydrotestosterone?

The main objectives of this work were: first to evaluate the contribution of testicular testosterone versus its 5 α -reduced metabolite, dihydrotestosterone (DHT) on AMH repression after puberty and its relation with spermatogenesis, and second to discuss the potential perspectives concerning the implication in human reproduction and solutions for fertility preservation.

In this respect I analyzed by histology and immunohistochemistry, testicular paraffin-embedded samples of a postpubertal case of 5 α -reductase type 2 deficiency (5ARD) and compared them with a case of complete androgen insensitivity syndrome (CAIS), and with histologically normal (with complete spermatogenesis) adult testicular samples. The results have been confronted to the very few existent data in the literature related to the histology of postpubertal testis in 5 α -reductase type 2 deficiency patients.

Previous data from the literature confirmed that in both pubertal and adult subjects with CAIS and 5ARD, testicular morphology, studied in few cases, was generally characterized by severely altered spermatogenesis (Steger, Rey et al. 1999; Hannema, Scott et al. 2006).

We believe that severe impairment of germ cell development and maturation in 5ARD might be related not only to the bilateral cryptorchidism, associated with both conditions (5ARD and

CAIS) (Steger, Rey et al. 1999; Hannema, Scott et al. 2006), but also to the altered levels of intratesticular androgenic action or production.

We, therefore, compared the testicular morphology as well as the AR and AMH expression in a postpubertal case of 5ARD, a postpubertal case of CAIS and an adult with complete spermatogenesis. We analyze and comment our findings to data from the literature describing the histological testicular aspects of postpubertal individuals considered to be 5 α - reductase type 2 deficient, but lacking SRD5A2 gene sequencing.

The results of this study have been recently accepted for publication (May 23, 2014), in BMC Endocrine Disorders, therefore, are presented in the form of the published paper.

CASE REPORT

Open Access

Testicular histological and immunohistochemical aspects in a post-pubertal patient with 5 alpha-reductase type 2 deficiency: case report and review of the literature in a perspective of evaluation of potential fertility of these patients

Lavinia Vija^{1,2,3,4}, Sophie Ferlicot⁵, Diana Paun³, Hélène Bry-Gauillard⁶, Gabriela Berdan⁷, Issam Abd-Alsamad⁸, Marc Lombès^{1,2,6} and Jacques Young^{1,2,6*}

Abstract

Background: Testicular morphology and immunohistochemical studies have never been reported in genetically documented adult patients with 5 alpha-reductase type 2 deficiency (5α-R2 deficiency).

Case presentation: We describe the testicular histopathology of a 17-year-old XY subject with 5α-R2 deficiency caused by the recurrent homozygous Gly115Asp loss of function mutation of the *SRD5A2* gene. We also performed an immunohistochemical analysis in order to further study the relationship between seminiferous tubules structure, Sertoli cell differentiation and androgenic signaling impairment in this case. We thus evaluated the testicular expression of the anti-Müllerian hormone (AMH), androgen receptor (AR) and 3β-hydroxysteroid dehydrogenase (3βHSD). Histological analysis revealed a heterogeneous aspect with a majority (92%) of seminiferous tubules (ST) presenting a mature aspect but containing only Sertoli cells and devoid of germ cells and spermatogenesis. Focal areas of immature ST (8%) were also found. Testicular AR and 3βHSD expression were detected in adult male control, 5α-R2 deficiency and CAIS subjects. However, AMH expression was heterogeneous (detectable only in few AR negative prepubertal ST, but otherwise repressed) in the 5α-R2 deficiency, conversely to normal adult testis in which AMH was uniformly repressed and to an adult CAIS testis in which AMH was uniformly and strongly expressed.

Conclusion: Intratesticular testosterone can repress AMH by itself, independently of its metabolism into dihydrotestosterone. We also compare our results to the few post pubertal cases of 5α-R2 deficiency with available histological testicular description, reported in the literature. We will discuss these histological findings, in the more general context of evaluating the fertility potential of these patients if they were raised as males and were azoospermic.

Keywords: 5α-reductase type 2 deficiency, Testicular histology, Sertoli cell, Anti-Müllerian hormone, Androgen receptor

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Background

The 46, XY disorders of sex development (DSD) are presently classified in three main categories [1]: disorders of gonad development such as gonadal dysgenesis, disorders of androgen biosynthesis and metabolism and disorders related to androgen sensitivity (the androgen insensitivity syndrome, AIS). Within the second category, genetic causes have been identified, such as loss of function mutations of the LH receptor gene (*LHCGR*), 17 β hydroxysteroid dehydrogenase deficiency type 3, related to mutations of the *HSD17B3* gene and loss of function mutations of the *SRD5A2* gene, responsible for 5 α -reductase type 2 deficiency which is one of the three 5 α -reductase isoforms expressed in humans [2]. In the third category, androgen receptor (*AR*) mutations have been identified in patients with mild, partial or complete androgen insensitivity syndromes (AIS) [1].

AIS have variable phenotypic presentations, mainly related to the severity of the deleterious effects of AR mutations. Indeed, subjects with complete androgen insensitivity (CAIS) present as girls and women with feminine aspect of external genitalia but with absent pubic hair, blind ending vagina and uterine agenesis. In partial androgen insensitivity syndrome (PAIS) phenotypes, there are varying degrees of masculinization, ranging from perineoscrotal hypospadias to minor forms of male infertility, more or less associated with gynecomastia, undervirilization, hypotrophic gonads and micropenis [1].

5 α -R2 deficiency phenotype is also variable, ranging from a complete female phenotype at birth to a more or less complete virilization of genitalia [3]. In girls with 5 α -R2 deficiency, spontaneous virilization occurring at the onset of puberty usually reveals the condition [3].

In both pubertal and adult subjects with CAIS and 5 α -R2 deficiency, very few studies of testicular histology showed severely altered spermatogenesis [4,5]. The severe spermatogenesis impairment in CAIS and 5 α -R2 deficiency could be related in part to the bilateral cryptorchidism [6] found in both conditions, but also by either altered levels of testicular testosterone action or the lack of metabolic activation to a more active androgen due to mutated 5 α -reductase type 2.

An interesting cellular marker of Sertoli cells, used for the evaluation of the androgen signaling within seminiferous tubules is the anti-Müllerian hormone (AMH). AMH expression is usually recognized as a marker of differentiation of Sertoli cells and considered to be negatively regulated by androgens at puberty and adulthood [7,8].

The first objective of this work was to evaluate the contribution of testicular testosterone versus its 5 α -reduced metabolite, dihydrotestosterone (DHT), on AMH repression after puberty and its relation with spermatogenesis. We, therefore, compared the testicular morphology, as well as the AR and AMH expression in a postpubertal

case of genetically demonstrated 5 α -R2 deficiency with that of a postpubertal case of CAIS. We also compared our findings to published data describing the histological testicular features of post pubertal individuals considered to be 5 α -R2 deficient, but in which the diagnosis was not genetically confirmed. Finally, we discuss the testicular histological findings in the more general context of the fertility potential of these patients in adult life, if they were assigned to a male gender.

Case presentation

The testicular samples of the 5 α -R2 deficiency patient were obtained immediately after the bilateral gonadectomy performed when she was 17-years-old. The patient was a post-pubertal XY female with primary amenorrhea, failure of pubertal breast development and virilization, clitoromegaly and bilateral cryptorchidism, with testes located in the inguinal canals. This phenotype was related to a recurrent [3], homozygous, missense deleterious mutation in the exon 2 of the *SRD5A2* gene (c.344G > A; Gly115Asp). The residual 5 α -reductase type 2 activity in cells transfected with this mutant was of less than 0.2% [9]. The detailed clinical and hormonal characteristics of this patient (testosterone-T: 7.2 ng/mL (normal range in post-pubertal males: 3.5-8.5), DHT: 0.16 ng/mL (0.25-1.1), FSH: 14.5 IU/L (2.7-7.4), LH: 4.2 IU/L (2.6-6.5), AMH: 65 pmol/L (15-89) as well as the results of the pelvic magnetic resonance imaging (MRI) have been previously described [10].

We comparatively analyzed the testicular histological aspect of this case with those of a CAIS and in a man with obstructive azoospermia but normal testicular spermatogenesis (see below). The CAIS patient was an 18-year-old, XY female, with primary amenorrhea (FSH: 3 IU/L, LH: 18 IU/L) and absent axillary and pubic hair and high testosterone (19 ng/mL) and AMH (170 pmol/L) levels. On clinical and ultrasound examinations were observed a blind ending vagina, absent uterus and bilateral cryptorchidism (the left gonad was situated in the pelvis while the right one was located in the inguinal canal). In this patient, the diagnosis of CAIS was confirmed by the identification of a missense mutation in the exon 5 of the *AR* gene. This recurrent, loss of function mutation (c.2194G > A, p.Asp732Asn or D732N upon a previous classification), has already been reported in several CAIS patients [11] and its deleterious character has been previously demonstrated, as functional analysis revealed that this mutant had lost 95% of the wild-type transcriptional activity [11].

The testicular sample, used as a normal control, was obtained from a 29-year-old patient, with normal testosterone and gonadotropin levels (T: 6.8 ng/mL, FSH: 4.5 IU/L, LH: 4.2 IU/L, AMH: 33 pmol/L) undergoing testicular biopsy for obstructive azoospermia. Indeed, for ethical

reasons, it was not possible to perform testicular biopsies in healthy men. However, as already reported, more than 86% of patients with obstructive azoospermia present normal spermatogenesis on testicular biopsies [12]. For this patient, the histological analysis of the testicular biopsy showed, as expected, normal structures and ongoing spermatogenesis within seminiferous tubules.

Histology and immunohistochemistry

All subjects gave their informed written consent to allow the study of testicular samples. This study was approved by the corresponding local ethics committees and was in accordance with the French Bioethics law No.2004-800. All paraffin samples from the three patients were studied by the pathologist (SF), without knowledge of the underlying conditions. For each sample, testicular morphology was assessed on haematoxylin-eosin staining, followed by immunohistochemical analysis for the expression and localization of AR, AMH and β -hydroxysteroid dehydrogenase (β HSD). The immunohistochemistry techniques used were previously reported in detail [13-15]. Immunonegative controls were performed substituting primary antibodies with corresponding preimmune immunoglobulins from the same species. These sections were all immune-negative.

Macroscopic aspects

The adult testicular samples used as a control, were harvested from testes with intrascrotal localization, presenting normal volume (18 cm³) and morphology. Macroscopically, both testes of the patient with 5α -R2 deficiency exhibited a similar hypotrophic aspect. Thus, the left testis had a 6 cm³ volume and weighted 7 g while the right testis volume was 7 cm³ and weighted 7 g. The testicular samples of the patient with CAIS obtained after bilateral gonadectomy, exhibited similar size (11 and 9 cm³) and macroscopic aspect than the 5α -R2 deficiency patient's testis.

Histological aspects

Haematoxylin-eosin stained paraffin sections from the adult testicular biopsy showed large seminiferous tubules, outlined by spindle-shaped peritubular myoid cells. All seminiferous tubules contained mature Sertoli cells lining the basal membrane and ongoing spermatogenesis (Figure 1A).

In the 5α -R2 deficiency patient's testis, haematoxylin-eosin staining revealed heterogeneous seminiferous tubules, surrounded by few peritubular myoid cells and a thickened basement membrane (Figure 1B). The majority of these seminiferous tubules displayed central lumina and normal diameters for the age. They contained two types of Sertoli cells: either mature Sertoli cells, with limited borders and visible nucleoli, or involuting Sertoli

cells, with lobulated shapes, irregular borders and inconspicuous nucleoli (Figure 1B). Besides these mature tubules, there was a focal area of seminiferous tubules, representing 8% of the seminiferous tubules, with small tubular diameters and lack of central lumina consistent with an immature, prepubertal pattern (Figure 1B, thick black arrow). These immature seminiferous tubules were characterized by a pseudostratified distribution of Sertoli cells; this aspect was similar to the testicular histology described by Regadera et al. [16] in patients with secretory azoospermia caused by hypogonadotropic hypogonadism. Some prepubertal seminiferous tubules also contained few spermatogonia. In order to specify the nature of Sertoli cells histological aspects in this 5α -R2 deficiency patient, we performed a selected magnification (Figure 2). We thus observed some seminiferous tubules, with flattened aspect, where Sertoli cells exhibited an oncocyctic cytoplasm (Figure 1B, white arrow and Figure 2, black arrow). The interstitial space in this sample contained large areas of Leydig cells presenting an intense eosinophilic and granular cytoplasm and single round nuclei, consistent with Leydig cell hyperplasia (Figure 1B, thin black arrow).

In the CAIS patient, the testicular sample stained with haematoxylin-eosin showed dispersed seminiferous tubules with a homogeneous immature aspect characterized by small tubular diameters and lack of central lumina. All seminiferous tubules were surrounded by a thickened basal membrane (Figure 1C, thick black arrow) and by peritubular myoid cells. The majority of seminiferous tubules in this testicular sample contained exclusively Sertoli cells with a pseudostratified distribution, consistent with immature seminiferous tubules [16]. Spermatogenesis was absent in all seminiferous tubules in this CAIS subject; spermatogonia were present only in few tubules (data not shown). Likewise the testicular sample of the 5α -R2 deficiency patient, we observed few seminiferous tubules containing several Sertoli cells with an oncocyctic aspect (Figure 1C, white arrow). Finally, the interstitial space in the CAIS testis contained focal areas of important Leydig cell hyperplasia (Figure 1C, thin black arrow).

Immuno-histochemical studies

To clarify the relationship between abnormal Sertoli cell differentiation, androgen signaling and spermatogenesis defect, we studied the expression of two Sertoli cell markers, AR and AMH in testicular sections in both 5α -R2 deficiency and CAIS patients compared to the control sample (Figure 1, panels D to I).

The AR protein was expressed in Sertoli, Leydig and peritubular myoid cells in the control adult testis sample (Figure 1D) in accordance to previous data reported by us and others [7,8,15]. Similarly, AR was expressed in Sertoli, Leydig and peritubular myoid cells in both 5α -R2 deficiency (92% of tubules) and CAIS (100% of

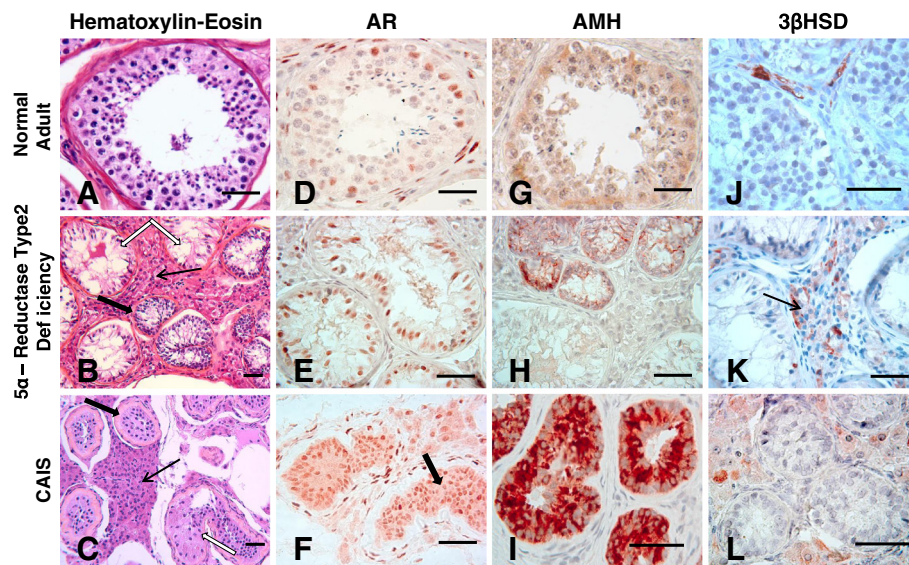


Figure 1 Histological characteristics and immunohistochemical detection for AR, AMH and 3βHSD expression, in testicular paraffin-embedded sections obtained from a 29 year old human adult with normal testicular structures (Figure 1: panels A, D, G, J), a 17 year-old teenager with 5 α-R2 deficiency (Figure 1: panels B, E, H, K) and a 18 year-old CAIS teenager (Figure 1: panels C, F, I, L). Panels A, B, C- histology of testicular samples on haematoxylin-eosin staining. Panel A shows complete spermatogenesis in a seminiferous tubule section of the normal adult testis; Panel B shows heterogeneous seminiferous tubules (ST), in the 5 α-R2 deficiency, such as tubules presenting large diameters and lumina (white arrows) and other tubules with small diameter lacking lumina (thick black arrow); Panel C, shows, in a CAIS patient, used for comparison, a homogeneous pattern, with small diameter, immature STs. In this CAIS patient, STs are delineated by a thickened basal membrane (thick black arrow). In one ST, Sertoli cells with an oncocyctic transformation of the cytoplasm are indicated (white arrow). The interstitial compartment contains an area of Leydig cell hyperplasia (thin black arrow). Panels D, E, F- show AR immune detection in testicular sections obtained from the three subjects. AR is immunodetected in Sertoli cells, Leydig cells and peritubular myoid cells. Panel F evidentiates positive AR immunostaining in ST of the CAIS patient's testicular sample. Panels G, H, I- show AMH immune detection in STs of the testicular sections obtained from the three subjects. Panels J, K, L- show 3βHSD immune detection in Leydig cells of the testicular sections obtained from the three subjects. Scale bars -50 μm.

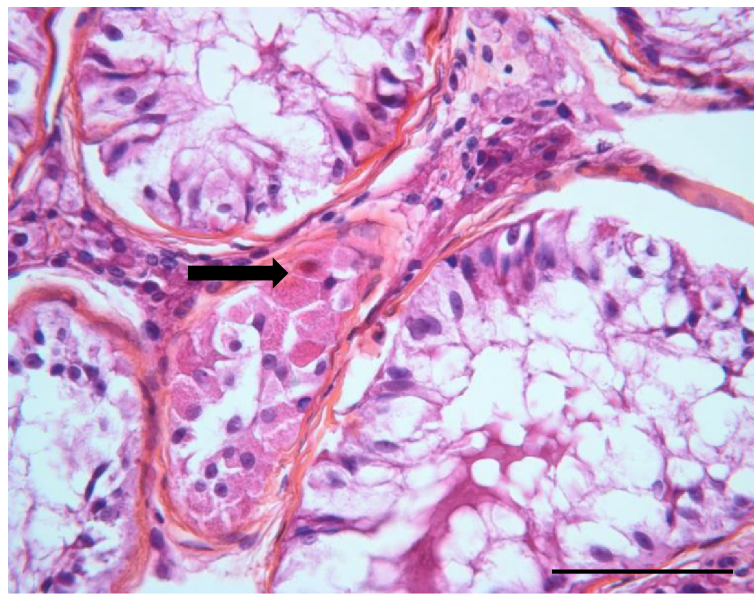


Figure 2 Testicular section magnification, on hematoxylin-eosin staining, of the 17 year-old teenager with 5 α-R2 deficiency. Seminiferous tubules with large diameter and containing only Sertoli cells, surround a flattened section of a tubule, containing Sertoli cells with oncocyctic transformation of the cytoplasm (thick black arrow). Scale bar-50 μm.

tubules) testes (Figure 1, panels E, F). However, in the 5 α -R2 deficiency patient's testis, the AR immuno-expression was absent in a minority (8%) of seminiferous tubules, with a prepubertal aspect.

As previously reported [7,13,17,18], AMH was barely detected in the normal adult seminiferous tubules with ongoing spermatogenesis (Figure 1G). In the 5 α -R2 deficiency patient's testis, AMH expression was heterogeneous, with no detectable immunostaining in 92% of tubules with larger lumina and AR expression. However, AMH was clearly expressed in focal areas (8% of tubules) corresponding to immature tubules with no AR expression (Figure 1H).

Contrasting with the morphological aspect of the 5 α -R2 deficiency patient's testis, all seminiferous tubules studied in the CAIS patient intensively expressed AMH (Figure 1I). This AMH expression pattern was similar to those previously described in CAIS patients by many groups [7,18].

Finally, in order to specify the Leydig cell steroidogenic status, we also evaluated the 3 β -hydroxysteroid dehydrogenase (3 β HSD) expression by immunostaining (Figure 1, panels J-L). Positive immunolabeling within interstitial Leydig cells was observed not only in the normal adult testicular sample (Figure 1 panel J), but also in the 5 α -R2 deficiency case (Figure 1 panel K) as well as in the CAIS sample (Figure 1 panel L).

Conclusions

Herein, we report the histological and immunohistochemical testicular analysis of a postpubertal patient with 5 α -R2 deficiency; the diagnosis was considered in front of the clinical presentation, associated with a high T/DHT ratio, and confirmed by the genetic analysis, which revealed a homozygous missense mutation in the *SRD5A2* gene (c.344G > A; Gly115Asp). The deleterious character of this recurrent mutation was clearly demonstrated by Wigley et al. [9] who showed a 99% loss of the enzymatic activity of the mutated protein. To our knowledge, we present the first testicular histological description in a postpubertal patient, with a genetic confirmation of the 5 α -R2 deficiency. Indeed, the eight previous publications (Table 1) describing with more or less details the testicular histology in postpubertal patients, putatively affected by this pathology, are relatively old and lack any information related to patients *SRD5A2* gene analysis [5,19-25].

Overall, histological features in those postpubertal patients with 5 α -R2 deficiency seem to be rather heterogeneous, varying from complete lack of spermatogenesis to apparently normal spermatogenesis (Table 1).

The histological analysis of the testicular samples obtained from our patient revealed a heterogeneous aspect with a wide majority of seminiferous tubules presenting

a mature aspect but a severely altered spermatogenesis, and containing only Sertoli cells. In a minority of seminiferous tubules of prepubertal appearance, where Sertoli cells exhibited a pseudostratified distribution, few germ cells were identified. Overall, the histological aspect of our patient's testicular samples was similar to that described by Steger et al. [5], in three postpubertal patients (Table 1, patients 2, 9 and 10), presenting with clinical features resembling a 5 α -R2 deficiency. The combined histological study of our case, and the analysis of the available literature (Table 1) also showed a strong percentage of impaired spermatogenesis (7/8, 87%) in cryptorchid 5 alpha-reductase type 2 deficiency patients [5,21,22]. These findings suggest that cryptorchidism by itself could be deleterious for spermatogenesis. Similarly, we compared serum dihydrotestosterone (DHT) levels in subjects with histologically normal spermatogenesis with those measured in patients presenting with impaired spermatogenesis (Table 1). Within the first group of patients (n = 4), mean DHT levels were of 0.26 \pm 0.05 ng/mL whereas in the second group mean DHT levels were 0.12 \pm 0.07 ng/mL. We therefore noticed a trend toward reduction in serum DHT levels in patients with impaired spermatogenesis suggesting that a more severe impairment in 5 alpha-reductase type 2 activity could also contribute to the spermatogenesis alteration.

Histological analysis of the testicular samples of the 5 α -R2 deficiency patient reported here also revealed an oncocyctic transformation of Sertoli cell cytoplasm in very few seminiferous tubules. This minority peculiar histological feature is similar to that described in several infertile subjects [26]. The pathophysiological significance of this feature is still unknown, but it remains to be determined whether this oncocyctic transformation may be associated to testis tumor development. Important areas of Leydig cell hyperplasia were also observed in our case; such morphological observation has been previously found in 5 α -R2 deficient testis [22,24,25].

Similarly, Leydig cell hyperplasia has been already reported in CAIS postpubertal patients [4], or in men with Klinefelter syndrome [27]. In all these different pathological conditions, Leydig cell hyperplasia could be related to chronic Leydig cell stimulation induced by the long-lasting LH excessive secretion [14,24,27,28]. The risk of progression of these lesions to a Leydig cell tumor is unknown in 5 α -R2 deficiency but deserves to be monitored if the testes are preserved.

The immunohistochemical analysis also revealed that in our patient, the androgen receptor (AR) was expressed in all three testicular compartments (Sertoli cells, Leydig cells and peritubular myoid cells), suggesting the possibility of a global cellular response to the intratesticular testosterone in this case of 5 α -R2 deficiency. Most notably, AR was expressed in 92% of the seminiferous tubules,

Table 1 Phenotypical and histological characteristics in postpubertal patients with 5 α -reductase type 2 deficiency

Patient number	Age (years)	Caryotype	Clinical presentation	TV (ml)/TW (g)*	FSH (IU/L)/(xULN)**	T (ng/ml)	T/DHT ratio***	SRD5A2 sequencing	Histology	Reference (publication year)
1	14	46,XY	Female PA; cryptorchidism	7/NA	20.0/(2.0)	3.6	36	NA	SGA	[22] (1986)
2	16	46,XY	Female PA; PPH; clitoromegaly cryptorchidism [§]	NA	38.3/(5.0)	NA	NA	NA	PST (1%) SCA (14%) SGA (25%) SCO (60%)	[5] (1999)
3	16	46,XY	Female PA; PPH; 4 cm phallus cryptorchidism [§]	3/ 15.5	1.0/(0.5)	5.8	29	NA	SCA SGA SCO	[22] (1986)
4	16	46,XY	Female PA; PPH; 3 cm phallus cryptorchidism	11/ 22	4.5/NA	11.4	34.5	NA	Normal	[22] (1986)
5	16	46,XY	Female PA; cryptorchidism	NA	8.7/(1.0)	9.0	225	NA	SGA	[22] (1986)
6	17	46,XY	Female PA; PPH; 3 cm phallus	3/NA	20/(1.0)	7.2	NA	NA	SCO	[24] (1980)
7	18	46,XY	Female PA; PPH; 3 cm phallus	3/NA	56/(3.1)	6.8	NA	NA	SCO	[24] (1980)
8	18	46,XY	Female PA; PPH; 1.5 cm phallus	15/NA	5.5/(1.1)	10	39	NA	Normal	[19] (1980)
9	18	46,XY	Female PA; PPH; clitoromegaly cryptorchidism [§]	NA	9.0/(1.3)	NA	NA	NA	PST (10%) SCO (90%)	[5] (1999)
10	18	46,XY	Female PA; PPH; clitoromegaly	NA	13.0/(2.0)	NA	NA	NA	PST (4%) SCO (96%)	[5] (1999)
11	25	46,XY	Female PA; PPH; 3 cm phallus	NA	NA	11	42	NA	Normal	[23] (1979)
12	35	46,XY	Female PA	8/NA	32.0/(3.2)	6.7	33.5	NA	Normal SCO	[22] (1986)
13	45	46,XY	NA	NA	NA	NA	NA	NA	Normal	[25] (1977)
14	65	46,XY	Male perineal hypospadias; 6 cm phallus cryptorchidism	NA	40/(4.0)	5.9	38	NA	SCO (100%)	[21] (1980)
15	NA	46,XY	NA	NA	NA	NA	NA	NA	Normal	[20] (1982)
Our case	17	46,XY	Female PA; PPH; clitoromegaly cryptorchidism	9/ 8	14.5/(2.0)	7.2	45	Gly115Asp	PST (8%) SCO (92%)	present paper

*TV /TW: mean testicular volume in milliliters/ mean testicular weight in grams; ** (xULN): ratio between the measured value and the upper limit of normal for the corresponding FSH assay; ***T/DHT ratio: testosterone/ dihydrotestosterone ratio; the ratio cutoff for the diagnosis of 5 α -reductase type 2 deficiency was set at 10 [3].

PA: primary amenorrhoea; PPH: pseudovaginal perineoscrotal hypospadias; [§]For these individuals cryptorchidism was not specified in the references, but it was suspected, given the female phenotype; NA: not available.

SGA: spermatogenic arrest at the level of spermatogonia; PST: prepubertal seminiferous tubules; SCA: spermatogenic arrest at the level of spermatocytes; SCO: Sertoli cell only; Normal: normal spermatogenesis.

namely in those with a mature aspect. However, AR expression was below detectable levels in 8% of the seminiferous tubules, specifically those with an immature aspect. Along this line, it is worth noting that AMH immunodetection was rather heterogeneous, as previously reported in a case of 5 α -R2 deficiency with "Sertoli cell only" histology [5]. We showed that AMH was repressed in the majority of the seminiferous tubules, whereas AMH was only detectable in immature tubules lacking AR expression. These results suggest that intratesticular testosterone seems sufficient to repress AMH, when AR is expressed, in spite of the 5 α -R2 deficiency, and thus irrespective of the conversion of testosterone into dihydrotestosterone. The absence of AMH repression in few seminiferous tubules, in which AR was lacking, is in accordance with previous data published [7,8], supporting the need of AR expression in Sertoli cells for AMH repression. Interestingly, in our patient, AMH was repressed in Sertoli cells harboring mature morphology, even in the absence of germ cells. This suggests that testosterone could exert direct inhibitory effects on AMH expression, independently from spermatogenesis initiation [29]. Alternatively, the absence of spermatogonia in most seminiferous tubules of our patient might have led to functional alterations in Sertoli cells, disrupting their ability to synthesize AMH.

In our patient with 5 alpha-Reductase type 2 Deficiency, serum AMH level was within normal range for postpubertal male as reported in few cases with this condition [30]. This contrasts with the very high serum AMH levels we observed in our CAIS patient, which were in line with the increase in AMH levels reported in adult CAIS patients [31]. This difference in serum AMH levels in 5 alpha-Reductase type 2 Deficiency versus CAIS reinforces the hypothesis that testosterone conversion into dihydrotestosterone seems not essential for AMH repression.

Identification of various spermatogenesis stages in patients with 5 α -R2 deficiency is of importance in the context of sex assignment and fertility preservation. Indeed, due to recent progress in the assisted reproduction techniques, successful results with testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) are now reported in azoospermic patients, either with androgen insensitivity [14] or with Klinefelter syndrome [27]. Concerning 5 α -R2 deficient patients, normal spermatogenesis with spontaneous fertility is extremely rare (two published cases) [32]. On the same way, spermatozoa detection in the seminal fluid is also a very rare event, found in only nine 5 α -R2 deficient cases [33-35], allowing either intrauterine insemination of the partner in one case [33], or an in vitro fertilization by ICSI, as recently reported for two patients [34,35]. Except these uncommon cases, fertility remains an important issue for 5 α -R2 deficient patients, notably those harboring a severe enzymatic

defect that precludes spontaneous parenthood and sperm recovery in the ejaculate (i.e. azoospermic patients). In these latter cases, of unknown prevalence, the only fertility therapeutic possibility could be the testicular sperm extraction (TESE). This therapeutic option seems reasonable since as indicated above, TESE has been used as a successful therapeutic strategy in a number of patients with non obstructive azoospermia of various origins, including those with cryptorchidism [36]. In this context, a detailed testicular histological analysis is crucial since this is currently the only reliable method to know if there is spermatozoa in the testis and if spermatozoon are present, to carry out a cryopreservation for ICSI. Finally, a direct relationship between the *SRD5A2* gene mutation severity and the presence of spermatozoa still remains an unsolved question, opening further studies with regards to the prognostic and risk stratification in relation with the severity of the enzymatic defect.

Consent

Written informed consent was obtained from the patient with 5 α -R2 deficiency, as well as from both the CAIS patient and the individual with obstructive azoospermia, for genetic and histological analyses and for publication of this case report and the accompanying images. Copies of written consents are available upon request for review by the Journal Editor.

Abbreviations

5 α -R2 deficiency: 5 α -reductase type 2 deficiency; AIS: Androgen insensitivity syndromes; AMH: Anti-Müllerian hormone; AR: Androgen receptor; 3 β HSD: 3 β -hydroxysteroid dehydrogenase; TESE: Testicular sperm extraction; ICSI: Intracytoplasmic sperm injection.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

LV, ML and JY led the conception and design, acquisition of data, review of literature, and drafted the manuscript. HBG and DP critically reviewed the manuscript. SF, IAA and GB provided the paraffin embedded samples and advised on the pathology pictures. All authors read and approved the manuscript.

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2. Discussion and perspectives

Histological analysis of postpubertal 5 α -reductase type 2 deficiency testicular biopsies has been rarely performed.

We aimed at reporting the histological and immunohistochemical testicular analysis of a postpubertal patient with 5ARD deficiency and reviewed the histological descriptions of 15 postpubertal patients with steroid 5 α -reductase type 2 deficiency (Peterson, Imperato-McGinley et al. 1977; Kuttann, Mowszowicz et al. 1979; Cantu, Corona-Rivera et al. 1980; Imperato-McGinley, Peterson et al. 1980; Okon, Livni et al. 1980; Imperato-McGinley, Peterson et al. 1982; Johnson, George et al. 1986; Steger, Rey et al. 1996; Steger, Rey et al. 1999). Of these 15 patients, one was 14 years old, four were 16 years old, one was 17, four were 18, three were aged between 25 and 45 years, one was 65 years old, while for a last patient with histological testicular description no clinical or biological data were available (see Table 1 of the published paper, or Table 9). Six patients exhibited normal spermatogenesis in almost all seminiferous tubules, while the other 9 biopsies exhibited a wide range of seminiferous tubules with spermatogenic impairment varying from arrest at the stage of spermatocytes to lack of maturation beyond the stage of spermatogonia and up to a complete aplasia of germ cells (Table 1, published paper, Table 9).

For our knowledge, we present the first testicular histological description in a postpubertal patient, presenting a genetic confirmation of the 5 α -reductase type 2 deficiency. The diagnosis was taken in consideration in front of the clinical presentation, associated with a high T/DHT ratio, further confirmed by the genetic analysis which revealed a homozygous missense mutation in the *SRD5A2* gene (c.344G>A; Gly115Asp). The deleterious character of this recurrent mutation was previously demonstrated by Wigley et al. (Wigley, Prihoda et al. 1994) who showed a 99% loss of the enzymatic activity of the mutated protein. Indeed (Table 1, published paper), the eight previous publications having described in more or less detail the testicular histology in postpubertal patients with this pathology are ancient and lack any information related to patients *SRD5A2* genetic analysis (Peterson, Imperato-McGinley et al. 1977; Kuttann, Mowszowicz et al. 1979; Cantu, Corona-Rivera et al. 1980; Imperato-

McGinley, Peterson et al. 1980; Okon, Livni et al. 1980; Imperato-McGinley, Peterson et al. 1982; Johnson, George et al. 1986; Steger, Rey et al. 1999).

The histological analysis of the testicular samples obtained from our patient revealed a heterogeneous aspect with a wide majority of seminiferous tubules presenting a mature aspect but severely altered spermatogenesis, as seminiferous tubules contained only Sertoli cells. In a minority of seminiferous tubules of prepubertal aspect, as Sertoli cells presented a pseudostratified distribution, few germ cells were identified.

It was of interest to compare our histological observations with those previously described in postpubertal patients with a suspicion of 5 α -reductase type 2 deficiency. Overall, the histological aspect of our patient's testicular samples was similar to that described by Steger et al. (Steger, Rey et al. 1999) in three postpubertal patients (Table 1, published paper, patients 2, 9 and 10) presenting clinical features of 5 α -reductase type 2 deficiency, but lacking data related to T/DHT ratios, or any genetic analysis of the *SRD5A2* gene. This aspect was different from five other cases reported by Johnson et al. (Johnson, George et al. 1986) in 1986, where the diagnosis was confirmed by both the clinical features and the elevated T/DHT ratios, though still lacking any genetic confirmation. In two patients (Table 1, published paper, patients 1 and 5), Johnson revealed seminiferous tubules with spermatogenesis arrest at the level of spermatogonia. In two other cases (Table 1, published paper, patients 3 and 12), the same authors described a heterogeneous aspect, including tubules containing exclusively Sertoli cells associated with tubules harboring more or less complete blocks in spermatogenesis at either the level of spermatogonia or spermatocytes, and even rare tubules containing spermatids (Johnson, George et al. 1986). It is interesting to note that in a 16 year old subject, Johnson reported normal spermatogenesis, although it is not forementioned whether this aspect was focal or concerned the majority of the seminiferous tubules (Table 1, published paper, patient 4).

In very ancient studies, Imperato-McGinley et al. described the aspect of Sertoli cell only in a 65 year old male subject 65 ans (see published paper, Table 1) with a clinical and hormonal evoking the 5 α -reductase deficiency, with the lack of genetic confirmation (Imperato-McGinley, Peterson et al. 1980). Moreover, the same authors presented a different histological aspect, in another postpubertal case with spermatogenesis evolving to the stage of spermatids, still lacking genetic confirmation (Imperato-McGinley, Peterson et al. 1982). In 1980, Okon et al. (Okon, Livni et al. 1980) described two siblings, of 17 and 18 years, in

whom diagnosis was suspected by the loss of the enzymatic activity in fibroblasts obtained from perineal skin. These patients presented complete absence of spermatogenesis, with only Sertoli cells within seminiferous tubules (Okon, Livni et al. 1980). Conversely, within the same year, Cantu et al. published the description of an 18 year old subject with clinical and T/DHT ratio raising the suspicion of this pathology (Cantu, Corona-Rivera et al. 1980; Onate, Tsai et al. 1995), who presented normal testicular volume and normal spermatogenesis. Finally, Kuttenn et al., described in 1979 a 45 year old patient with elevation of the T/DHT ratio raising the suspicion of the 5 α -reductase type 2 deficiency (Table 1, published paper, patient 11) (Kuttenn, Mowszowicz et al. 1979). The histological description is rather brief but suggests that spermatogenesis was present, with spermatids detected. In 1977, Peterson et al described the testicular histology of a 45 year old patient with the diagnosis argued by the clinical features and the T/DHT ratio. Although brief, the testicular description specifies a complete spermatogenesis, without details related to the proportion of the seminiferous tubules with this aspect (Table 1, published paper, patient 13).

Overall, the histological aspects in postpubertal patients with 5 α -reductase type 2 deficiency seem heterogeneous, varying from complete absence of spermatogenesis to apparently normal spermatogenesis. The majority of publications did not specify the proportion of each type of seminiferous tubules so as to have precise information related to the histology of the whole testis; however, only one study, besides ours, includes such details, allowing to better acknowledge the heterogeneous aspect (Steger, Rey et al. 1999).

The combined histological study of our case, and the analysis of the available literature (Table 1, published paper) also showed a strong percentage of impaired spermatogenesis (7/8, 87%) in cryptorchid 5 alpha-reductase type 2 deficiency patients (Imperato-McGinley, Peterson et al. 1980; Johnson, George et al. 1986; Steger, Rey et al. 1999). These findings suggest that cryptorchidism by itself could be deleterious for spermatogenesis. Similarly, we compared serum dihydrotestosterone (DHT) levels in subjects with histologically normal spermatogenesis with those measured in patients presenting with impaired spermatogenesis. Within the first group of patients (n=4), mean DHT levels were of 0.26 ± 0.05 ng/mL whereas in the second group mean DHT levels were 0.12 ± 0.07 ng/mL. We therefore noticed a trend toward reduction in serum DHT levels in patients with impaired spermatogenesis suggesting that a more severe impairment in 5 alpha-reductase type 2 activity could also contribute to the spermatogenesis alteration.

Another interesting aspect is related to the detectable levels of DHT in our subject, although inferior to the lower limits for a normal postpubertal male (0.16 ng/ml for normal limits between 0.25 and 11), witnessing for the activity of the 5 α -reductase type 1 enzyme, active at puberty, detected not only at the cutaneous levels, but also in the testis, localized in postpubertal Leydig and Sertoli cells (Aumuller, Eicheler et al. 1996).

Moreover, neither male mouse models with mutations in the 5 α -reductase type 1 gene (*SRD5A1*), nor male mouse models invalidated for both 5 α -reductase type 1 and 2 genes (*SRD5A1* and *SRD5A2*) have any alterations of sperm production or fertility, and none of these models present cryptorchidism (Mahendroo, Cala et al. 2001). Although it is difficult to extrapolate from mice to human, murine models of double invalidation of both 5 α -reductases, suggest that cryptorchidism only might be responsible for the spermatogenesis impairment.

Just like in mice, intratesticular testosterone concentration and bioactivity in adult fertile men is by far more important than that of dihydrotestosterone, justifying that, as in mice, testosterone and not its metabolite, dihydrotestosterone, is the only responsible for maintaining the androgenic regulation of some adult testicular functions (spermatogenesis and AMH repression). Indeed, Jarow et al, demonstrated on testicular samples obtained by testicular percutaneous aspiration from adult fertile men, that in human testis DHT represents 2% of the concentration of intratesticular T, while despite the increased affinity of DHT for AR, as 70% of testosterone is bioactive, it still remains significantly preponderant and the principal androgen in the human testis (Jarow and Zirkin, 2005).

The histological analysis of the testicular samples of the 5ARD deficiency patient allowed us to observe an oncocytic transformation of Sertoli cell cytoplasm in very few seminiferous tubules. This minority histological aspect is similar to the one described in several infertile subjects (Nistal and Paniagua 1984). The significance of this aspect is still unknown, as, at present, there are no published references indicating whether the oncocytic transformation would prevail the development of testicular tumors.

In order to provide more details related to the characteristics and degree of maturation of the cellular components of the seminiferous tubules in 5 α -reductase type 2 deficiency, in relation with the spermatogenesis stages, we are the first, to our knowledge to provide an immunohistochemical characterization of the AR distribution in this pathology (Table 9).

AR was detected in the nuclei of Sertoli, Leydig and peritubular myoid cells (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Rey, Musse et al. 2009; Rey and Grinspon 2011),

suggesting the possibility of a global cellular response to the intratesticular testosterone in this case of 5ARD. Most notably, AR was expressed in 92% of the seminiferous tubules, namely in those with a mature aspect. However, AR expression was below detectable levels in 8% of the seminiferous tubules, specifically those with an immature aspect. AR expression was not altered in postpubertal seminiferous tubules, which suggests that the absence or severely impaired dihydrotestosterone synthesis did not affect androgen signaling and AR expression in human postpubertal testis.

Along this line, it is worth noting that AMH immunodetection was rather heterogeneous, as previously reported in a case of 5ARD with a “Sertoli cell only” histology (Steger, Rey et al. 1999).

In our case, few of the seminiferous tubules expressed high AMH levels, specifically those seminiferous tubules with an immature aspect, while AR expression was uniformly present in all mature seminiferous tubules, irrespective of the degree of spermatogenic impairment.

These results suggest that intratesticular testosterone seems sufficient to repress AMH, when AR is expressed, in spite of the 5ARD, so independent of the conversion of testosterone into dihydrotestosterone. The lack of AMH repression in the minority of seminiferous tubules, in which AR was not expressed, is in agreement with previous data that we and others published (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009), underlying the necessity of AR expression in Sertoli cells for AMH repression.

AMH expression was switched off in the majority of the seminiferous tubules, represented by those seminiferous tubules that lacked germ cells and/or evidence of spermatogenesis. These results suggest that testosterone could exhibit inhibitory effects on AMH suppression independently from spermatogenesis, implying a possible complete maturation and full differentiation of Sertoli cells followed by germ cell destruction, as described in the majority of cryptorchid testes, irrespective of their etiology (Johnson, George et al. 1986; Rajpert-De Meyts, Jorgensen et al. 1999; Regadera, Martinez-Garcia et al. 2001; Chemes, Rey et al. 2008).

Moreover, Steger (Steger, Rey et al. 1996) analyzed AMH immunoreactivity in Sertoli cells of seminiferous tubules exhibiting various degrees of spermatogenic impairment, in testicular biopsies obtained from azoospermic patients, but also in a prepubertal case of

5ARD. The testicular biopsy from the 7 year old, 5- α reductase type 2 deficient patient contained seminiferous tubules lacking germ cells; however, on semi-quantitative evaluation, AMH was present only in 45% of the Sertoli cells (Table 9). Steger suggested that the heterogeneity of AMH expression, despite complete absence of germ cells, or stages of spermatogenesis was related to the fact that Sertoli cells presented a mosaic pattern of differentiation (Steger, Rey et al. 1996).

Alternatively, the absence of spermatogonia in the majority of seminiferous tubules of our patient might have led to functional alterations in Sertoli cells, disrupting their ability to synthesize AMH.

In our patient with 5 alpha-reductase type 2 deficiency, serum AMH level was within normal range for postpubertal male as reported in few cases with this condition (Stuchi-Perez, Hackel et al. 2005). This contrasts with the very high serum AMH levels we observed in our CAIS patient, which were in line with the increase in AMH levels reported in adult CAIS patients (Rey, Mebarki et al. 1994). This difference in serum AMH levels in 5 alpha-reductase type 2 deficiency versus CAIS reinforces the hypothesis that testosterone conversion into dihydrotestosterone seems not essential for AMH repression.

The identification of different stages of spermatogenesis in patients with 5 α -reductase type 2 deficiency is important in the perspective of sex assignment and fertility preservation. Indeed, due to recent progress in the assisted reproduction techniques, it is now possible to obtain successful results with testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) in azoospermic patients, including those with androgen insensitivity and Klinefelter syndrome (Massin, Bry et al. 2012; Aksglaede and Juul 2013). Indeed, spontaneous fertility is exceptional in patients with 5 α -reductase type 2 deficiency (Ivarsson 1996), as well as the presence of spermatozoa on the seminal fluid (Katz, Kligman et al. 1997) allowing spontaneous fatherhood or intrauterine insemination of the conjoint. Only two patients with 5 α -reductase type 2 deficiency are reported as present to have achieved paternity by ICSI (Matsubara, Iwamoto et al. 2010; Kang, Imperato-McGinley et al. 2011). As fertility remains an important issue for 5 α -R2 deficient patients, notably those harboring a severe enzymatic defect that precludes spontaneous parenthood and sperm recovery in the ejaculate (i.e. azoospermic patients), the only fertility therapeutic option could be the testicular sperm extraction (TESE). TESE seems a reasonable therapeutic procedure, as it has

already been used as a successful therapeutic strategy in a number of patients with nonobstructive azoospermia of various origins, including those with cryptorchidism (Raman and Schlegel 2003). In this context, a detailed testicular histological analysis is crucial since this is currently the only reliable method to know if there is sperm in the testis and if sperm are present, to carry out a cryopreservation for ICSI. Finally, a direct relationship between the *SRD5A2* gene mutation severity and the presence of spermatozoa still remains an unsolved question, opening further studies with regards to the prognostic and risk stratification in relation with the severity of the enzymatic defect.

We also analysed data from the literature concerning prepubertal cases of 5ARD with documented testicular histology. Testicular histological aspect of seminiferous tubules revealed various aspects, ranging from normal to lack of maturation beyond the stage of spermatogonia (Table 9) and up to a complete aplasia of germ cells (Sertoli-cell only pattern) in two neonates of 2 and 8 months respectively, 5 infants (aged between 1 and 4 years) and three prepubertal children of 9, 10 and 11 years (Table 9).

Our observations generally agree with previous reports and confirm that testosterone and dihydrotestosterone levels as well as meiotic entry are not the only factors implicated in the complex regulatory mechanism of AMH expression.

Moreover, the similarity of seminiferous tubules histology observed in both infant and postpubertal conditions of 5α -reductase type 2 deficiency (Table 9), might suggest that the severity of 5α -reductase type 2 deficiency present in our patient has not exerted a specific pathological impact on the development of germ cells, besides the effects of cryptorchidism itself.

In conclusion, 5α -reductase type 2 deficiency represents a very interesting human model of androgen action on testicular maturation and spermatogenesis. Studies on larger sample sizes would allow a better understanding of the intricate and complex mechanisms involved in the maturation of Sertoli cells and their roles in spermatogenesis.

	Age (years)	T/DHT ratio*	FSH (IU/L)/ (xULN)**	Histology	AMH (%)	AR (%)	Ref.
Prepubertal							
1	2 months	18.7	nd	Prepubertal NSPG	nd	nd	<i>Fernandez-Cancio et al, (2003)</i>
2	8 months	20.1	na	SGA	nd	nd	<i>Hadziselimovic et al, (2004)</i>
3	1	na	0.5	Prepubertal NSPG	100	nd	<i>Steger et al, 99</i>
4	1(14 months)	na	0.7	Prepubertal NSPG	100	nd	<i>Fernandez-Cancio et al, (2004)</i>
5	2	24.4	na	SGA	nd	nd	<i>Hadziselimovic et al, (2004)</i>
6	2	na	0.2	Prepubertal NSPG	100	nd	<i>Steger et al, 99</i>
7	4	29.8	na	SGA	nd	nd	<i>Hadziselimovic et al, (2004)</i>
8	7	na	na	SCO	45	nd	<i>Steger et al.(1996)</i>
9	9	38.7	na	low no of spermatogonia	nd	nd	<i>Hadziselimovic et al, (2004)</i>
10	10	na	0.1	Prepubertal NSPG	100	nd	<i>Steger et al,(1999)</i>
11	11	31.2	na	low no of spermatogonia	nd	nd	<i>Hadziselimovic et al, (2004)</i>

Postpubertal							
11	14	36	20	SGA	nd	nd	Johnson et al, (1986)
12	16	na	38.3	SCO, SGA	50, 85	nd	Steger et al, (1999)
13	16	34.5	4.5	NSPG	100	nd	Johnson et al, (1986)
14	16	29	11	SCO, SGA	nd	nd	Johnson et al, (1986)
15	16	225	8.7	SGA	nd	nd	Johnson et al, (1986)
16	17	na	20	SCO	nd	nd	Okon et al, (1980)
17	18	na	13.5	SCO, NSPG	100	nd	Steger et al, (1999)
18	18	na	9	SCO, NSPG	100	nd	Steger et al, (1999)
19	18	na	56	SCO	nd	nd	Okon et al, (1980)
20	18	39	5.5	Normal	nd	nd	Cantu et al, (1980)
21	25	42	na	Normal	nd	nd	Kuttenn et al, (1979)
22	35	33.5	32	Normal, SCO	nd	nd	Johnson et al, (1986)
23	45	na	na	Normal	nd	nd	Peterson et al, (1977)
24	65	38	40	SCO	nd	nd	Imperato-McGinley et al, (1980)
25	na	na	na	Normal	nd	nd	Imperato-McGinley et al, (1982)
our case	16	45	14.5	SGA, SCO, SSCR	8	92	present paper

*T/DHT ratio: testosterone/dihydrotestosterone ratio; the ratio cutoff for the diagnosis of 5 α -reductase type 2 deficiency was set at 10. FSH** (xULN): folliculo-stimulating hormone ratio between the measured value and the upper limit of normal for the corresponding FSH assay; nd, not determined; na, not available.
 prepubertal NSPG, prepubertal normal spermatogenic progression; SGA-arrest at the level of spermatogonia; SCO-Sertoli cell only; NSPG, normal spermatogenic progression; SSCR- severe Sertoli cell regression.

Table 9. Immunohistochemical detection of anti-Mullerian hormone (AMH) and androgen receptor (AR) within Sertoli cells in the presented patient with 5 α -reductase type 2 deficiency (index case) compared to prepubertal (n=11) and postpubertal (n=15) cases described in the literature.

Paper 4

Healthy birth after testicular extraction of sperm and ICSI from an azoospermic man with mild androgen insensitivity syndrome caused by an androgen receptor partial loss-of-function mutation.

Nathalie Massin, Hélène Bry, Lavinia Vija, Luigi Maione, Elisabeth Constancis, B. Haddad, Yves Morel, Frank Claessens, Jacques Young.

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As already mentioned, during fetal life and during the “mini-puberty” period (1 to 4 months postnatally), the transient coexistence of high concentrations of androgens and AMH, related to a physiological androgen insensitivity was explained by the absence of functional AR expression in SC during this developmental periods (Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009; Vija, Meduri et al. 2013).

Moreover, AMH expression significantly decreases in adulthood, in association with the androgenic stimulation of a functional AR present on Sertoli cells associated to the meiotic entry of spermatocytes.

In order to better apprehend the particularities of AMH expression in human pathologic situations associated with defectuous androgen signaling and infertility, I performed the immunohistochemical description of AR and AMH immunoexpression in an adult patient, with a MAIS, presenting for infertility related to azoospermia. The patient presented a phenotype of hypogonadism, but with high testosterone and androgen insensitivity index (ASI), associated with a point mutation (F754S) in the ligand-binding domain of the AR gene, leading to impaired transcription although unaffected ligand binding, suggesting that this AR residue is involved in allosteric communications between the AR-DBD and AR-LBD (Helsen, Dubois et al. 2012).

The histological and immunohistochemical analysis of the testicular biopsy obtained of this MAIS patient revealed rare (less than 5%) seminiferous tubules presenting spermatogenesis on testicular biopsy. In those few seminiferous tubules with ongoing spermatogenesis, AMH was repressed, even though the AR mutation affected all somatic cells, contrasting with the majority of seminiferous tubules devoid of spermatogenesis, where AMH was strongly expressed (Massin, Bry et al. 2012). This new evidence supports the importance of the indirect negative role of meiotic development of germ cells in AMH expression, as already reported (Rey, al-Attar et al. 1996).

For this patient, testicular sperm extraction, followed by intracytoplasmic sperm injection and embryos implantation, succeeded in obtaining two pregnancies at two year interval.

This success brings new hopes to these patients with androgen insensitivity syndromes, most frequently considered sterile, to have their biologic children.

ORIGINAL ARTICLE

Healthy birth after testicular extraction of sperm and ICSI from an azoospermic man with mild androgen insensitivity syndrome caused by an androgen receptor partial loss-of-function mutation

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Summary

Context The androgen receptor (AR) is essential for the development and maintenance of the male phenotype, and for spermatogenesis. Mutations in the AR gene cause a wide variety of androgen insensitivity syndromes (AIS), ranging from complete feminization to phenotypic males with infertility.

Objective We report the first birth achieved after intracytoplasmic sperm injection (ICSI) with sperm from an azoospermic man with an AR mutation associated with mild AIS (MAIS).

Patients and methods A couple with primary infertility was referred to our centre. The man had azoospermia with testicular hypotrophy and an undervirilized phenotype despite a normal plasma testosterone level. His androgen sensitivity index and serum anti-mullerian hormone (AMH) levels were elevated, pointing to AIS. Molecular analysis of the AR gene revealed a point mutation resulting in an F754S substitution (renumbered F755S in the 2012 McGill University AR gene database), in the ligand-binding domain of the protein, and further analysis indicated impaired receptor function.

Results After genetic counselling of the couple, oocytes were retrieved after controlled ovarian hyperstimulation, and sperm were obtained simultaneously by testicular extraction for ICSI. Nine embryos were obtained. Two were transferred and two were suitable for cryopreservation. A pregnancy was obtained and a healthy girl, carrying the F754S AR mutation, was born at 37 weeks of gestation. AR and AMH were detected by immuno-

histochemistry in the patient's testicular specimens. AMH immuno-staining was intense in tubules without spermatogenesis and weak in those with ongoing spermatogenesis.

Conclusion A healthy child can be obtained by testicular extraction and ICSI despite azoospermia in MAIS. The parents must be informed of the X-linked transmission of the mutation to their descendants. The relationship between AR signalling, testicular AMH expression and spermatogenesis in this patient is discussed.

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Introduction

Androgens are key steroid hormones responsible for the male phenotype. Their actions are mediated through a single androgen receptor (AR), which, upon ligand binding, translocates to the nucleus to regulate the expression of androgen-responsive genes.¹ Mutations in the AR gene, located on the X-chromosome, cause complete feminization of 46 XY individuals if they totally disrupt AR function, resulting in a full androgen insensitivity syndrome (AIS).^{2,3} Mutations that do not completely abrogate AR activity can cause a broad spectrum of milder AIS, from ambiguous genitalia (disorder of sex development, DSD) in newborns to idiopathic male infertility with no obvious phenotypic abnormalities.^{2–6} Spermatogenesis is often impaired (from azoospermia to oligospermia), and most men with a mutated AR are infertile.^{2–6}

Intracytoplasmic sperm injection (ICSI), a procedure in which a spermatozoon is injected into an ovum *in vitro*, is an effective treatment for male-factor infertility.⁷ However, nonobstructive azoospermia necessitates testicular extraction of sperm (TESE). We report the case of an infertile couple in which the man had

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Clinical Trial Registration Number: not applicable.

mild AIS with azoospermia. The couple underwent TESE and ICSI, resulting in the birth of a healthy girl and a current ongoing pregnancy.

Case report

A 29-year-old man and a 27-year-old woman were evaluated after 2 years of primary infertility. The man had a history of late puberty and bilateral gynaecomastia, which had been treated surgically at age 16 years. He had an undervirilized phenotype, with a gynecoid habitus, scant facial and pubic hair, a high-pitched voice, micropenis and bilaterally hypotrophic testes (9 and 10 ml; normal 15–30 ml) but no hypospadias. Genital ultrasound examination confirmed the hypotrophic testes (9.6 and 10.0 cm³) and also showed prostate hypotrophy (15 cm³; normal: 16–25 cm³) but normal vas deferens. Serum hormone levels, measured as previously reported,^{8–10} are shown in Table 1. Thus, despite this phenotype, plasma testosterone levels were at the upper limit of the normal range, basal serum estradiol was increased, and basal and GnRH (100 µg IV)-stimulated LH levels were high. The androgen sensitivity index (ASI) was elevated, pointing to AIS.⁶ Serum anti-mullerian hormone (AMH), measured as previously described,¹⁰ was also increased. Other investigated hormonal axes were normal. Analysis of four semen specimens showed azoospermia, with a low volume (2–2.5 ml; normal 3–5 ml). His karyotype was normal (46, XY). His wife's findings were normal.

Methods and results

All the participants (or parents) gave their written informed consent for hormonal exploration and/or genetic analyses, in

Table 1. Hormone values in the patient with partial AIS and the AR F754S mutation

			Normal range in men
Basal FSH	1.8	IU/l	3–7
GnRH-stimulated FSH (peak)*	2.8	IU/l	2.8–8.0
Basal LH	12	IU/l	3–8
GnRH-stimulated LH (peak)*	56	IU/l	<25
Total testosterone	24.5	nM	12–29
Bioavailable testosterone†	14.3	nM	3.4–14.5
ASI‡	294	IU × nM ²	6–139
Estradiol	160	pM	50–120
Inhibin B	107	pg/ml	90–350
AMH	76	pM	22–38

AIS, androgen insensitivity syndromes; AMH, anti-mullerian hormone; ASI, androgen sensitivity index; AR, androgen receptor.

*100 µg IV.

†Serum concentrations of bioavailable testosterone were calculated with a validated algorithm based on equations described by Vermeulen as previously reported,⁸ using measured total testosterone and SHBG concentrations, an assumed constant for albumin (43 g/l) and affinity constants of SHBG and albumin for testosterone.

‡Androgen sensitivity index (LH × T).⁶

keeping with the provisions of the French Bioethics Law and the Declaration of Helsinki and after approval by the Bicêtre Hospital ethic committee (Comité de protection des personnes, Ile de France, Hôpital Bicêtre).

Molecular analysis of the androgen receptor gene

Molecular analysis of the patient's AR gene was carried out as previously described¹¹ and revealed a nucleotide point mutation that caused a phenylalanine to serine substitution (F754S mutation) in the ligand-binding domain at position 754 (Fig. 1), based on the AR gene nucleotide and amino acid numbering used in ref. 11. However, we have to underline here that the McGill University AR gene database has very recently moved the numbering to the NCBI reference sequence NM_000044.2 and has consequently changed the amino acid numbers in the database, so this codon 754 will be numbered 755.¹² This recurrent¹³ mutation was absent in 500 control men. To exclude Kennedy's syndrome, the number of CAG repeats in exon 1 of the AR gene was determined as previously reported.¹⁴ This showed 19 CAG repeats (normal range 11–35).

Functional in vitro analysis of the androgen receptor F754S mutant

The functional analysis of AR F754S was performed in COS-7 cells transiently transfected with a pSG5-based expression vector carrying wild-type AR (pSG5AR) or the F754S AR mutant (pSG5ARmut), a TAT-GRE-based reporter gene and the beta-galactosidase gene, as described in.¹⁵

The F754S mutation did not affect steady-state AR expression in transfected cells (Fig. 2a), and the mutant AR showed ligand affinities similar to those of the wild-type AR (Fig. 2b). However, AR F754S was less potent than the wild-type receptor at transactivating a TAT-GRE-based luciferase reporter gene, even

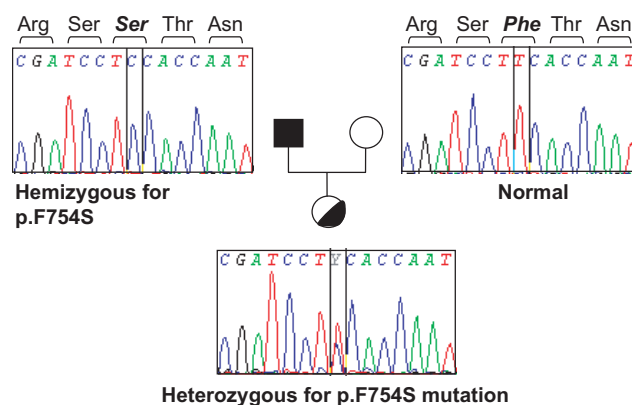


Fig. 1 Mutational analysis of the androgen receptor gene in the patient and his daughter. The patient carries a nucleotide point mutation affecting the ligand-binding domain of AR, with an amino acid change from phenylalanine to serine at position 754 (F754S). The father is hemizygous and the daughter is heterozygous for F754S. As indicated in the text, this amino acid is now numbered 755 in the McGill University AR gene database (see ref. 12).

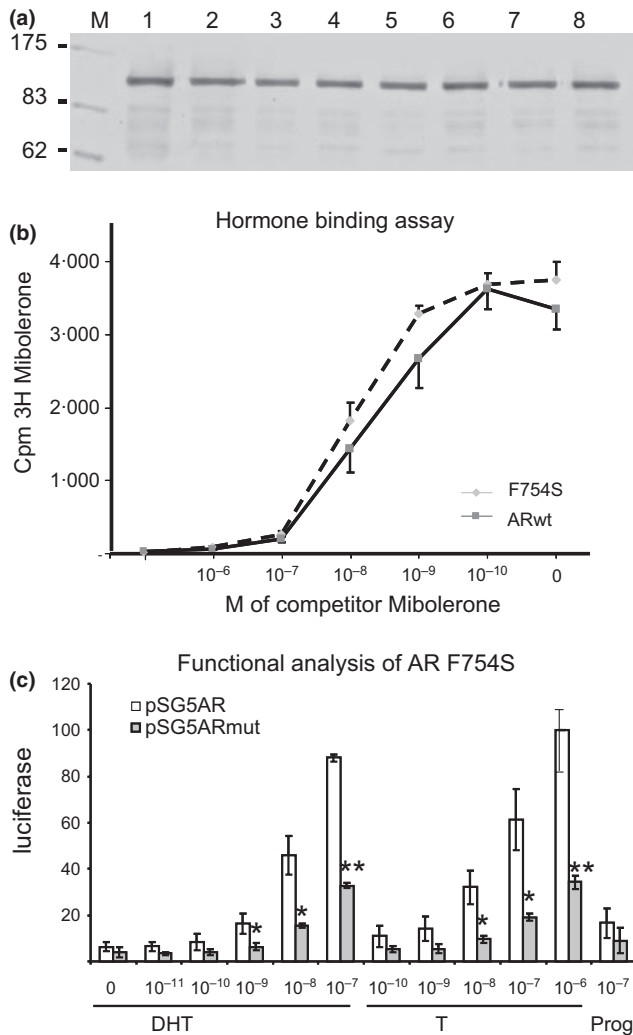


Fig. 2 *In vitro* functional analysis of F754S AR mutant. (a) Western blot analysis demonstrated equal expression of wt AR (lanes 1, 3, 5, 7) and the AR mutant (lanes 2, 4, 6, 8) independently of hormone treatment (no hormone: lanes 1 and 2; 10⁻¹⁰ M DHT: lanes 3 and 4; 10⁻⁹ M DHT: lanes 5 and 6; 10⁻⁸ M DHT: lanes 7 and 8). Molecular weight markers are indicated on the left. (b) The whole-cell hormone-binding assay was performed with transfected COS-7 cells, as described in.¹⁵ After binding of excess labelled mibolerone, increasing concentrations of cold competitor mibolerone were added as indicated on the X-axis. The amount of radioactive mibolerone retained by the cells after extensive washing is indicated on the Y-axis. Bars indicate the standard deviation. (c) Functional analysis of the F754S AR mutant. COS-7 cells were transfected as described in.¹⁵ Dihydrotestosterone, testosterone and progesterone were added to the medium at the indicated concentrations, and luciferase was assayed in cell extracts, with correction for transfection efficiency based on beta-gal activity. The values obtained with the wild-type AR at 10⁻⁶ M testosterone were attributed a value of 100. The error bars represent the standard deviation of three independent experiments performed in triplicate. **P* < 0.05; ***P* < 0.01 (nonparametric Wilcoxon test).

at higher hormone concentrations (Fig. 2c). The estimated loss of function was 60% at a testosterone concentration of 10⁻⁶ M, corresponding to a normal intratesticular concentration.

TESE and ICSI procedures

After genetic counselling, we offered the couple-assisted reproduction technology (ART). With their written informed consent, a first attempt of ICSI was made, with sperm obtained by bilateral testicular extraction. The woman underwent controlled ovarian hyperstimulation with long agonist protocol.¹⁶ After chorionic gonadotropin injection, 14 oocytes were retrieved by US-guided transvaginal needle aspiration. Bilateral testicular biopsy was performed simultaneously. Examination of fresh tissue at higher magnification in the ART laboratory identified enough mature living spermatozoa to fertilize each of the 13 mature oocytes. Nine oocytes were successfully fertilized. Two embryos were transferred into the uterine cavity 2 days after fertilization, and another two embryos were suitable for cryopreservation. The woman was given 400 mg of natural progesterone intravaginally each day for 2 weeks. On day 12 after embryo transfer, the serum chorionic gonadotropin concentration was 176 IU/l, and ultrasound examination at 4 weeks showed a singleton pregnancy. Ultrasound examination at 22 weeks showed a normal female foetus. At week 37, the woman gave birth to a healthy girl (3240 g and 48 cm). Genetic analysis of blood taken from the umbilical cord confirmed that the girl had inherited the F754S AR mutation.

The couple returned 2 years later for implantation of the two cryopreserved embryos. The women failed to get pregnant and a second ICSI attempt was carried out with the cryopreserved sperm from the TESE. The fresh cycle failed again, but a current pregnancy is ongoing with the cryopreserved embryos of the second ICSI. Ultrasound at 24 weeks showed a normal foetus.

Microscope and immunohistochemical analysis of the patient's testicular biopsy

Microscopic analysis of the patient's testicular biopsy sample obtained during the TESE procedure showed about 100 seminiferous tubules, half of which contained only poorly differentiated Sertoli cells (Fig. 3a, black arrow), while the remainder contained a few spermatogenic elements. About 5% of the tubules exhibited ongoing spermatogenesis, including late spermatid stages (Fig. 3b, white arrows).

The immunohistochemical status of the AR and AMH in the patient's testis, performed as previously described,¹⁷ is shown in Fig. 4. AR protein was detected in Sertoli cells nuclei as well as in Leydig and myoid peritubular cells (Fig. 4a). The AR staining was similar in Sertoli cells in tubules with ongoing spermatogenesis and in those without, whereas AMH immunostaining was weak (but detectable) in tubules with ongoing spermatogenesis (Fig. 4b, black arrows), and very intense in tubules without spermatogenesis (Fig. 4b, white arrows).

Discussion

The severity of AIS ranges from the testicular feminization syndrome to the undervirilized male syndrome.^{2,6} The patient described in this study was referred to our ART center for infertility because of azoospermia. The contrast between his clinical

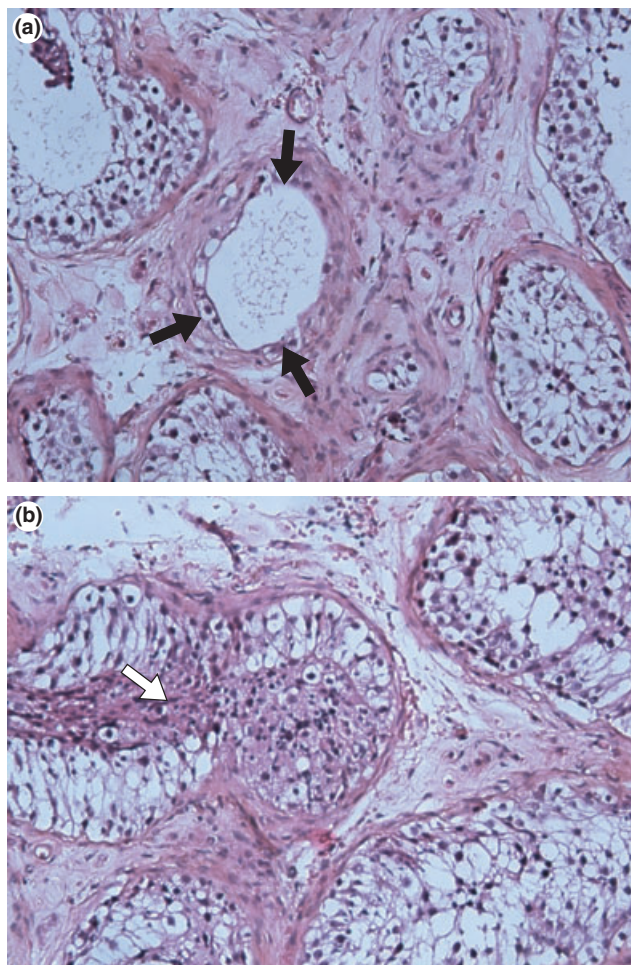


Fig. 3 Testicular biopsy of a man with an AR gene mutation. The biopsy specimen was fixed in Bouin solution and stained with haematoxylin–eosin–safran. Half of the tubules contained only poorly differentiated Sertoli cells (a, black arrow), while the remainder contained few spermatogenic elements. About 5% of the tubules exhibited ongoing spermatogenesis, including late spermatid stages (b, white arrows). Magnification $\times 20$.

signs of hypogonadism and his normal testosterone and LH levels led us to conduct further investigations. The ASI⁶ pointed to AIS. Molecular analysis of the patient's AR gene revealed a F754S mutation, and its role in the patient's phenotype was confirmed by *in vitro* studies showing that the mutant AR was functionally impaired at the transcriptional level. Although hormone binding was comparable to that of wild-type AR, transactivation capacity of the F754S mutant was clearly impaired when tested in COS-7 cells. The partial loss of function of F754S AR matched the patient's mild AIS phenotype, as in a phenotypically similar patient with AIS described by Tadokoro *et al.*,¹³ who also carried this recurrent mutation.

Despite the phenotypic variability associated with AR mutations, probably reflecting different degrees of disruption of AR interactions with DNA or proteins,^{2–6,12} major impairment of sexual activity¹⁸ and infertility are consistent features. Infertility in patients with AIS is related to the inability of their testes to

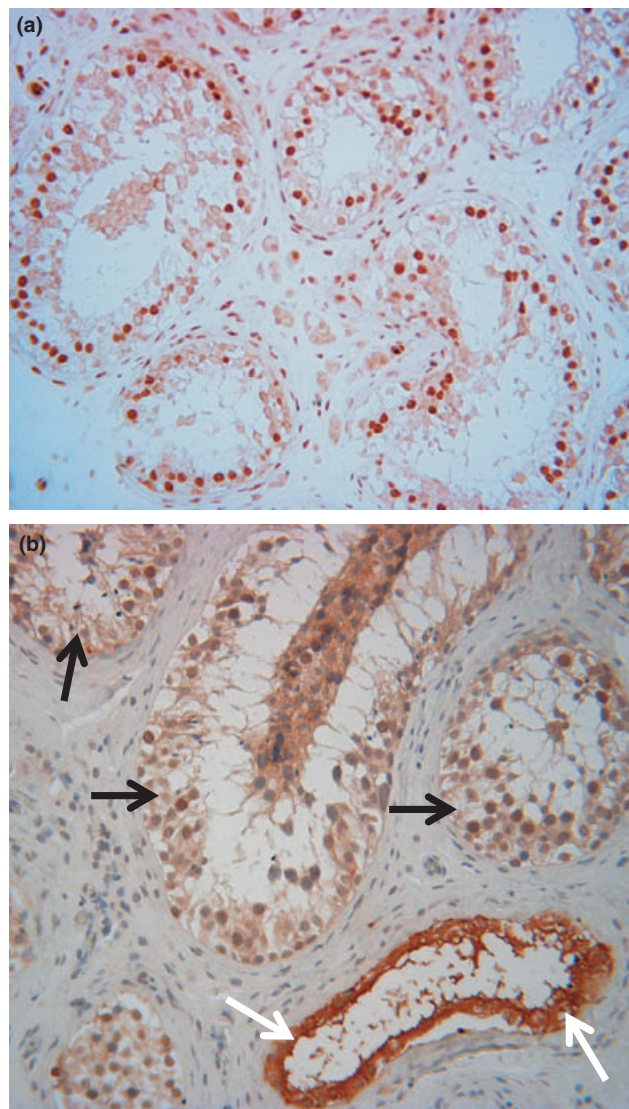


Fig. 4 Androgen receptor and anti-müllerian hormone (AMH) expression in the MAIS patient testis. (a) The biopsy specimen was fixed in Bouin solution and immunostained with the anti-AR antibody (Santa Cruz, sc-816) at 1/50 dilution. Seminiferous tubules exhibiting ongoing spermatogenesis (including late spermatid stages) showed a similar number of Sertoli cells with positive nuclear AR staining than seminiferous tubules without spermatogenesis (a) (magnification $\times 20$). (b) The biopsy specimen was fixed in Bouin solution and immunostained with the AMH antibody (kindly provided by Dr Jean Yves Picard) at 1/4000 dilution.¹⁷ Biopsy fragments were also immunostained with haematoxylin to identify nuclei and the stage of spermatogenesis. Seminiferous tubules containing ongoing spermatogenesis (including late spermatid stages) contained weak, but detectable, Sertoli cells AMH-positive immunostaining (Fig. 4b, black arrows), whereas AMH was very abundant in seminiferous tubules without spermatogenesis (b, white arrows) (magnification $\times 20$).

produce sperm cells.^{4–6,17–19} Many studies in rodents, nonhuman primates and humans have shown the crucial role of testicular androgen signalling for the initiation of spermatogenesis. Moreover, interruption of spermatogenesis in the testes of mice with AR knock-out specifically in Sertoli cells, as reported by

independent teams,²⁰ shows that it is in fact the expression of a functional AR by this cell type that is crucial for this process. More recent work has shown that the integrity of the DNA-binding domain of AR expressed by Sertoli cells is necessary for the inductive effect on spermatogenesis, suggesting the latter is mediated by classical genomic mechanisms.²¹

It is important to note that the testicular histology of the patient described here was heterogeneous, most seminiferous tubules showing no spermatogenesis but a small minority (<5%) showing complete spermatogenesis. This heterogeneity has previously been described in almost all cases of nonobstructive azoospermia²² and highlights the complex regulation of testicular sperm production. In this patient, the similar AR expression in both seminiferous tubules with or without ongoing spermatogenesis indicates that this histological heterogeneity is not explained by a simple reduction in the AR protein availability.

Despite rare cases of fatherhood,^{23,24} almost all men with partial AIS are considered to be sterile by many paediatricians and endocrinologists and are usually advised to consider adoption or the use of donor sperm. Pharmacological restoration of AR function with testosterone has been reported in men with minimal AIS,^{25,26} but it is rarely used and usually unsuccessful.

Intracytoplasmic sperm injection is an effective option for severe male-factor infertility, but it requires at least one living spermatozoon. We report the first case of successful TESE and ICSI despite functional impairment of the male partner's AR. In men with nonobstructive azoospermia, spermatozoa can sometimes be recovered from a testicular biopsy sample, but there is no reliable way of identifying men in whom sperm will be found.²⁷ Partial AIS can raise the issue of sex assignment at birth.¹⁸ In such cases, paediatric endocrinologists should be aware that male assignment does not necessarily lead to untreatable sterility. Testicular extraction of sperm can be an effective option, but, if the man or the woman has a genetic anomaly, then the couple should first receive genetic counselling and a preimplantation diagnosis should be proposed when possible and available. As the AR gene is located on the X-chromosome, AIS is an X-linked inheritable disorder. The parents should be told that their offspring will be normal, except that daughters will carry the father's mutation and might transmit AIS to their sons. Because of the lack of a strict genotype–phenotype correlation, at least in mild forms of AIS,^{2–4,11} the phenotypic consequences of the mutation in offspring cannot be reliably predicted.

Data from several laboratories suggest that AR point mutations contribute to about 2% of cases of male infertility.^{5,6,28–32} Infertile men with azoospermia or severe oligospermia and other clinical manifestations of AIS such as cryptorchidism, gynecomastia, low virilization or hypospadias should be systematically screened for AR gene mutations.³³ The ASI, calculated by multiplying the LH and testosterone plasma levels, has been described as a sensitive marker for identifying patients at risk of AR gene mutations.⁶ However, no clinical or hormonal features have been consensually proposed to reliably preselect patients at a higher risk of AR gene mutations.^{28–32} Serum AMH assay might be useful for identifying such azoospermic patients with AR mutations. Indeed, the patient described here had a high AMH

level, as previously described in patients with subnormal testicular androgen signalling due either to decreased production of testicular testosterone secondary to low gonadotropin levels^{10,17} or to testosterone insensitivity, as in subjects with AIS.^{17,34} AMH expression, as detected by immunohistochemistry, was effectively high in Sertoli cells of seminiferous tubules without spermatogenesis, which predominated in our patient. Interestingly, AMH expression was low in the minority of seminiferous tubules that exhibited advanced spermatogenesis, even though the AR mutation affects all somatic cells. This further supports the importance of the indirect negative role of meiotic development of germ cells in AMH expression by Sertoli cells, as suggested by Rey *et al.*³⁵

In conclusion, we report the first case of paternity after testicular extraction of sperm-intracytoplasmic sperm injection in a patient with androgen receptor mutation, giving potential hope to these patients with androgen insensitivity syndromes, usually considered to be sterile, to father children.

Acknowledgments

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Competing interests/financial disclosure

Nothing to declare.

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Androgenic regulation in Sertoli cells

Discussion, **conclusions and perspectives**

General discussion, conclusions and perspectives

Sertoli cells (SC) are key players in the androgen regulation of spermatogenesis, *via* the androgen receptor (AR)-mediated signaling. In adulthood, SC create and maintain a specific environment favorable for the entry in meiosis of primary spermatocytes (Sharpe, McKinnell et al. 2003), being also involved in the hormonal regulation of spermatogenesis.

The androgen receptor (AR) is a ligand-activated transcription factor that is crucial for male sex development, virilization and regulation of spermatogenesis. AR displays distinct expression profiles in SC during testis development, tightly correlated to the stages of spermatogenesis. AR is absent in neonatal SC while its expression becomes maximal at puberty and in adulthood, concomitantly with the progression of spermatogenesis, unlike constant AR expression in Leydig and peritubular myoid cells in both prepubertal and postpubertal testicular developmental stages.

In adult human testis, androgens initiate spermatogenesis, a complex cellular differentiation process that does not occur in fetal and newborn testis despite sustained levels of intratesticular androgen production, due to a physiological postnatal insensitivity to testosterone, explained by the absence of AR in SC in neonatal testis (Rey 2003; Chemes, Rey et al. 2008; Boukari, Meduri et al. 2009).

The general objective of this work was to decipher several molecular mechanisms by which AR regulates physiological functions in SC and supports germ cell development and spermatogenesis.

Although studies performed on various cell-selective AR deficiency animal models brought interesting data related to potential androgen-regulated genes and androgenic mechanisms of regulation in a testicular environment, it still remains difficult to identify direct androgen-dependent target gene in SC, due to the complexity of the paracrine and metabolic pathways existing among the multiple cellular components of the testis.

For this purpose, in order to provide better comprehension and study the molecular mechanisms related to physiological and developmental aspects of androgenic regulation of the AR in Sertoli cells, we characterized and studied a novel murine mature immortalized Sertoli cell line, called ST38c. Unlike all other immortalized Sertoli cell models, ST38c Sertoli cells harbor substantial expression of endogenous AR that conserves its androgen-dependent transcriptional activation and exhibits agonist-dependent transcriptional and posttranslational regulation. We also identified by microarray studies, 72 up-regulated and 74 down-regulated genes, while some of them were validated by real-time quantitative PCR.

Interestingly, functional annotation clustering analysis (DAVID) revealed that the up-regulated genes preferentially influenced regulation of transcription and apoptosis, whereas androgen down-regulated genes differentially impacted cytokine and various extracellular signaling pathways (for further details, see the Array Express database (European Bioinformatics Institute, <http://www.ebi.ac.uk/arrayexpress/>), where the microarray was registered with the accession number E-MTAB-1732).

We first used this cellular model in order to test the hypothesis that AMH suppression in mature Sertoli cells would be directly androgen and AR mediated.

As a matter of fact, my PhD predecessor, Kahina Boukari demonstrated, by means of cellular transfection in an immature Sertoli cell model, SMAT1, transiently transfected with hAR expression vector, that the negative androgenic regulation on AMH is mediated by AR. ST38c, as a cellular model harbouring an important and stable endogenous expression of AR represents an optimal study tool in order to better understand this aspect.

However, transfection assays using AMH promoters were not successful, as this cell line presents a particular resistance to transfection, including for techniques such as, electroporation or viral infection. Furthermore, successful assays of AR mRNA and protein silencing, using a siRNA approach, didn't show any AMH augmentation of expression after AR inhibition. These data, although, preliminary, corroborated with recent publications, allow concluding that AMH suppression in postpubertal Sertoli cells is a complex process, which does not imply only AR, in an independent manner.

However, ST38c cells continue to represent a very interesting cellular model which may be used for further studies, such as the effects of endocrine disruptors on Sertoli cells and spermatogenesis. As ST38c do not express FSH-R, we tried to co-culture them with primary cultures of murine germ cells. Although, we didn't succeed in revealing any FSH-R or FSH responsiveness, we believe that further studies are needed in order to understand or overcome this problem.

Another hypothesis related to the factors that might be responsible for the physiological androgen resistance in the neonate is related to a differential expression and contribution of several AR co-regulators, which would modulate AR transactivating capacity.

Although, in early August 2013, in the NURSA registry there were more than 300 AR coregulators, the majority have not yet been examined neither in animal models nor in human pathologies, therefore their true physiological relevance in normal and pathological conditions remains to be established.

Therefore, another objective of this work was the study of the expression of two AR coregulators during murine and human ontogeny and postnatal development, as well as in pathologies associated with androgen action or AR impairment (such as: androgen insensitivity syndromes, representing a human model of AR invalidation; congenital hypogonadotropic hypogonadism, which is a situation of reduced testicular androgenic exposure and 5-alpha reductase type 2 deficiency, which is a situation of reduced testicular exposure to dihydrotestosterone).

We selected two coregulators, SRC-2 and HBO1, as bibliographic research revealed them as interesting candidates, exhibiting relevant characteristics and implications for testicular development and fertility.

We performed a descriptive analysis of testicular specific expression of murine and then human SRC-2 and HBO1 from fetal life to adulthood. Moreover, coregulator expression has also been studied using transcript qPCR analysis and immunohistochemistry, in human testicular samples with androgen signaling defects (androgen insensitivity and cases of hypogonadotropic hypogonadism and 5 α -reductase type 2 deficiency).

We provided the cartography of SRC-2 and HBO1 expression during human testicular postnatal different stages. SRC-2 presented a stable expression contrasting with the progressive evolution profile of the AR signaling, suggesting that Sertoli SRC-2 expression was independent of the androgen signaling. The lack of coexpression of AR and SRC-2 during the neonatal period of development suggests that SRC-2 might exert important coactivating control of other nuclear receptors, such as ER β , given the physical interaction, already reported, between ER β and SRC-2 (Warnmark, Almlof et al. 2001).

Interestingly, HBO1 and AR presented a similar maturation profile for expression during human testis development. This temporal and positively correlated expression pattern suggests that HBO1 expression would be related to a functional AR signaling in the Sertoli cell. In order to validate this hypothesis we studied HBO1 transcript expression in hAR transfected SMAT1 cells and observed that androgen stimulation significantly increased HBO1 expression. Moreover, we observed that HBO1 was not immunodetected in testicular biopsies obtained from 7 patients with complete androgen insensitivity syndrome and deleterious AR mutations. We consider that it would be interesting to assess by molecular biology techniques (CHIP) whether ST38c cells, or HBO1 promoter constructs, respectively, express androgen responsive elements (ARE). Further studies are needed to understand how HBO1 impacts androgenic regulation of spermatogenesis.

As we observed when performing double immunofluorescence labeling, HBO1 is a nuclear protein not only expressed in Sertoli cells, but also in spermatogonia. We propose that the physiological role of HBO1 in germ cells would be distinct from that exerted in Sertoli cells given that it is well established that AR is not expressed in spermatogonia in humans. We propose that HBO1 might affect other steroid receptor signaling such as PR or ER although their expression in human germ cells remains still controversial. Alternatively, given that HBO1 plays an important role on DNA replication and regulated cellular proliferation, its expression in spermatogonia could be related to the fact that spermatogenesis is represented by a series of cellular mitotic and meiotic divisions. It would be interesting to explore whether HBO1 could be used as a marker for spermatogonia.

In vitro, transactivation experiments showed that SRC-2 behaves as a potent AR coactivator in a Sertoli cell like context while cotransfection of HBO1 with AR markedly reduced DHT-stimulated AR transactivation, consistent with its corepressor property. Collectively, these findings suggest that the coactivating activity of SRC-2 on AR function in the Sertoli cell could play a pivotal role for the sustained production of spermatozooids in human adults, while HBO1 corepressor activity in Sertoli cells would be a molecular factor attenuating Sertoli cell activation in response to very high intra-testicular testosterone exposure (Coviello, Matsumoto et al. 2005). Alternatively, HBO1 expression in adult Sertoli cells could determine a partial testicular resistance to testosterone and thus explain the necessity of very high intratesticular testosterone levels needed to induce spermatogenesis in men (Schaison, Young et al. 1993; Coviello, Matsumoto et al. 2005; Jarow and Zirkin 2005).

Generally, these results indicate that modulation of SRC-2 and HBO1 abundance within the Sertoli cells might constitute molecular markers for altered spermatogenesis. Given the opposite coregulatory properties of SRC-2 and HBO1 on AR signaling, it would be an interesting perspective to examine whether infertile patients might harbor either loss of function or gain of function deleterious mutations of these two coregulators.

A third objective was related to the study of the relative contribution of testosterone versus dihydrotestosterone to spermatogenesis maintenance and to AMH suppression in the adult testis. Therefore I analyzed the AR and AMH expression in postpubertal testis of a case of 5ARD. There was a heterogeneity of seminiferous tubules aspects with 92% of tubules with a mature aspect expressing AR and lacking AMH. These results suggest that intratesticular testosterone seems sufficient to repress AMH, when AR is expressed, in spite of the 5ARD deficiency, so independent of the conversion of testosterone into dihydrotestosterone. We also presented a review of the literature related to histological aspect of postpubertal testis in

5ARD, as well as an analysis of the potential perspectives for fertility (mainly TESE with ICSI) for these patients.

In conclusion, this work brings up interesting and original data related to human testicular physiology and pathologies related to androgen/AR impairment.

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Androgen Receptor signaling in Sertoli cells

Abstract

Sertoli cells (SC) have essential roles in the androgen regulation of spermatogenesis, *via* the androgen receptor (AR)-mediated signaling. This work aimed at identifying the molecular mechanisms related to the androgenic regulation of the AR and its molecular partners in Sertoli cells during different testicular developmental stages.

We first characterized and studied a novel murine mature immortalized Sertoli cell line, called ST38c, which harbors substantial expression of endogenous AR, conserves its androgen-dependent transcriptional activation and exhibits agonist-dependent transcriptional and posttranslational regulation, as well as posttranslational stability.

We used this cellular model in order to test the hypothesis that anti Müllerian Hormone (AMH) suppression in mature Sertoli cells would be directly androgen and AR mediated.

Next, we hypothesized that the physiological androgen resistance in the neonate would also be related to the differential expression of several AR coregulators. Therefore, we analysed the differential expression and contribution of two AR co-regulators (SRC-2 and HBO1) during human testicular ontogeny, as well as in pathologies associated with androgen action or AR impairment (such as androgen insensitivity syndromes).

Using *in vitro* transfection assays, we showed that SRC-2 is an AR coactivator while HBO1 is an AR corepressor in Sertoli cell models. We provided the cartography of SRC-2 and HBO1 expression during human testicular postnatal different stages and showed that SRC-2 presented a stable expression contrasting with the progressive evolution profile of the AR signaling, suggesting that Sertoli SRC-2 expression was independent of the androgen signaling. Interestingly, HBO1 and AR presented a temporal and positively correlated maturation profile, suggesting that HBO1 expression would be related to a functional AR signaling in the Sertoli cell. Moreover HBO1 expression is induced by androgens in the presence of the AR. Unlike SRC-2, HBO1 is not only expressed in Sertoli cells, but also in spermatogonia, being an interesting germ cell marker.

Finally, we also studied AR and AMH immunoexpression in postpubertal cases of 5- α reductase type 2 deficiency and minimal androgen receptor resistance, in respect with the spermatogenesis status of seminiferous tubules, and androgen induced AMH suppression in order to assess the differential contribution of testosterone versus dihydrotestosterone and gather more information about the fertility perspectives in this particular pathologies.

Key words: Androgen receptor, human testis, androgen receptor coregulators, SRC-2, HBO1, 5- α reductase type 2 deficiency, androgen insensitivity syndromes

Signalisation androgénique dans les cellules de Sertoli

Résumé

Les cellules de Sertoli (CS) jouent des rôles essentiels pour la régulation de la spermatogenèse, via la signalisation modulée par le récepteur aux androgènes (RA). Les objectifs de ce travail ont été d'identifier les mécanismes moléculaires de la régulation androgénique et des rôles des partenaires moléculaires dans la régulation androgénique des cellules de Sertoli pendant le développement testiculaire, du fœtus à l'âge adulte, pendant différentes stades du développement.

Nous avons caractérisé et étudié une nouvelle lignée immortalisée, mature, de CS, ST38c, présentant une expression substantielle de RA endogène, une activation transcriptionnelle du RA induite par les androgènes, ainsi qu'une régulation et une stabilisation de la protéine RA par des mécanismes post traductionnels. Ce modèle a été utilisé afin de tester l'hypothèse que la suppression de l'hormone antimüllérienne (AMH) est modulée directement par les androgènes, via la RA, dans les cellules matures de Sertoli. En parallèle, nous avons testé l'hypothèse que la résistance physiologique aux androgènes du nouveau-né est liée à l'expression différentielle de quelques corégulateurs du RA. Ainsi, nous avons analysé l'expression et la contribution de deux corégulateurs du RA (SRC-2 et HBO1) pendant l'ontogenèse testiculaire humaine et pour des pathologies liées au dysfonctionnement de l'action des androgènes ou du RA (syndromes d'insensibilité aux androgènes).

Nous avons démontré, après des essais de transfection *in vitro*, que SRC-2 est un coactivateur, alors que HBO1 est un corepresseur du RA dans un modèle de cellules de Sertoli. Nous avons cartographié l'expression testiculaire humaine de SRC-2 et HBO1, pendant différents stades du développement pré et postnatal et nous avons démontré que SRC-2 présente une expression stable, contrastant avec le profil d'expression différentielle, progressive avec l'âge de RA dans la cellule de Sertoli, suggérant que l'expression du SRC-2 est indépendante de la signalisation androgénique. Par contre, HBO1 et le RA présentent un profil de maturation et d'expression temporelle corrélé, suggérant que l'expression du HBO1 serait liée à une signalisation du RA fonctionnelle dans les cellules de Sertoli. Nous avons aussi démontré que l'expression du HBO1 est induite par les androgènes en présence du RA. Contrairement au SRC-2, HBO1 est non seulement exprimé dans les cellules de Sertoli, mais aussi dans les spermatogonies, pouvant représenter un marqueur potentiellement intéressant des cellules germinales.

Enfin, nous avons aussi étudié l'immuno-expression testiculaire de RA et AMH chez des patients post pubères avec un syndrome de déficit en 5 α -réductase type 2, et insensibilité minimale aux androgènes (MAIS), afin d'étudier la contribution des androgènes (testostérone versus dihydrotestostérone) pour la spermatogenèse, la répression de l'AMH ainsi que pour mieux comprendre les perspectives de fertilité chez ces patients.

Mots clefs: Récepteur aux androgènes, testicule humain, corégulateurs du récepteur aux androgènes, SRC-2, HBO1, déficit en 5 α -réductase de type 2, syndrome d'insensibilité aux androgènes