SEXUAL STEROID REGULATION OF SPERMATOGENESIS: NEW ACTORS ENTER THE STAGE

Sandra Filipa Tomás Sequeira Laurentino

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Orientadores: Prof. Doutor José Eduardo Brites Cavaco
(Faculdade de Ciências da Saúde, Universidade da Beira Interior)
Prof. Doutora Sílvia Cristina da Cruz Marques Socorro
(Faculdade de Ciências da Saúde, Universidade da Beira Interior)

Co-orientador: Prof. Doutor Mário Manuel da Silva Leite Sousa
(Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto)

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“Displeasure is the source of all great discoveries.”

Kim Nasmyth

Aos meus pais e avós.

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Resumo

A espermatogénese é o processo biológico que resulta na formação e emissão dos gâmetas masculinos, *i.e.* os espermatozóides, o qual requer uma estricta regulação hormonal para que possa decorrer com sucesso. A importância dos androgénios (como a testosterona e a 5α-di-hidrotestosterona) para a regulação da espermatogénese é bem reconhecida. Porém, mais recentemente, a importância dos estrogénios (como o 17β-estradiol) também tem sido demonstrada. Estes esteróides sexuais actuam através de factores de transcrição activados por ligando, os receptores de estrogénios α (ERα) e β (ERβ) e o receptor de androgénios (AR), respectivamente. A maioria das acções destas hormonas são alcançadas através da regulação de genes-alvo. Os dois receptores de estrogénios têm efeitos diferentes e por vezes opostos na regulação dos seus genes-alvo e a acção estrogénica irá depender da interacção entre eles, quando co-expressos na mesma célula. A expressão de ERα e ERβ no testículo humano tem sido objecto de forte debate, e uma resposta definitiva sobre se apenas o ERβ ou ambos os ERs são expressos seria fundamental para a compreensão das acções dos estrogénios na espermatogénese humana. Vários variantes de ERα e ERβ gerados por processamento alternativo de RNA mensageiro foram descritos no testículo, desempenhando papéis importantes na regulação dos respectivos receptores protótipo. Em contraste, apenas um variante do AR foi descrito até agora no testículo humano, apesar de se esperar a existência de mais variantes responsáveis por acções reguladoras ou não-clássicas. A definição do transcriptoma regulado pelos estrogénios e androgénios é também de suma importância para a compreenção dos papéis desempenhados pelas hormonas esteróides sexuais no testículo. Na presente tese, os objectivos foram clarificar a expressão do ERα e ERβ no testículo humano, procurar variantes alternativos do AR, e identificar e caracterizar novos genes regulados por estrogénios e androgénios com potencial importância no controlo da espermatogénese. Os resultados apresentados demonstram inequivocamente que tanto o ERα como o ERβ são expressos no testículo humano e clarificam a sua distribuição celular. Foi confirmada a existência de formas alternativas do AR no testículo, com a identificação de quatro novos variantes, dois deles apresentando-se conservados ao longo da linha evolutiva dos vertebrados, indicando uma importância funcional. No que se refere ao transcriptoma regulado pelos esteróides sexuais, foram identificados dois novos genes, um regulado por estrogénios e outro por androgénios. O inibidor da apoptose e modulador da resposta a danos no DNA Aven foi identificado como um novo gene-alvo para os estrogénios no testículo. Pela primeira vez a sua expressão e distribuição celular foi caracterizada no testículo humano e de rato, encontrando-se presente em células de Sertoli e células germinativas. Talvez mais importante ainda, demonstrámos que os níveis de expressão de Aven no testículo humano estão positivamente correlacionados com a qualidade da espermatogénese. Relativamente ao gene regulado por androgénios, a Regucalcina (RGN), foi caracterizada a sua expressão em resposta à 5α-di-hidrotestosterona, e demonstrou-se que se localiza em todos os tipos celulares do testículo humano e de rato. A RGN está envolvida no controlo do cálcio intracelular e na regulação da proliferação celular e apoptose, processos cuja regulação tem

uma importância fulcral para o controlo da espermatogénese. Os estrogénios e androgénios são reconhecidos factores de sobrevivência das células germinativas e regulam os mecanismos de controlo da apoptose testicular. Pensamos que tanto o Aven como a RGN estarão envolvidos nos mecanismos de sobrevivência das células germinativas, que são controlados por estrogénios e androgénios. Em conclusão, esta tese contribuiu para aumentar o conhecimento acerca das acções estrogénicas e androgénicas no testículo. A identificação de "novos actores" no "elenco" do "drama" que é o controlo hormonal da espermatogénese abre novas perspectivas de abordagem na investigação da espermatogénese nos mamíferos e permite clarificar as bases moleculares da infertilidade masculina.

Resumo Alargado

A espermatogénese é um processo complexo que culmina com a produção dos gâmetas masculinos, os espermatozóides. Este processo deve ser fortemente regulado de modo a que ocorra normalmente. O principal mecanismo de controlo da espermatogénese é o eixo hipotálamo-pituiária-gónada. Em síntese, o hipotálamo produz a hormona libertadora de gonadotrofinas que actua na pituitária induzindo a libertação de hormona luteinizante (LH) e hormona estimuladora de folículo (FSH). A LH e FSH chegam ao testículo através da circulação, onde vão actuar em tipos celulares distintos. A LH actua nas células de Leydig (LC) que se localizam no espaço intersticial, estimulando o processo de esteroidogénese ou seja, produção de esteróides sexuais. A FSH actua nas células de Sertoli (SC), localizadas nos túbulos seminíferos (a unidade fundamental da espermatogénese), estimulando-as a produzir diversos factores necessários à manutenção das células germinativas. As LC produzem diversos esteróides sendo o principal a testosterona, a qual pode ser convertida nos tecidos alvo num androgénio mais potente, a 5α-di-hidrotestosterone pela enzima 5-α-reductase, ou no estrogénio 17β-estradiol pela enzima P450 aromatase, que também é expressa nas LC, para além de outras células testiculares. Estrogénios e androgénios actuam através de receptores membros da super-família dos receptores nucleares, que funcionam como factores de transcrição activados por ligando. Estão descritos dois receptores diferentes para os estrogénios, o receptor de estrogénios α (ERα) e o β (ERβ), enquanto que para o receptor de androgénios (AR) existe apenas uma forma. O mecanismo de acção dos estrogénios e androgénios envolve basicamente os mesmos passos. O esteróide entra na célula, onde se liga ao seu receptor, que por sua vez irá sofrer alterações conformacionais que possibilitam a sua translocação para o núcleo. Aí, os receptores irão ligar-se sob a forma de dímeros à região reguladora dos genes-alvo, estimulando ou reprimindo a sua transcrição. Por actuarem através deste mecanismo, a maioria das acções dos estrogénios e androgénios são causadas pela regulação da expressão de genes-alvo o que se traduzirá na síntese de novas proteínas responsáveis pelos efeitos atribuídos a estas hormonas. Apesar de apresentarem grande homologia, o ERα e ERβ têm efeitos diferentes e até por vezes opostos nos seus genes-alvo, para além de que podem formar heterodímeros, estando por isso a resposta estrogénica dependente dos níveis de expressão de cada um dos receptores e também da interacção entre eles, quando expressos em conjunto na mesma célula. Por isso é determinante, conhecer os níveis de expressão e a distribuição celular dos dois tipos de ER para cada tecido específico. Tem havido um grande debate acerca da expressão de ERα e ERβ no testículo humano. Alguns autores sugerem que apenas o ERβ é responsável pelas acções estrogénicas neste tecido, e como tal na espermatogénese, apesar de os dados obtidos em roedores e também a descrição de um homem com uma disrupção do gene do ERα sugerirem o contrário. A clarificação da expressão e distribuição dos ERs no testículo humano é fundamental para compreender a acção dos estrogénios sobre genes-alvo, principalmente tendo em conta o papel fundamental dos estrogénios na espermatogénese. Os receptores de esteróides sexuais têm frequentemente variantes originados por processamento alternativo de RNA mensageiro. No

caso dos ERs já foi descrita uma grande quantidade destes variantes, os quais se encontram presentes em vários tecidos, entre os quais o testículo. A maioria destes variantes tem uma função de regulação da actividade dos receptores protótipo correspondentes. Em contraste, até ao momento apenas foi descrito um variante do AR no testículo, o qual apresenta funções reguladoras do AR protótipo. Sendo assim, prevê-se que existam por identificar no testículo outros variantes do AR, que poderão desempenhar importantes funções reguladoras ou ser responsáveis por acções não-clássicas, isto é, não relacionadas com a regulação da expressão génica. Apesar de ser aceite que as principais acções de androgénios e estrogénios são causadas pela regulação da expressão de genes-alvo, ainda não se conhece totalmente a pletora de genes regulados por estas hormonas com possível importância na espermatogénese. No entanto o estudo destes genes reveste-se de fundamental importância, já que permite conhecer mais detalhadamente os papéis desempenhados pelas hormonas esteróides na espermatogénese e poderá indicar potenciais alvos terapêuticos e de diagnóstico que ajudem a decifrar as causas da infertilidade masculina idiopática. Os principais objectivos desta tese foram portanto, clarificar a expressão do ERα e ERβ no testículo humano tal como a sua distribuição celular, procurar variantes do AR com importância funcional no testículo, identificar e caracterizar novos genes regulados por estrogénios e androgénios com potencial importância na regulação da espermatogénese. Os resultados apresentados confirmam inequivocamente que tanto ERα como ERβ são expressos no testículo humano. Para além disso a sua distribuição celular também foi clarificada sendo que o ERα encontra-se localizado nas LC, SC, espermatogónias, espermatócitos, espermátides redondas e alongadas, enquanto o ERβ é expresso nas mesmas células excepto SC e espermatogónias. No que se refere ao AR, foram identificados quatro variantes em testículo humano, sendo que dois deles também se encontravam presentes em fígado, pulmão, rim e coração humanos. Para além disso procurou-se saber se algum destes novos variantes do AR no testículo humano seriam expressos no testículo de outras espécies. Dois dos variantes encontrados no testículo humano encontravam-se de facto conservados ao longo da linha evolutiva de vertebrados, indicando uma potencial importância funcional. No que se refere a genes-alvo de esteróides no testículo, foram identificados e caracterizados dois novos alvos; um novo gene regulado por estrogénios, o Aven, e um novo gene regulado por androgénios, a Regucalcina (RGN). O Aven é um inibidor da apoptose que também actua como modulador da resposta a danos no DNA. Os resultados demonstram que em túbulos seminíferos de rato cultivados *ex vivo* os estrogénios estimulam a expressão do Aven. A distribuição celular do Aven no testículo humano e de rato foi caracterizada, verificando-se que se localiza principalmente nas SC e espermatócitos e menos nas espermatogónias. Também se verificou que a expressão de Aven se correlaciona com a qualidade da espermatogénese, apresentando um máximo quando a espermatogénese se apresenta conservada, tendo por isso um potencial no estudo da infertilidade masculina. O facto de o Aven ser um poderoso inibidor da apoptose parece indicar que a disrupção da espermatogénese pode ter na sua génese um aumento da morte de células germinativas, o que está de acordo com estudos anteriores. Por outro lado demonstrou-se que a expressão de RGN em túbulos seminíferos de rato cultivados *ex vivo* é

estimulada por androgénios. Verificou-se ainda que a sua distribuição celular no testículo é extremamente ubíqua, sendo expressa em todos os tipos de células tanto em testículo humano como no de rato. A RGN é um regulador da concentração intracelular de cálcio que actua através da modulação da actividade de canais e transportadores de cálcio na membrana celular e do retículo endoplasmático e mitocôndria. Já foi também demonstrado que a RGN desempenha um papel na proliferação celular e controlo da apoptose. Sabe-se que a sobrevivência das células germinativas está dependente de uma correcta acção de estrogénios e androgénios. Assim sendo, os dados apresentados sugerem que, tanto o Aven como a RGN, poderão estar envolvidos nos mecanismos de regulação da sobrevivência das células germinativas masculinas, mediados pelos esteróides sexuais. Em conclusão, esta tese mostra dados importantes para decifrar os mecanismos de acção de estrogénios e androgénios no testículo e na espermatogénese através dos seus receptores, ERα, ERß e AR e dos genes regulados por estes dois grupos de hormonas. Para além disso são apresentados "novos actores" que podem desempenhar papéis importantes na regulação da espermatogénese pelos esteróides sexuais, o que abre novas linhas de investigação no estudo da espermatogénese e consequentemente da fertilidade masculina.

Abstract

Spermatogenesis, the process of male gamete (*i.e.* spermatozoa) production, requires tight hormonal regulation in order to proceed successfully. The importance of androgens (like testosterone and 5α-dihydrotestosterone) to the regulation of spermatogenesis is well recognized. However, more recently, the importance of estrogens (like 17β-estradiol), has also been demonstrated. These sexual steroids act through ligand-activated transcription factors, estrogen receptors α and β (ERα and ERβ) and androgen receptor (AR), respectively. Most actions of these hormones are achieved through the regulation of target genes. The two ERs have different and sometimes opposing effects on the regulation of target genes, and estrogenic action will ultimately depend on interplay between them, when co-expressed in the same cell. The expression of ERα and ERβ in human testis has been strongly debated and a definite answer to whether only ERβ or both ERs are expressed is pivotal to understanding the estrogenic actions in human spermatogenesis. Several splice variants for ERα and ERβ have been described in testis, playing important roles in the regulation of their prototype receptors. In contrast, only one AR variant has been described so far in human testis, although the existence of more variants responsible for regulatory and non-classical actions is highly expected. The definition of the estrogen and androgen regulated transcriptome is of pivotal importance to understand the precise roles of sexual steroid hormones in testis. The main objectives of this thesis were to clarify the expression of ERα and ERβ in human testis, search for alternatively spliced AR variants, and to identify and characterize novel estrogen and androgen regulated genes with a potential importance in the control of spermatogenesis. The results presented herein demonstrate unequivocally that both ERα and ERβ are expressed in human testis, and clarify their cellular distribution. The existence of alternatively spliced testicular AR variants was confirmed with detection of four new AR forms in human testis, two of them conserved along the vertebrate evolutive line indicating a relevant functional importance. Conserning the sex steroid regulated transcriptome, two novel genes were identified, one regulated by estrogens and the other by androgens. Apoptosis inhibitor and modulator of DNA-damage response Aven was identified as a novel estrogen target gene in testis. Its expression was for the first time characterized in human and rat testis, as well as its cellular distribution to Sertoli and germ cells. Perhaps more importantly, it was shown that the expression levels of Aven in human testis are positively correlated with quality of spermatogenesis. Concerning androgen regulated gene, the expression of Regucalcin (RGN) in response to 5α-dihydrotestosterone was characterized, and RGN shown to be expressed by all cells in rat and human testis. Regucalcin is involved in the control of intracellular calcium concentration and regulation of cell proliferation and apoptosis, processes whose regulation is of pivotal importance in the control of spermatogenesis. Estrogens and androgens are well recognized as germ cell survival factors and are known to regulate control mechanisms for testicular apoptosis. Therefore, we believe that both Aven and RGN are involved in mechanisms of germ cell survival, which are controlled by androgens and estrogens. In conclusion, this thesis has contributed to increase the knowledge about estrogenic and

androgenic action in testis. The “new actors cast” to the drama that is the hormonal control of spermatogenesis open new storylines in the research of mammalian spermatogenesis and perheaps male fertility.

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List of Abreviations

ABP – androgen binding protein

AF1 – transactivation function 1

AF2 – transactivation function 2

AR – androgen receptor

ARE – androgen response elements

ArKO – aromatase knockout mouse

AT – annealing temperature

Bp – base pair

C – number of cycles

Ca^{2+} - Calcium

$[Ca^{2+}]_i$ - intracellular calcium

cDNA – complementary DNA

CREM - cAMP-responsive element modulator

DBD – DNA binding domain

DHT – 5α-dihydrotestosterone

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

E_2 – 17ß-estradiol

ERα – estrogen receptor α

ERß – estrogen receptor ß

ERE – estrogen response element

GnRH – gonadotropin releasing hormone

H – heart

HP – hypospermatogenesis

HSP – heat shock protein

IHC – immunohistochemistry

K – kidney

L – liver

LBD – ligand binding domain

LC – Leydig cell

Lg – lung

LH – luteinizing hormone

MA – meiotic arrest

Met – methionine

mRNA – messenger RNA

OAZ – obstructive azoospermia

NR – nuclear receptor

NTD – amino-terminal domain

PCR – polymerase chain reaction

qPCR – quantitative (real-time) PCR

RGN – regucalcin

RNA – ribonucleic acid

RNA P II – RNA polymerase II

RT-PCR – reverse transcription PCR

Sa – round spermatids

SC – Sertoli cell

SCOS – Sertoli cell-only syndrome

Sd – elongated spermatids

SeT – seminiferous tubules

SG – spermatogonia

SR - Sex steroid hormone

ST – spermatocytes

STF – seminiferous tubule fluid

Sz – spermatozoa

T - testosterone

TNFα - tumour-necrosis factor-α

WB – western blot

WT – wild type

Zn – zinc

1.GENERAL INTRODUCTION

Cellular and Molecular Overview of Mammalian Spermatogenesis

Spermatogenesis is a complex process occurring in testis involving cell division and maturation that culminates with the production of the male gametes, spermatozoa. Besides spermatogenesis, the testes are also the place for the synthesis of steroids. Testes are enclosed by a fibrous capsule, *tunica albuginea*, composed of an outer visceral peritoneum and an inner layer of fibroblasts, collagen fibers, and smooth muscles cells. Inside each testis there are several coiled hairpin-shaped seminiferous tubules (SeT) with both ends emptying into a structure called *rete testis*[1]. The SeT is the functional unit of spermatogenesis and contains the somatic Sertoli cells (SC) and various types of germ cells (Figure 1.1). The tubules are surrounded by peritubular tissue composed of connective tissue and peritubular myoid cells, which move immature spermatozoa towards the *rete testis*[2,3]. The synthesis of steroids occurs mainly in the interstitial space, which consists of the Leydig cells (LC), macrophages, blood and lymph vessels, and nerves. The SeT open into transitional zones in the *rete testis* lined by cells resembling Sertoli cells which appear to form a valve or plug.

Spermatogenesis can be defined as the sequence of cytological events that result in the formation of mature spermatozoa from precursor cells[4]. It is characterized by three functional phases: proliferation, meiosis, and differentiation[5]. The process requires spermatogonial stem cells renewal and amplification by mitosis and differentiation. Spermatogonial stem cells respond to differentiation signals and undergo mitosis without completing cytokinesis. Spermatogonia then differentiate into primary spermatocytes, which proceed to meiosis and originate secondary spermatocytes. These divide to become haploid spermatids and are transformed into spermatozoa (Sz) by spermiogenesis[5]. For spermatogenesis to occur a unique environment within the SeT is necessary, which is achieved by the blood-testis barrier, formed by adjacent SC[6].

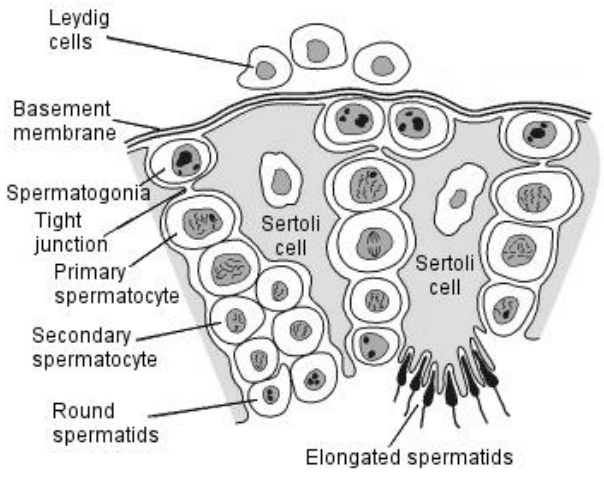

Figure 1.1 Schematic view of the testicular histology (modified from [7])

The somatic cells

There are two main types of somatic cells in the testis: SC and LC. While SC are an integrant part of the seminiferous epithelium and have nursing functions, LC are localized in the interstitial space between the tubules and their main function is testicular steroidogenesis[4].

Sertoli cells play an essential role in spermatogenesis as they supply the developing germ cells with nutrients and growth factors, and the number of SC determines testicular size and sperm output[8]. The critical importance of SC to the survival of germ cells is also demonstrated by the fact that although SC-only tubules exist, in cases of Sertoli cell-only syndrome (SCOS), there are no cases describing germ cell-only tubules[9]. Moreover, because germ cells do not express androgen receptor (AR), the action of androgens on germ cells is achieved through SC[10-12]. In fact targeted deletion of AR in Sertoli cells results in complete disruption of spermatogenesis[13-15], while deletion in germ cells causes no effect on male fertility[16] (see also Androgens and Androgen Receptor, page 16). Sertoli cells were first described by Enrico Sertoli in 1865 as individual elements extending from the basement membrane to the lumen of the seminiferous tubule involving the many clusters of associated germ cells[17]. These cells regulate the flow of nutrients and growth factors to the germ cells while protecting them from harmful agents and the host's immune system[18]. This is done by the so-called blood-testis barrier, formed by tight junctions between neighbouring SC (Figure 1.1)[18]. The barrier divides the basal compartment containing diploid cells from the adluminal compartment, containing haploid germ cells and bathed by the SeT fluid (STF) secreted by SC. Also, they are responsible for the phagocytosis of apoptotic germ cells and residual bodies, which result from the spermiation (release) of spermatids into the lumen of the tubules[19,20].

Sertoli cells produce numerous molecules, including the androgen binding protein (ABP) which enables the achievement of high concentrations of androgens in the STF and the excurrent ductal system[21].

The proliferation of SC is stimulated by follicle-stimulating hormone (FSH)[22], which also induces SC to produce factors such as ABP, inhibin, lactate and transferrin[23-26] (see also Regulation of spermatogenesis, page 7). Not only androgens but also estrogens act on SC and in addition, immature SC are known to express active P450 aromatase, which converts testosterone into 17β-estradiol (E_2), making SC simultaneously a source and a target for estrogens (see also Estrogens and Estrogen Receptors: α and β, page 11).

Leydig cells are localized in the interstitial spaces between SeT (Figure 1.1)[4]. These cells contain lipid droplets containing cholesterol esters, which are used for the synthesis of testosterone. Leydig cells are, in fact, the main site of steroidogenesis in the testis and the main source of testicular androgens[27]. They have well developed smooth endoplasmic reticulum (SER), which contains membrane-bound steroidogenic enzymes[28,29]. Only one step of the steroidogenic pathway occurs outside of the SER, the conversion of cholesterol to

pregnenolone (Figure 1.2) happening in the membrane of the mitochondrial cristae[29,30]. The SER is therefore the main site of steroidogenic enzymes in LC, and testosterone production is actually directly proportional to the volume of this organelle[31]. In LC, testosterone can be further metabolized to a more potent androgen, 5α-dihydrotestosterone (DHT), by the action of 5α-reductase (Figure 1.2) within the microsomes of SER, although testosterone is the prevalent intratesticular androgen[29,32,33]. Alternatively, testosterone can also be metabolized by P450 aromatase complex into the E_2 (Figure 1.2), which locates to the mitochondria of the LC[34,35].

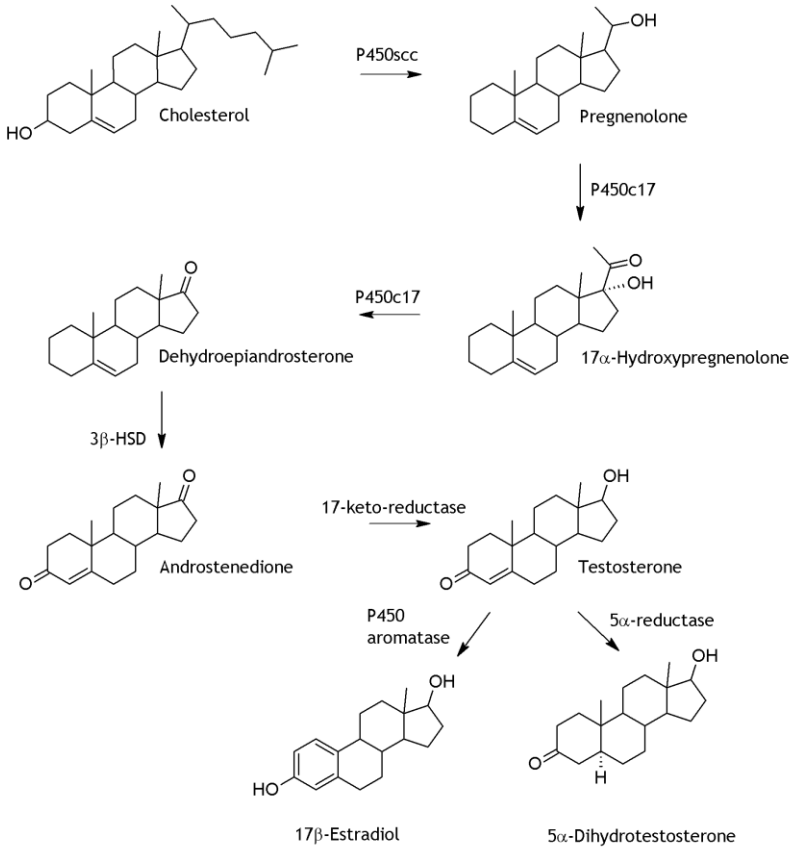

Figure 1.2 Steroidogenic pathway leading to the synthesis of testosterone from cholesterol. Testosterone can be further metabolized to estrogen 17Β-estradiol or to more potent androgen 5α-dihydrotestosterone. Legend: P450scc – cholesterol side-chain cleavage enzyme; P450c17 – 17α-hydroxylase/17,20-lyase; 3Β-HSD – 3Β-hydroxy steroid dehydrogenase.

The germ cells

The germinal epithelium is constituted by germ cells in different developmental stages nested in invaginations of SC[4]. The earliest cells to appear in the germinal epithelium are spermatogonia (Figure 1.3). In order for spermatogenesis to be continuous spermatogonia must renew themselves by mitosis in addition to proceeding to meiosis, originating two types of cells: type A and type B spermatogonia[4]. As they undergo mitosis they remain connected by intracellular cytoplasmic bridges, due to incomplete cytokinesis[36]. These bridges, which persist until Sz are released into the SeT lumen, enable the movement of several macromolecules including mRNAs and proteins, allowing the transport of Y chromosome

encoded gene products to X-bearing cells and *vice versa*[37]. While type A spermatogonia continue proliferating and remain in the periphery of the tubules, type B spermatogonia progressively loose contact with the basal membrane and are able to enter the process of meiosis by differentiating into primary ST. At this point, primary spermatocytes pass through the blood-testis barrier into the adluminal compartment.

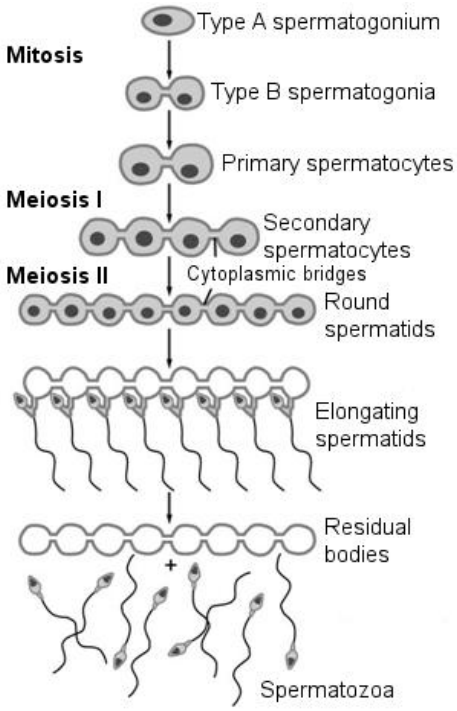

Figure 1.3 Cell division and differentiation events occurring on spermatogenesis (modified from [38])

Meiosis involves a round of DNA replication followed by two cell divisions (Figure 1.3). Meiosis I begins with primary spermatocytes and results in secondary spermatocytes, bearing a haploid number of chromosomes and diploid DNA content. Meiosis II is similar to a mitotic division and occurs in secondary ST resulting in haploid spermatids. Secondary spermatocytes have a very short life-span, and are therefore very rare in testis tissue sections. These cells are located close to the lumen and have a spherical shape. They undergo meiosis II and each one gives rise to two spermatids (Figure 1.3). Early spermatids are smaller than secondary spermatocytes and can be found in the lumen of SeT. The transformation of spermatids into Sz does not involve further cell division, it is a cellular restructure process called spermiogenesis[4]. This involves formation of the acrosome (Golgi originated lysosomal structure that undergoes acrosome reaction at fertilization)[39], nuclear changes (from central to eccentric position, condensation of chromatin), development of the flagellum, reorganization of cytoplasm and organelles and spermiation (release of the spermatozoon by Sertoli cells into the SeT lumen)[4].

Regulation of spermatogenesis

Spermatogenesis is a process that requires tight control for its maintenance. This is achieved by hormonal and non-hormonal regulators. The major mechanism for hormonal control of spermatogenesis is the hypothalamic-pituitary-gonadal axis[40] (Figure 1.4). The hypothalamus releases gonadotropin releasing hormone (GnRH) which acts on the pituitary inducing the release of luteinizing hormone (LH) and FSH. In the testis, LH will act on LC, stimulating the synthesis of testosterone (T), while FSH will act on SC stimulating spermatogenesis and the production of several signalling factors[41].

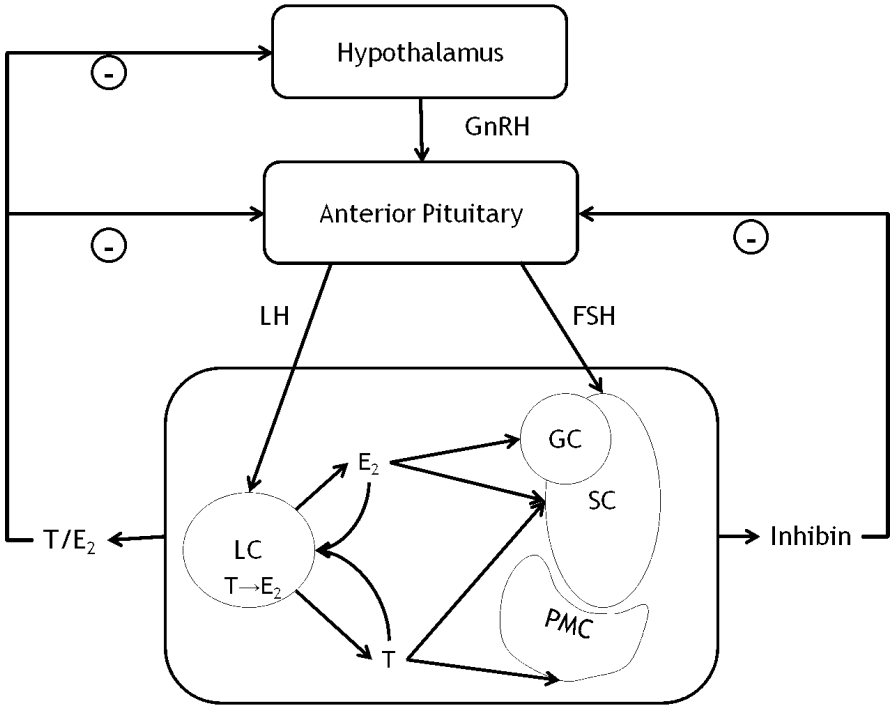

Figure 1.4 Overview of the hypothalamic-pituitary-testicular axis. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; LC, Leydig cell; SC, Sertoli cell; PMC, peritubular myoid cell; GC, germ cell; T, testosterone; E_2, 17β-estradiol.

After being produced by LC, T diffuses into the SeT where it acts on SC, or enters circulation, acting in numerous target organs[42,43] (more on this subject in section Androgens and Androgen Receptor, page 16). Testosterone can be converted (Figure 1.2) into E_2 (more details in the section Estrogens and Estrogen Receptors: α and β, page 11) or into a more potent androgen DHT. Testosterone/DHT/E_2 can act in a negative feedback mechanism to inhibit the hypothalamic release of GnRH and pituitary release of LH (Figure 1.4). Testes also produce inhibins, mostly inhibin B, part of the transforming growth factor β (TGF-β) family which will exert a negative feedback effect on the production and release of FSH by the pituitary[44]. Inhibin B is a dimeric glycoprotein which is formed by an α subunit, produced mainly by SC and to a lesser extend by LC[25,45], and a β_B subunit, produced mainly by germ cells[46,47]. Because of this inhibin B is a combined product of somatic and germ cells and therefore its levels reflect the quality of spermatogenesis[48,49].

Other factors from the TGF-ß family besides inhibin B play a role in the regulation of spermatogenesis. For example, stem cell factor and glial cell line-derived neurotrophic factor, which seem to be important for the survival and differentiation of spermatogonia[9,50,51]. Moreover, anti-mullerian hormone (AMH) is secreted by SC until puberty and induces the regression of the Mullerian duct in male embryos[52]. An interesting part of the control of spermatogenesis is played by cytokines. For example, tumour-necrosis factor-α (TNFα) is produced in the testis by a variety of cells including SC[53,54] and several germ cells[54,55], and is capable of inhibiting LC steroidogenesis[56] and increasing AR expression in SC[57]. The expression of TNFα receptors in SC is hormonally controlled by FSH[58].

Sertoli cells produce other factors necessary for successful spermatogenesis. As previously said they produce ABP, which enables a high concentration of androgens on the adluminal compartment milieu. Other factors synthesized by SC are, for example, transferrin, ceruloplasmin, proteases, protease inhibitors and structural components of the basement membrane[9,41]. Transferrin in particular has been a marker for SC number and function, and its concentration in seminal fluid has been correlated with sperm production[59-61]. This protein transports iron to the germ cells in the adluminal compartment[62,63]. Although germ cells seem to have high iron requirements for proliferation and differentiation[64,65], its levels must be tightly regulated as excessive iron is damaging to spermatogenesis[66]. Iron is not, however, the only inorganic regulator of spermatogenesis. There has been some data indicating that calcium (Ca^{2+}) has an important role to play in the regulation of spermatogenesis. This ion is essential for the maintenance of SC tight junctions forming the blood-testis barrier[67] and modulates the activity of enzymes interfering in SC architecture[68,69]. A tight regulation of intracellular Ca^{2+} concentration is essential for LC steroidogenesis, for example by controlling the expression of steroidogenic acute regulatory protein[70,71]. Moreover, it has been shown that administration of Ca^{2+} channel blockers has deleterious effects on mammalian spermatogenesis, being associated with reversible infertility[72-78].

The Steroid Hormone Receptor Superfamily

Sex steroid hormone (SR) receptors act mainly through nuclear receptors (NR), a superfamily comprising receptors which act as transcription factors[79,80]. However, ligands were only identified for 24 members of the family, the rest being called orphan receptors[79]. The NR superfamily can be divided into six subfamilies or classes based on evolutionary data[79,81,82]:

- Subfamily 1 has 21 members and includes among others thyroid hormone receptors α and ß, retinoic acid receptors α, ß, and γ, and vitamin D receptor.
- Subfamily 2 is slightly smaller, with 13 members, including retinoid X receptors α, ß, and γ, and testis receptors α and ß.
- Subfamily 3 includes all SR in addition to estrogen-related receptors α, ß, and γ (Table 1.1).

- Subfamily 4 is smaller, having only three members (nerve growth factor-induced clone B α, β, and γ).
- Subfamily 5 has only two members, steroidogenic factor 1 and Fushi Tarazu factor 1.
- Subfamily 6 has only one member, germ cell nuclear factor.

Table 1.1 Subfamily 3 of the NR superfamily. Adapted from [79,82]

Receptor	Denomination	Subtypes	Nomenclature	Ligand
ER	Estrogen receptor	α β	NR3A1 NR3A2	E_2, tamoxifen, raloxifen, various synthetic compounds
ERR	Estrogen-related receptor	α	NR3B1	None
		β γ	NR3B2 NR3B3	Diethylstilbestrol, 4-hydroxy-tamoxifen
GR	Glucocorticoid receptor		NR3C1	Cortisol, dexamethasone, RU486
MR	Mineralocorticoid receptor		NR3C2	Aldosterone, apirolactone
PR	Progesterone receptor		NR3C3	Progesterone, medroxyprogesterone actate, RU486
AR	Androgen receptor		NR3C4	Testosterone, flutamide

The first SR to be identified, albeit by biochemical methods, was a receptor for estrogens in 1962 [83]. However, it took 26 years for estrogen receptor α (ERα) to be cloned[84,85].

All SR share common structural features, consisting of basically the same functional domains (Figure 1.5): amino-terminal domain (NTD; A/B region), DNA-binding domain (DBD; C region), a hinge region (D region), and ligand binding domain (LBD; E region). Estrogen receptors also contain a region F of yet unknown function. The NTD, which contains the transcription activation function AF1, is the most variable region. In contrast, the DBD is the best conserved, as this is the domain by which SR bind to DNA at specific sequences called hormone response elements. The DBD contains two cystine-rich zinc (Zn) fingers (Figure 1.6) which fold to form a compact structure with two perpendicular α-helices that are important for DNA recognition and binding[86-88]. The first Zn-finger contains the P-box, a sequence of five amino acids which is involved in DNA recognition, while the second contains the D-box, also formed by five amino acids which is involved in receptor dimerization[89].

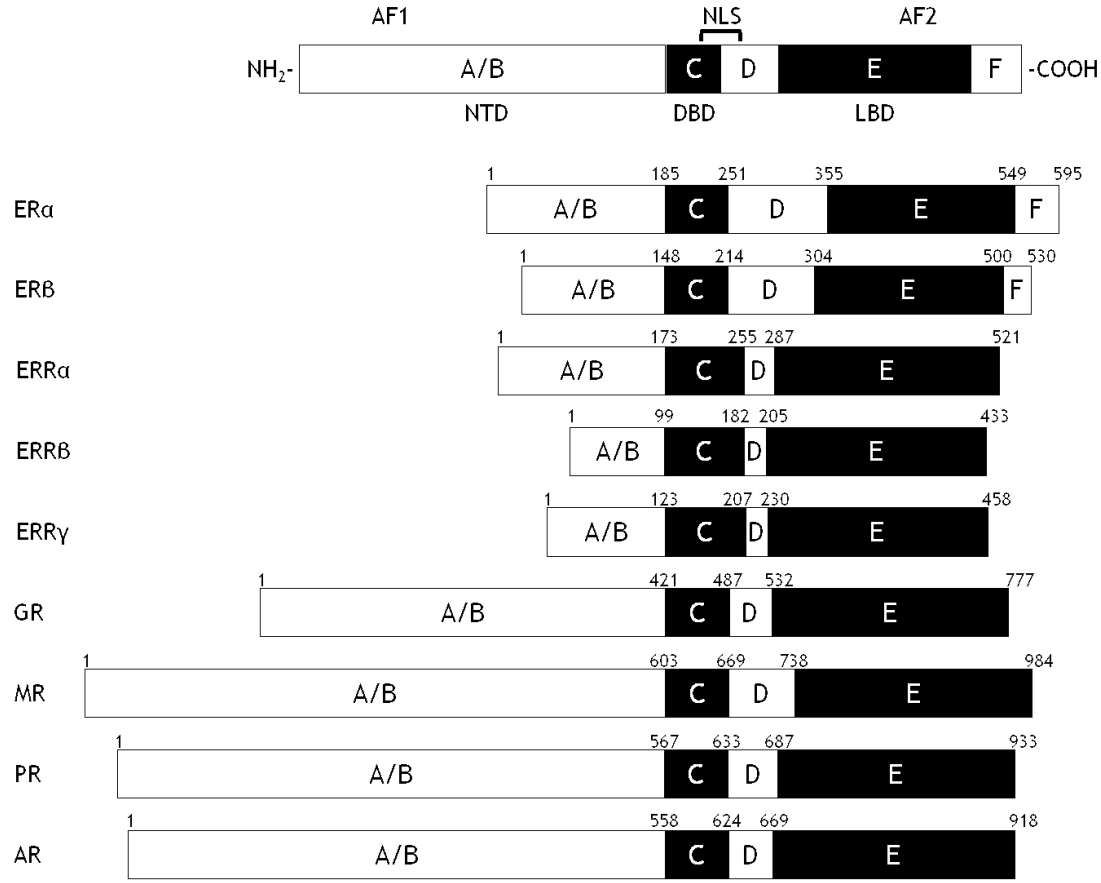

Figure 1.5 The upper panel shows a schematic representation of a typical nuclear receptor. The A/B region contains the amino (NH_2)-terminal domain (NTD), the C region contains the DNA-binding domain (DBD), the hinge is located in region D, while region E constains the ligand binding domain (LBD) in the carboxy(COOH)-terminal end of the protein. In some members a region F with unknown function may also be present. Two activation functions, AF1 and AF2, are localized on the NTD and LBD, respectively and a nuclear localization signal is localized between the DBD and the hinge region D. The schematic representation of the members of subfamily 3 of the nuclear receptor superfamily are also shown (ERα and ERβ, estrogen receptors α and β; ERRα, ERRβ, and ERRγ, estrogen-related receptors α, β, and γ; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor). The amino acid position of each region is shown with arabic numerals and refers to the human proteins[90-92]

The DBD is linked to the LBD through the hinge region, a poorly conserved domain which often contains a nuclear localization signal. The LBD is less conserved than the DBD, however it is responsible for ligand binding, dimerization, interaction with heat-shock proteins, and contains ligand-dependent transactivation function AF2[93,94].

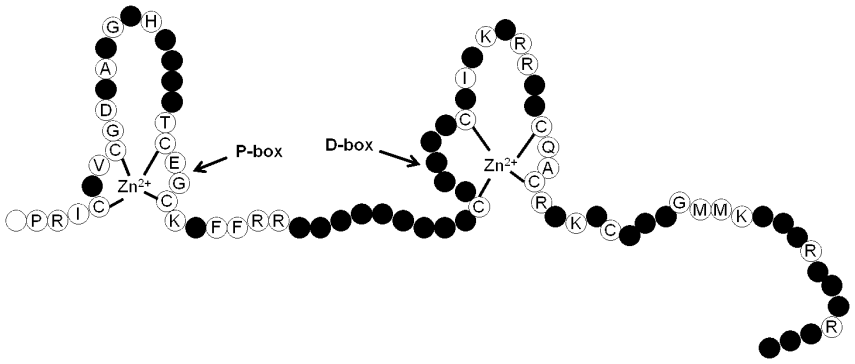

Figure 1.6 Schematic view of DNA binding domain with 2 zinc fingers and corresponding cystine residues. Aminoacids common between nuclear receptors are indicated by their corresponding letters, while non-conserved residues are indicated in black (based on [82]. Localization of P-box and D-box is pointed.

Hormone response elements are located on the regulatory regions of target genes and consist of a 6 bp consensus motif[82]. For SR the sequence of the motif is AGAACA separated by three spacer nucleotides however, naturally occurring response elements can show variation from this sequence[82,95]. Most receptors will bind as dimers to these response elements, which are usually made from two of these hexameric motifs (called half-sites), arranged into palindromes, inverted palindromes, or direct repeats[82]. The exception is the ERs, which bind to palindromes of the half-site GGTCA[96].

Is this thesis, attention will be focused on two groups of sex steroid hormones, androgens and estrogens, and their respective receptors.

Estrogens and Estrogen Receptors: α and β

Estrogens are steroid hormones synthesized from C19 androgens (*e.g.* T) by aromatase encoded by the *CYP19* gene[97]. The most prevalent estrogen in humans is E_2, although smaller amounts of several metabolites are present. Estrogens act through ERα and ERβ, sex steroid receptors, through various mechanisms in order to exert their effects[98]. Although classically viewed as "female" hormones, estrogens have been shown to play pivotal roles in male physiology, including in reproduction[99]. Estrogens and ERs have also been suggested to play important parts in the pathophysiology of numerous types of cancer[100-103], including prostate cancer[104].

ERα and ERβ genes and proteins

The first evidence of a receptor for estrogens appeared in the 1960's[83] based on the specific binding of E_2 in the uterus. In 1986 the cDNA for estrogen receptor (ER) was cloned by two groups[84,85]. However, 10 years later a second ER was cloned and named ERβ, while the first ER cloned was re-named ERα[105,106]. The two ER subtypes are encoded by genes in different chromosomes. In humans, ERα is encoded by a gene in chromosome 6[107,108] while ERβ gene is located on chromosome 14[109]; in rat ERα and ERβ are encoded by genes on chromosomes 1[110]

and 6[111], respectively. Both the genes encoding human ERα and ERβ are organized into eight exons[109,112]. Expression of the ERα/β gene is regulated by estrogens[113-115], similar to what happens with the AR and other SR[116,117].

The human ERα gene encodes a 595 aa protein[85] while ERβ protein is 485 aa long[105]. Both ERα and ERβ proteins have the typical structure of a NR with functional domains from A/B to F (Figure 1.5). The NTD (domain A/B) is the least conserved between α and β subtypes and contains a transactivation domain while only 58% of the LBD is conserved between the two receptors[98] (Figure 1.7). Alike other members of NR superfamily, ERs have two transactivation functions, AF1 (located in NTD) and AF2 (located in LBD)[118]. Similarly to what happens with the AR, binding of ligands to the LBD will cause a conformational change that will render the AF2 domain fully active[93,119]. Between the DBD and the LBD localizes the flexible hinge region, which is poorly conserved between the two receptors[106] and may account for differences in receptor-induced changes in DNA structure necessary for gene transcription[120]. The hinge region also harbours the nuclear localization signal[121]. Both receptors have a high degree of homology concerning their DBD[98] (Figure 1.7), which contain the two typical Zn-fingers responsible for interaction with estrogen response elements (ERE) in the regulatory sequences of target genes, and the P-box (involved in DNA recognition) is identical in both receptor proteins (Figure 1.6)[105].

Figure 1.7 Percentage of amino acid identity between human estrogen receptors α (ERα) and β (ERβ)[92,106]

Several ERα and ERβ have been described, some of which originated by alternative splicing[122-124], however whether all of them are translated into proteins or have important biological functions remain to be determined[125]. However, some variants have been showed to modulate the activity of the prototype ERs[126,127].

Mechanisms of estrogen action

Estrogens can act through two main groups of mechanisms: genomic and non-genomic actions. In the classical mechanism, estrogens bind to its receptor triggering a series of conformational changes and events that ultimately lead to changes in the transcription rate of estrogen-regulated genes (Figure 1.8). Although this process is not entirely understood, it is very similar to what happens with other SR, namely AR, and it involves release of chaperone proteins, phosphorylation[128], dimerization of the estrogen-bound receptor, interaction with DNA, recruitment of transcription factors and coregulators, and assembly of the transcriptional complex[129,130]. Phosphorylation regulates ER activation[131,132], and dimerization[133], and ligand-independent activation of ERs through phosphorylation has been

described[134,135]. Estrogen receptors bind to EREs in the regulatory regions of target genes, containing the consensus motif GGTCA organized in palindromic repeats with a variable 3 bp spacer[96,136]. Both ERs bind these ERE with similar affinity, however the addition of a nucleotide to the consensus, forming palindromes of AGGTCA with a 3 bp spacer, determines the affinity with which ERα binds to the ERE[137]. The activity of ERs can be modulated by several factors, including alternatively spliced variants[126,127]. More importantly, ERβ acts as a dominant negative regulator of ERα transcriptional activity[138]. Therefore, in cells and tissues expressing both ERs, estrogenic actions are determined by the balance between ERα and ERβ. ERα and ERβ sometimes show opposite transcriptional regulatory activities. A classical experiment which involved binding of agonist raloxifene to ERβ induced an increase in reporter gene expression, however binding to ERα resulted in minimal activation[139]. Opposing actions of ERα and ERβ also occur in the regulation of cyclin D1 expression[140]. Curiously ERs bind DNA not only as ERα/ERα or ERβ/ERβ homodimers but also as ERα/ERβ heterodimers, adding new levels of complexity to estrogenic action[141,142]. It has been demonstrated that these heterodimers bind to DNA with higher affinity than ERβ homodimers[142] and are able to bind not only to classical but also to specific EREs in the genome resulting in specific gene expression profiles different from those regulated by homodimers[143].

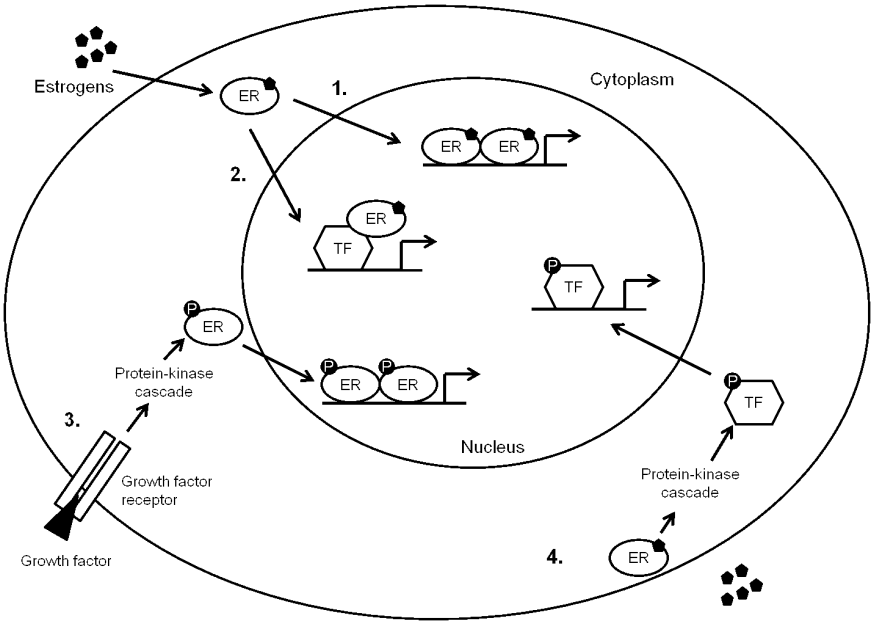

Figure 1.8 Mechanisms of estrogen control of gene expression. 1. Classical mechanisms – estrogens enter the cell, bind to their cognate nuclear receptors (ER) which travel to the nucleous and bind, as dimmers, to estrogen responsive elements in the genome, controlling the rate of gene expression. 2. Estrogen-bound ER can bind and activate other transcription factors, that in turn bind to their responsive elements on the genome, controlling gene expression. 3. Estrogen-independent ER-mediated mechanism – growth factors can bind to their membrane receptor, triggering a protein-kinase cascade that phosphorilates and activate ER, which enters nucleus and binds to estrogen responsive elements, controlling gene expression. 4. Membrane ER action – estrogens can bind to ERs located at the cell membrane, triggering the activation of protein-kinase cascades, that will end with the phosphorilation of transcription factors that bind to their own response elements in genome, thereby controlling gene expression.

Estrogen receptors can also regulate gene expression by non-classical mechanisms[144] (Figure 1.8), the so-called nongenomic-to-genomic pathways, through interaction of the receptors with transcription factors which in turn bind to their own response elements in DNA[144-146]. Also, ERs can activate protein-kinases that activate transcription factors, thereby influencing gene expression[147]. These pathways are sometimes initiated in the plasma membrane, which has sparked the debate about the localization of classical ERs to the plasma membrane versus existence of novel ER forms which are specifically membrane-bound[148-150]. Also, a transmembrane G-protein coupled receptor (GPR30) has been shown to be able to mediate estrogen signalling[151], however the true contribution of this receptor to estrogenic signalling has been questioned (reviewed by [152]). There are also evidences indicating that ERs, like AR, can have ligand-independent transcriptional activity[153,154] (for an extensive review on this subject read [155]).

Non-genomic ER actions are faster since they do not involve the activation of RNA transcription or translation into protein, which is a common feature among steroid receptors[149,156,157]. They usually involve the activation of protein-kinase cascades[158-160], mobilization of intracellular Ca^{2+} [161,162], increase in cAMP concentration[163], modulation of nitric oxide release[162], amongst other effects. Some of the non-genomic estrogenic actions occur through membrane-bound ERs, as shown also by the use of membrane-impermeant forms of estrogens[164,165]. It is interesting to note that the opposite actions of ERα and ERβ are also recognisable in these non-genomic effects, for example whereas ERβ activates c-Jun N-terminal kinase, ERα inhibits its activation[149].

Role of estrogens/estrogen receptors in male reproduction

Although classically viewed as "female hormones", the first evidence of the presence of estrogens in male gonad was known in 1934[166]. In recent times the importance of estrogens to males as been stressed by many studies regarding male-specific physiological functions, such as spermatogenesis (for review see [167] and [168]). In testes, E_2 is produced through the aromatization of T mostly in LC, but also several different germ cells, and even spermatozoa have been shown to contain active aromatase[169-172]. Intratesticular E_2 reaches levels that can be up to 100-400x higher than the serum levels[173-175]. Estrogens act in a negative feedback fashion in the hypothalamus and pituitary, regulating the secretion of GnRH and FSH, respectively (Figure 1.4).

The localization of ERα and ERβ in testis has been matter of much debate. In rodents, ERα seems to be expressed by LC[176-178] and peritubular myoid cells[179], although expression in some germ cells has also been described[176]. On the other hand, ERβ has been localized to LC[179,180], SC[176,179,181,182], peritubular myoid cells[179,180], and some germ cells[179-181]. In humans, the controversy surrounding ERα and ERβ testicular localization is even greater. Some authors have localized ERα to LC[183,184], SC[184], and some germ cells[185-189]. Espression of ERβ was detected in LC[183,190], SC[183,190], peritubular myoid cells[190], and some germ cells[185,187-189,191,192].

Some authors have not detected ERα expression in human testis[190-192] which, together with data from immunolocalization studies in rodent testis, led to the idea that ERß is the main mediator of estrogenic action in the testis. However, data from ER mutations and KO animals does not corroborate this theory.

Contrarily to AR, whose defects cause dramatic consequences to male phenotype and fertility, very little is known of the effect of defective estrogenic action in humans. There is only one disruptive mutation of ERα known in humans[193]. This man had normal male phenotype, however he presented with low sperm counts and decreased sperm viability[193]. Until now, there have been no reports of ERß disruptive mutations in humans. There are few reports of men with disruptive mutations of aromatase gene, and therefore with an absence of endogenous estrogens, but not all of them had their reproductive parameters tested[194-196]. Although the reproductive phenotype varies slightly with the type of mutation, all of the aromatase-deficient men studied had normal male phenotype however, reproductive parameters showed impairment of fertility with decreased sperm motility[194-196], oligozoospermia[196], hypospermatogenesis and meiotic arrest[195]. The major contribution to the understanding of estrogenic action in male reproduction, and more importantly in spermatogenesis, came from the generation of KO mice for aromatase and both ERs. Table 1.2 shows the reproductive phenotypes of some estrogen related KO mice. Initially, male mice lacking aromatase (ArKO) are fertile and phenotypically normal, which indicates that estrogens are not necessary for development of male phenotype[197]. However, they progressively develop disruption of spermatogenesis[198]. Also, inclusion of phytoestrogens in the diet of these animals partially prevented disruption of spermatogenesis, which indicated a potentially important role for exogenous estrogens in the regulation of male fertility[199]. The non-requirement of estrogen action for normal male phenotype is confirmed by the development of KO mice for ERα, ERß, or both, which show normal male reproductive tract[200-204]. The absence of ERß does not cause changes in male fertility[202,203], in contrary to ERα lacking animals, in which a reduced sperm count can be observed[200,201]. However, these animals present progressive disruption of spermatogenesis, which was explained as a result of defective fluid resorption in the efferent tubules, causing SeT atropy due to fluid back pressure[205].

There has been increased attention given to the effects of estrogens in male reproduction in the past two decades because of the reports that exposure to environmental contaminants with estrogenic activity may have deleterious effects on male reproductive development and may be causing a decline in male fertility[206-208]. Although the issue is controversial, there is evidence that excessive estrogenic exposure can lead to disturbances of spermatogenesis, mainly through disruption of the balance between germ cell survival and death[209-212]. There are many studies relating estrogens to apoptosis in testis and identifying E_2 as a germ cell survival factor[185,213], highlighting the importance of estrogens to male fertility. The disruption of spermatogenesis in the ArKO mouse has been attributed to increased germ cell apoptosis

due to absence of E_2[198]. Therefore estrogens are thought to play a role in the delicate balance governing cell survival and death in the testis.

Table 1.2 Knockout mice for the study of estrogen action in spermatogenesis and male fertility

Animal	Type	Male phenotype (compared to WT)	Spermatogenic status	Reference
ArKO	Complete aromatase deficiency	Enlarged accessory sex glands, otherwise normal	Initially fertile, develop progressive infertility due to disruption of spermatogenesis	197,198
ERα insertional disruption	Complete ERα deficiency	Normal	Reduced sperm count	200
αERKO	Complete ERα deficiency	Normal	Reduced sperm count, progressive disruption of spermatogenesis	201,205
$ERβ^{-/-}$	Complete ERβ deficiency	Normal	Normal	202
βERKO	Complete ERβ deficiency	Normal	Normal	203
αβERKO	Complete ERα and ERβ deficiency	Normal	Similar to αERKO	204

Legend: ERα, estrogen receptor α; ERβ, estrogen receptorβ; KO, knockout; WT, wild type.

Although supportive and valuable evidences allow recognizing that estrogens have an important role in the regulation of spermatogenesis, there are gaps concerning the regulatory pathways involved in the testicular estrogenic functions. For instance, the cellular expression patterns of ERα and ERβ in human testis are not clearly defined, and the role of ERα in spermatogenesis remains to be clarified. On the other hand, the nature of estrogenic actions in testis, wether genomic or non-genomic, is slowly being uncovered. Regulation of kinase intracellular signalling pathways by estrogens has been described in testis[188,214,215]. Few studies have aimed at identifying genes regulated by estrogens in testis[216-219], and for most of the identified targets their roles in male fertility and the expression patterns within the testis remain unstudied[218,219]. Also, the administration of endocrine disruptors acting through ERs, which have been pointed as one of the reasons for the decline in human fertility, have been shown to alter testicular gene expression patterns[207,220,221], highlighting the importance of genomic estrogen action in the regulation of spermatogenesis. Therefore, it is important to know the targets of estrogenic action in order to better understand the regulation of spermatogenesis by this class of hormones.

Androgens and Androgen Receptor

Androgens have been classically viewed as predominantly "male hormones". Androgens are responsible for initiation and maintenance of spermatogenesis, maintenance of male phenotype, among other physiological effects in the male[222]. Testosterone, the most important androgen, is mainly synthesized in LC from cholesterol (Figure 1.2) in response to

stimulation by LH. Synthesized T can be secreted into circulation, metabolized into a more potent androgen DHT, or converted into E_2 by aromatization (Figure 1.2).

Androgens classically exert most of their actions by binding to their cognate nuclear receptor, AR (Table 1.1, Figure 1.5), which is expressed in androgen-target tissues such as the male reproductive tract, skin, and muscle, amongst others[10,223,224]. Moreover, AR has also been shown to play a part in the development of several cancer types, most notably prostate cancer[225-229]. Variations in androgenic action causes a wide array of consequences, which range from mild spermatogenic defects[230,231] to the severe complete androgen insensitivity syndrome (or testicular feminizing syndrome), which causes development of female phenotype in individuals with XY karyotype[232].

The AR gene and protein

The AR gene is located on the X-chromosome[233-235]. It is composed of eight exons and the mRNA codes for a protein with approximately 919 amino acids and 110kDa in humans, although precise length varies individually due to the existence of two variable polyglutamine and polyglycine stretches encoded by exon 1 (see section Variations on the AR gene and protein consequences)[233]. The human AR gene contains a TATA-less promoter[236]. The AR gene is itself androgen-regulated[237,238], and although there are no studies describing the existence of ARE in its promoter, an androgen regulated region containing AREs has been identified in its coding region[116] which is common within SR[113,117]. Although only one gene encoding a receptor for androgens has been found in mammals, Japanese eel (*Anguilla japonica*) has been demonstrated to have two ARs, eAR1 and eAR2, encoded by distinct genes[239]. However, the existence of two protein forms for AR in humans has been hypothesized due to the presence of a second immunoreactive band with ≈87kDa in western blots (WB) detecting AR protein[240]. This AR isoform (AR-A) is shorter than the prototype protein (AR-B), and apparently results from initiation of translation of the same transcript at an internal methionine residue (Met188)[240], therefore differing only on the N-terminal portion of the protein. Another explanation is that the AR-A form results from *in vitro* proteolysis[241]. Experimental studies have found functional differences between the two potential isoforms, which seems to indicate these two forms have physiological significance[242,243]. Nevertheless whether the two forms are simply an experimental artefact or represent truly functional distinct forms of AR remains to be fully determined.

Much like other members of the NR superfamily, AR protein has three fundamental structural domains (Figure 1.5, Figure 1.9): NTD (encoded mostly by exon 1), DBD (encoded mainly by exons 2 and 3) and LBD (encoded by part of exon 4, and exons 5, 6, 7, and 8). The NTD contains the transactivation domain AF1, considered to be the major activation domain for AR[244,245]. Similarly to other NRs, the DBD of AR consists of two Zn-fingers, which mediate the interaction between the receptor protein and its response elements in the genome[246]. The hinge region, a flexible linker connecting the DBD to the LBD and containing the nuclear

localization signal[247], is encoded by part of exon 4. The LBD also shares structural features with other NRs. It is constituted by eleven α-helixes, which form a ligand-binding pocket[248] and contains the transactivation function AF2, which is only complete and active upon ligand-binding[93,119]. The LBD not only interacts with AR agonists but also antagonists[249].

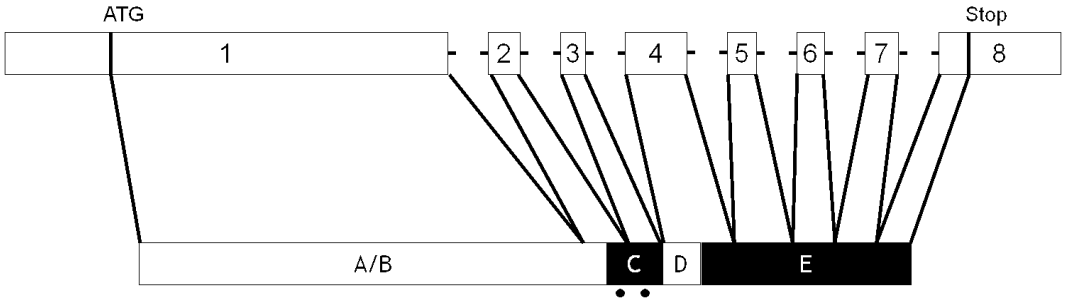

Figure 1.9 Structural organization of the AR gene (up) and protein (down). Exons are indicated by numbers. ATG and Stop show the localization of the initiation of translation and stop codon, respectively. A/B region represents the amino-terminal domain, C is the DNA-binding domain (two black dots show localization of zinc-fingers), D is the hinge region and E contains the ligand-binding domain[233,250]

Variations on the AR gene and protein consequences

The exon 1 of the AR gene contains two polymorphic regions, with variable numbers of trinucleotides CAG [$(CAG)_n$] and GGN [$(GGT)_3GGG(GGT)_2GGC_n$], encoding for a polyglutamine and polyglycine tract, respectively[233]. Longer polyglutamine tracts, even within normal range (9-36), are associated with lower transactivation activity[251,252]. Different numbers of GGN repeats have also been shown to have impact in the transactivation activity of AR, and a lower number of repeats is associated with stronger AR activity[253-255].

Similarly to other steroid receptors, several AR variants resulting from alternative splicing of the AR gene have been described. Most of them were detected in cancer tissues and cell lines, most notably hormone-refractory prostate cancer. Only one AR splice variant was detected in normal tissues, AR45, which was also the only AR splice variant to be found in testis[256].

Table 1.3 Published AR mRNA splice variants

Splice variant	Features	Splicing mechanism	Tissues/cells expressing
ARΔ3 [257]		Exon deletion	Breast cancer tissues and breast cancer cell lines: T47D, ZR-75-1, BT-20, MCF-7, BT-474, MDA-MB-231.
AR45 [256]		Alternative use of exons	T, K, L, U, P, Lg, Tr, M, B, H
AR23 [258]		Alternative 3' splice site	LnCaP cell line
$AR^{Ex3dup*}$ [259]	Results in longer protein, containing 3 Zn fingers.	Exon duplication	22Rv1 PCa cell line.
$AR^{Ex1/2/2b*}$ [259]	Truncated protein	Splicing of intronic cryptic exons	22Rv1 PCa cell line.
$AR^{Ex1/2/3/2b*}$ [259]	Truncated protein	Splicing of intronic cryptic exons	22Rv1 PCa cell line.
AR-V1, AR-V2, AR-V3, AR-V4, AR-V5, AR-V6, AR-V7 [260]	Truncated proteins	Splicing of intronic cryptic exons	Hormone-naive and hormone-refractory PCa tissues, CWR22Rv1 cell line
AR3 [261]	Truncated proteins	Splicing of intronic cryptic exons	LNCaP and LNCaP derivatives C-81, C4-2 and C4-2b, CWR-R1, 22Rv1. Benign and malignant prostate tissues
AR4 [261]			CWR-R1
AR5 [261]		Splicing of intronic cryptic exons, exon duplication	CWR-R1

* Expressed together; T, testis; K, kidney; L, liver; U, uterus; P, prostate; Lg, lung; Tr, trachea; M, muscle; B, breast; H, heart.

Mechanisms of androgen action

The classical mechanism of androgen action through AR involves a series of events initiated by androgen binding to a pocket on the LBD of AR, ultimately ending in its translocation to the nucleus, dimerization, recruitment of coregulators, and control of target-gene expression[262]. Ligand-binding causes a series of conformational changes that leads the release of the receptor from various chaperone proteins[263], phosphorylation by various kinases[264], and enables the translocation of androgen-bound AR to the nucleus[265]. Androgen receptor activation is therefore regulated by phosphorylation, which occurs both when ligand is present as well as in its absence[264,266] as it has been shown that phosphorylation causes ligand-independent activation of AR[267]. After entering the nucleus, AR dimers[268] will bind to androgen response elements (ARE) in regulatory regions of target genes[269,270] (Figure 1.10).

The first Zn-finger of each AR protein will interact with one half-site of the ARE, while the second Zn-finger will be involved, through the D-box, in receptor dimerization[271]. At this point, there will be recruitment of co-factors (co-activators or co-repressors)[272], and assembly of the transduction machinery[273], ultimately regulating the expression of target genes.

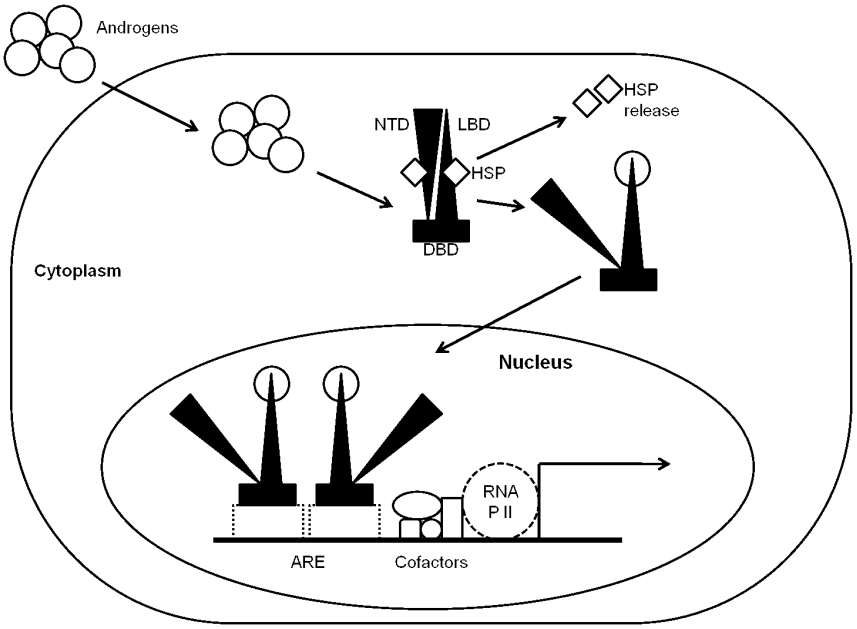

Figure 1.10 Classical mechanism of androgen action for the regulation of gene expression. Classical androgen receptor action involves androgen binding to androgen receptor, release of chaperone heat-shock proteins (HSP), conformational change, translocation to nucleus, binding to androgen response elements (ARE), followed by assembly of transcriptional machinery and transcription. NTD – aminoterminal domain; DBD – DNA binding domain; LBD – ligand binding domain, RNA P II - RNA polymerase II.

Androgen receptor binds to AREs that display the consensus motif common for steroid receptors (except ERs), AGAACA organized in palindromic repeats, with a 3 bp variable spacer[88,269]. However, AR can also bind to other type of response elements, known as specific AREs, which are organized as direct repeats of the consensus sequence rather than palindromic repeats[88,274].

Besides the classical genomic actions of androgens, AR also plays a part in other types of mechanisms. These are usually called non-genomic androgen actions, however this is a somewhat broad classification, as some of these non-genomic effects lead to changes in gene expression, and there is always possibility of action overlapping. Androgen-bound/activated AR can control gene expression without interacting with DNA, in a sort of "non-genomic to genomic" pathway[275]. This justifies why some genes whose expression is controlled by androgens do not have an ARE in their regulatory regions. Androgen receptor can interact with and activate other transcription factors, such as AP1, which can in turn bind to their own response elements on the regulatory regions of target genes, thereby regulating their expression[276,277]. Androgen receptor can also mediate truly non-genomic actions[278]. These are faster than genomic ones, as they do not depend upon transcription and translation mechanisms. They typically include events such as rises in intracellular Ca^{2+} concentration

($[Ca^{2+}]_i$)[279,280] and activation of intracellular transduction pathways[281,282]. Also, this type of action sometimes originates in the cell membrane, and can be induced even by androgens that have been rendered impossible to enter the cell (*e.g.* BSA-bound T) or in the absence of functional AR[283,284].

Role of androgens/androgen receptors in male reproduction

Androgens are not only essential for the development of male phenotype, they are also pivotal for the initiation and maintenance of spermatogenesis[285,286]. However, the precise functions of T on the regulation of spermatogenesis are not completely understood. Androgens are thought to act on spermatogenesis exclusively through the somatic cells of the testis, most notably SC, as most reports describe the localization of AR protein to be confined to these cell types of the testis[10,11,287,288]. Nevertheless there are reports describing the localization of AR to germ cells[12,178,223,289]. More recently, several authors have detected the presence of AR by in human sperm[189,282,290].

In humans, androgen secretion by LC is high until 3-6 months after birth, and then decreases and remains low until the onset of puberty[291,292]. Under the influence of LH, LC maturate and start secreting androgens, which causes the intratesticular androgen levels to rise stimulating the maturation of SC and the initiation of full adult spermatogenesis[292]. During fetal life, AR immunoexpression can be observed in the nucleus of LC and peritubular myoid cells, while SC remain AR-negative during fetal and early post-natal life[293,294]. While in rodents the expression of AR in SC increases from birth[295,296], along with a decrease in the secretion of AMH[296], in humans there is a longer period of latency (corresponding to infancy) when SC show very low AR expression[294,297]. Sertoli cell AR expression starts to increase between 4-8 years of age, and from 9-14 years of age all SC start to express AR[297], indicating the onset of puberty, the increase in intratesticular androgen levels and the initiation of spermatogenesis.

Inside SeT, T can achieve concentrations that are 50 to 100 times higher than in circulation[32,298-300]. Although the need for such high concentrations remains unknown it has been shown that reduced intratesticular T may cause apoptosis of testicular germ cells[301]. Apoptosis of testicular germ cells is controlled by androgens[302-304]. Testosterone inhibits apoptosis in adult human SeT culture *in vitro*[305]. Also, withdrawal of intratesticular T in rats causes defects in the progression of spermatogenic cells through later stages of spermatogenesis and spermiogenesis[306-310].

Defects in androgen actions have been shown to play a role in male infertility[286,311]. Notably, alterations in AR gene lead to a wide array of consequences, including male infertility, prostate cancer, spinal and bulbar muscular atrophy, and androgen insensitivity syndrome[312-314]. In both rodents and humans, null mutations in the AR gene cause complete androgen insensitivity syndrome, which is characterized by pseudohermaphroditism and sterility in 46,XY individuals[232,312,314]. An overwhelming number of mutations in the AR have been

described (organized in a monthly updated AR gene mutation database accessible through: http://androgendb.mcgill.ca/), causing a wide array of phenotypes[315]. Defects in androgen action through AR rather than altered androgen serum levels have been related to altered spermatogenesis[316]. Several mutations in the AR have been detected in patients with impairment of spermatogenesis (for example[317-322]). The importance of correct AR activity for male fertility has also been demonstrated by the development of full and cell-specific knockout mice. Table 1.4 shows the reproductive phenotype of some of the most important AR knockout mice, showing the importance of AR in distinct cell types of the testis. The analysis of these animals has highlighted the importance of AR function to the development of normal male phenotype and spermatogenesis. The importance of AR presence in testis, mainly in somatic SC and LC, has also been confirmed, and the inactivation of AR function in these two cell types causes disruption of spermatogenesis.

Table 1.4 Main knockout mice for the study of AR in spermatogenesis and male fertility

Animal	Type	Male phenotype (compared to WT)	Spermatogenic status	Reference
$AR^{Tfm/y}$	Complete AR deficiency	Female-like appearance	Disrupted spermatogenesis	323
$ARKO/AR^{-/y}$	Complete AR deficiency	Female-like appearance	Disrupted spermatogenesis	324
$AR^{flox(ex1\text{-}neo)/Y}$	Complete AR deficiency	Female-like appearance	Disrupted spermatogenesis	13
$S\text{-}AR^{-/y}$	SC specific	Smaller testes, otherwise normal	Disrupted spermatogenesis	15
SCARKO	SC specific	Smaller testes, otherwise normal	Disrupted spermatogenesis	14
$AR^{flox(ex1\text{-}neo)/Y}$;Amh-cre	SC specific	Normal	Disrupted spermatogenesis	13
$PM\text{-}AR^{-/y}$	Peritubular myoid cell specific	Smaller testes, otherwise normal	Oligozoospermia, but normal fertility	325
$G\text{-}AR^{-/y}$	Germ cell specific	Normal	Normal	16
$L\text{-}AR^{-/y}$	LC specific	Atrophied testes and epididymis, otherwise normal	Disrupted spermatogenesis	326

Legend: AR, androgen receptor; KO, knockout; LC, Leydig cell; SC, Sertoli cell; WT, wild type

Other variations in AR which are thought to contribute to male infertility are variations in the number of polymorphic repeats in exon 1 of the AR gene, CAG and GGN. It has been suggested that the size of CAG tract is inversely correlated with the transcriptional activity of AR protein[251,252]. This can have a physiological impact in a number of androgen-regulated events and organs. For instance, a negative correlation between the number of CAG and GGN repeats and prostate cancer risk has been suggested[327,328]. Also, the enlarged polyglutamine tract may also cause accumulation of toxic aggregates which may lead to spinal and bulbar muscular atrophy (over 40 repeats), a neurodegenerative disease affecting exclusively males which is accompanied by reproductive abnormalities[329,330]. Several studies have also correlated higher number of CAG repeats, albeit within the normal range, with male

infertility[331-338]. Higher number of repeats has even been associated with lower sperm counts[339] and teratozoospermia[340]. However, other studies found no association between CAG and male infertility and impaired spermatogenesis[341-349]. The contradicting results of the several studies have been attributed to several factors, including ethnicity and inconsistencies in inclusion criteria[230,342]. A meta-analysis has suggested that variations in exon 1 CAG repeat may indeed be related to infertility in otherwise healthy men[230]. Men with higher number of CAG repeats usually have raised serum T levels[350,351], which may compensate for lower AR activity. However, longer polyglutamine tracts have also been associated with higher E_2 levels which raised the hypothesis that the disruptive effects of this polymorphism on spermatogenesis can be attributed to differences in estrogen rather than androgen action[350].

All the effects of androgens on spermatogenesis are mediated by the AR. Some effects might be due to non-genomic pathways[352], such as the increase in Ca^{2+} concentration and the phosphorilation of kinases in SC[279,353,354]. These effects are also caused by FSH action, and therefore represent a synergistic way by which both FSH and androgens sustain and regulate spermatogenesis[355]. However, spermatogenesis can be maintained in the absence of FSH, provided testosterone is maintained at normal levels, while the opposite is not true. This is because of the androgen regulation of target gene expression, essential for the maintenance of spermatogenesis[355]. Both complete and SC-specific ablation of the AR gene in mice, which cause disruption of spermatogenesis (Table 1.4), affect the expression profile of many genes[356,357]. Also, the decrease in testicular androgen concentration caused by an over-expression of ABP, causes a profound effect on the expression of androgen-target genes[358]. Interestingly, most of the androgen regulated genes in testis were not found to have an ARE in their regulatory regions[356,359,360].

Although the identification of androgen targets on mammalian spermatogenesis is increasing rapidly, research in this area is important in order to understand the full spectrum of androgenic regulation of spermatogenesis. It might also provide clues to understand the causes of idiopathic male infertility, which causes 40-60% of all cases in male-factor infertility[361].

Aim and Outline of the Thesis

The importance of androgens to the successful occurrence of spermatogenesis is well established. Also several kinds of evidence have highlighted the role of estrogens in spermatogenic process. However, the mechanism by which these hormones and their receptors regulate spermatogenesis is yet to be completely established. The expression of AR in somatic testicular cells and its absence from the germ cell line is widely accepted. However, the expression of ERs in human testis is still a matter of much debate, as it is not clearly defined whether estrogens exert their effects through ERα, ERß or both. On the other

hand, the existence of several splice variants for ERα and ERβ, with a potential role in the regulation of spermatogenesis has been described. However, little is known about alternatively spliced AR forms in testis. Furthermore, deepening the knowledge of estrogen and androgen target genes in the testis will help to define the actions these hormones carry out in testicular physiology and spermatogenesis. The aim of this thesis was to study the expression and localization of ERα and ERβ in human testis, to analyse the existence of possible alternatively spliced variants of AR in testis, and to identify new estrogen and androgen target genes in testis and explore their putative function in spermatogenesis. Therefore, in order to fill these objectives the thesis was structured into the following chapters:

Chapter 2 – describes ERα and ERβ expression and localization in human testicular biopsies by reverse-transcription polymerase chain reaction (RT-PCR) and/or immunohistochemistry.

Chapter 3 – identifies AR splice variants in human testis and characterizes their expression in other tissues. The presence of these transcripts in testis along the vertebrate evolutive line is also evaluated, as well as their potential functional importance.

Chapter 4 –Using a suppressive subtractive hybridization strategy aimed at screening for novel estrogen targets in mammalian testis, we have identified Aven as an estrogen up-regulated gene in rat seminiferous tubules (SeT). Aven is an apoptosis inhibitor and modulator of DNA damage response. In this chapter we decided to characterize the expression of Aven in rat and human testis. The regulation of Aven expression by E_2 was confirmed in rat SeT cultured *ex vivo*, and its expression was evaluated by quantitative RT-PCR (qPCR) and WB. Also, the localization of Aven in rat and human testis was characterized by immunohistochemistry (IHC). In addition, the expression levels of Aven were studied in testis of men with different spermatogenic status and a correlation was established.

Chapter 5 – Regucalcin (RGN), a Ca^{2+}-binding protein which regulates intracellular Ca^{2+} homeostasis as well as cell apoptosis and proliferation, has been identified as a sex steroid regulated gene in rat reproductive organs such as breast and prostate. This chapter characterizes the regulation of RGN expression by DHT in rat SeT culture *ex vivo* identifying RGN as a new androgen target gene in male reproductive tract. The expression and/or localization of RGN in testicular cell types was evaluated by RT-PCR, WB, *in situ* hybridization, IHC, and qPCR.

Chapter 6 – reviews several aspects of RGN biology, with a special focus on its roles in male reproduction.

Chapter 7 – contains an integrative view of the results presented in the thesis and discusses the potential of these findings, with a special focus on the regulation of germ cell apoptosis.

Chapter 8 – states the final conclusions and the future perspectives.

2. ESTROGEN RECEPTORS α AND β IN HUMAN TESTIS: BOTH ISOFORMS ARE EXPRESSED

Published in: Cavaco, JE, SS Laurentino, A Barros, M Sousa, and S Socorro, *Estrogen receptors alpha and beta in human testis: both isoforms are expressed*. Syst Biol Reprod Med, 2009 **55**(4): 137-44.

Abstract

Currently, clinical and experimental evidence point to an essential role of estrogens and estrogen receptors in male fertility. The expression of estrogen receptor α (ERα) and ß (ERß) in human testis has been described. However, some studies were unable to detect ERα, while others report the expression of both isoforms, with ERß presenting a wide distribution within somatic and germinal testicular cells. This has suggested that estrogens may exert their testicular effects exclusively through ERß. The present work aims to study the expression of ERα and ERß in testicular biopsies of men with conserved and disrupted spermatogenesis, in order to better clarify the positive cell populations. Human testicular tissue was obtained from 10 men undergoing testicular biopsy for infertility relief due to azoospermia: two patients had secondary obstructive azoospermia with conserved spermatogenesis, five had Sertoli cellonly syndrome, two had hypospermatogenesis and one had meiotic arrest. Reverse-transcription polymerase chain reaction (RT-PCR) allowed the detection of both ERα and ERß mRNAs in all samples. Immunohistochemistry revealed that ERα was present in Leydig cells, Sertoli cells, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa, while ERß was present in the same cell types except spermatogonia and Sertoli cells. This study demonstrates ERa mRNA expression in human testis and describes its localization in somatic and germ cell subtypes. These findings suggest that both ER isoforms are involved in the control of testicular function.

Introduction

Estrogens and estrogen receptors (ERs) may play an important role in male fertility, as shown by presentations of inactivation of estrogen receptor α (ERα) and decreased sperm viability[193], patients with aromatase deficiency, which have altered testicular size, oligozoospermia and reduced sperm motility[195,196], and the relationship between ERs polymorphisms, male fertility and sperm output[362-365]. At the moment evidence is accumulating pointing towards a direct or indirect role of estrogens in the regulation of Leydig, Sertoli and germ cells development and function (see reviews of [168,366,367] and references therein).

The biological actions of estrogens are exerted by their interaction with ER subtypes α (ERα) and β (ERβ), and few studies aimed to determine their presence and location in the human testicular tissue have been undertaken. Some studies have shown ERα protein expression in Leydig cells[183], Leydig and Sertoli cells[184], spermatocytes[185], spermatids and spermatozoa[186,187], or in spermatozoa[188,189]. However, others were unable to detect ERα, which led to the idea that estrogens exert their testicular effects exclusively through ERβ[190-192]. The expression of the ERβ protein has always been detected in human testicular tissue, albeit also without concordant results. ERβ was immunolocalized to Leydig and Sertoli

cells[183], Leydig, myoid peritubular and Sertoli cells as well as spermatogonia[190], Sertoli cells, spermatogonia, spermatocytes and spermatids[191], spermatogonia, spermatocytes and spermatids[192], spermatocytes and spermatids[185], spermatids and spermatozoa[187], or in spermatozoa[188,189].

Although the cellular distribution of ERα and ERβ in the human testicular tissue has been described, the results were not concordant for the same type of cells under analysis and the presence of ERα has been questioned. The aim of the present study was to characterize the expression of ERα and ERβ in human testicular biopsies from azoospermic patients showing distinct germ cell populations in order to better clarify the positive cell populations.

Materials and Methods

Tissues

Human testicular tissue was obtained from 10 infertile men aged 29-48 years old, with normal karyotypes, undergoing a treatment testicular biopsy due to azoospermia[368]. Two patients had obstructive azoospermia due to inflammatory epididymal obstruction, with conserved spermatogenesis, five had Sertoli cell-only syndrome, two had hypospermatogenesis and one had meiotic arrest at the primary spermatocyte stage. All patients gave their informed consent and the study was approved by the local ethical committee. Tissue samples were immediately frozen in liquid nitrogen after collection and stored at -80°C for RNA extraction, or fixed in 4% buffered formalin at 4°C for 24 hours and embedded in paraffin for immunohistochemistry.

RT-PCR

Total RNA was extracted from testicular samples using Tri Reagent (Sigma-Aldrich, St-Louis, Missouri, USA) according to the manufacturer's instructions. Quality of the extracted RNA was assessed by ethidium bromide stained agarose gel electrophoresis and 28S/18S ratio. One µg total RNA was reverse transcribed in a final volume of 20 µL with 200 U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) according to the protocol provided by the manufacturer, using 250 ng random hexamer primers (Invitrogen) and 0.5 mM each dNTP (GE Healthcare, Buckinghamshire, UK). Intron spanning primer sets were used for the amplification of ERα and ERß cDNA fragments (Table 2.1). One µL of synthesized cDNA was amplified in a final volume of 50 µL, containing 1xPCR buffer (Promega, Wisconsin, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTP (GE Healthcare), 0.5 µM each primer (Metabion, Munich, Germany) and 1.25 U Taq DNA polymerase (Promega). For each set of PCR reactions a control was run in parallel with RNA not reverse transcribed instead of cDNA. Both the annealing temperature of the primers and the number of cycles required for the exponential phase of amplification of each fragment were optimized (Table 2.1). PCR reactions were carried out in triplicate and optical density values were determined using the BIO-1D 99.04 software

(Vilber-Lourmat, France). Expression of ERα and ERβ was normalized using 18S as internal control. The identity of the obtained amplicons was confirmed by DNA sequencing using the Sanger's Method (Stabvida, Oeiras, Portugal).

Table 2.1 Oligonucleotides and cycling conditions for PCR amplification of ERα and ERβ

Gene	Sequence (5'-3')	Amplicon size (bp)	AT (ºC)	C
ERα [109]	Sense: AATTCAGATAATCGACGCCAG Anti-sense: GTGTTTCAACATTCTCCCTCCTC	345	62	37
ERβ [109]	Sense: TAGTGGTCCATCGCCAGTTAT Anti-sense: GGGAGCCACACTTCACCAT	393	60	44
18S	Sense: AAGACGAACCAGAGCGAAAG Antisense: GGCGGGTCATGGGAATAA	149	56	26

AT: Annealing temperature; C: Number of cycles during the exponential phase of amplification.

Antibodies

The primary antibodies used were anti-ERα rabbit polyclonal antibody (MC-20: sc-542, Santa Cruz Biotechnology, Santa Cruz, USA) and anti-ERβ rabbit polyclonal antibody (06-629, Upstate Biotechnology, Lake Placid, USA) diluted 1:100 and 1:50, respectively. The specificity of these antibodies detecting ERα and ERβ proteins has been previously described in tissues known to express these receptors[369,370]. As secondary antibody against ERα and ERβ, a biotinylated goat anti-rabbit IgG antibody (Sigma-Aldrich) was used at a dilution of 1:20.

Immunohistochemistry

Briefly, 5µm paraffin sections of each testicular sample were deparaffinized and rehydrated in graded alcohols. Heat-induced antigen retrieval was performed in a 10 mM citric acid (pH 6.0) for 30 minutes, at 80-85°C. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 10 min. Sections were incubated overnight at 4°C with the primary antibody, diluted in phosphate buffered saline with 1% bovine serum albumin. Antibody binding was detected using ExtrAvidin Peroxidase staining kit (Sigma-Aldrich) and 3,3'-diaminobenzidine tetra-hydrocloride (Sigma-Aldrich) according to the manufacturer's instructions. After colour development, sections were slightly counterstained with hematoxylin, dehydrated and mounted using Entellan (Merck, Germany). The specificity of immunostaining was assessed by omission of the primary antibodies and usage of pre-absorbed antibodies. Blocking peptides for ERα (sc-542P, Santa Cruz Biotechnology) and ERβ (YAEPQKSPWCEARSLEHT, synthesised by STAB VIDA) antibodies were added to working solutions at 100-fold excess primary antibody concentration.

Results

Both ERα and ERβ mRNA are expressed in human testis

RT-PCR analysis allowed the detection of both ERα and ERβ mRNAs in all types of testicular samples from individuals presenting normal to disrupted spermatogenesis (Figure 2.1). genomic DNA amplification was excluded since primers were located in different exons, with the identity of the amplicons also being confirmed by sequencing. The expression of ERα (except for samples 7 and 9) was relatively constant among samples within the same spermatogenic phenotype, while ERβ expression was more variable as seen from the optical density values of normalized expression (Figure 2.1). In addition, the number of cycles necessary to reach the exponential phase of the amplification reaction for ERα was lower than that for ERß (Table 2.1), which suggests a higher expression of ERα in human testis.

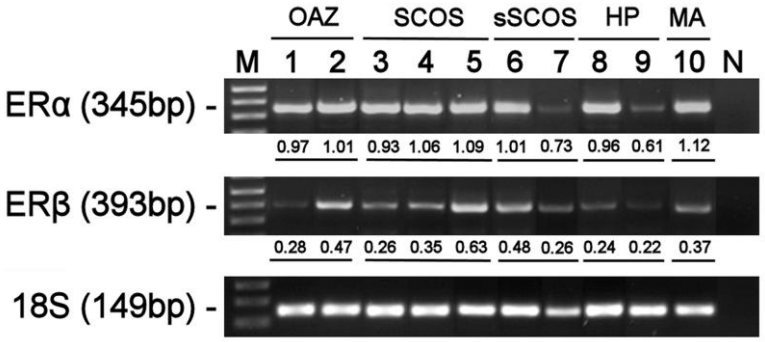

Figure 2.1 Expression of ERα and ERß mRNAs in human testicular tissues. Representative results of electrophoresis of PCR products are shown. Under each electrophoresis lane are indicated optical density values normalized to 18S (mean of triplicates). M, molecular weight marker (1 Kb Plus DNA ladder, Invitrogen); 1-2, samples from patients with secondary obstructive azoospermia (OAZ); 3-7, samples from patients with Sertoli cell-only syndrome (SCOS); 8-9, samples from patients with hypospermatogenesis (HP); 10, sample from patient with meiotic arrest (MA); N, negative control (cDNA not reverse transcribed).

ERα is expressed in somatic and germ cells of human testis

Positive staining for ERα (Figure 2.2, panels B, E, H and K) was detected in Sertoli and Leydig cells, and in all germ cell stages, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa regardless of spermatogenic phenotype. Negative controls obtained by pre-absorption or omission of the primary antibody showed complete absence of staining (Figure 2.2, panels A, D, G, J).

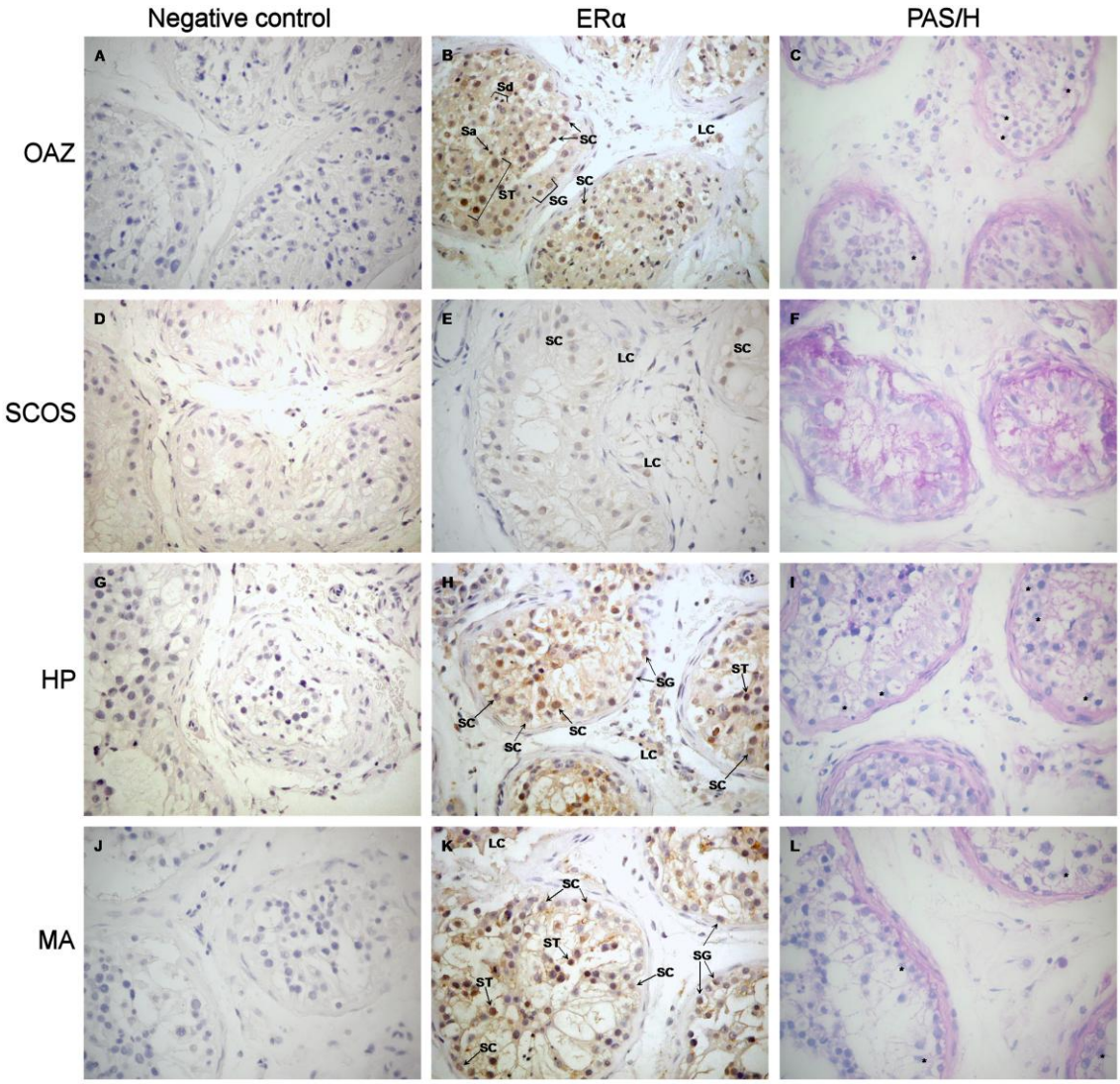

Figure 2.2 Immunohistochemical localization of ERα adult human testis from patients with secondary obstructive azoospermia (OAZ), Sertoli cell-only syndrome (SCOS), hypospermatogenesis (HP) and meiotic arrest (MA). All samples displayed the same staining pattern for the same antibody. The immunohistochemical procedure was repeated at least twice for each sample and the representative results are shown. ERα was detected in Leydig cells (LC), Sertoli cells (SC), spermatogonia (SG), spermatocytes (ST), round spermatids (Sa), and elongated spermatids/spermatozoa (Sd) (B,E,H,K). Negative controls obtained by pre-absorption of the primary antibodies show complete absence of staining (A,D,G,J). Periodic-acid Schiff and hematoxylin staining was performed on the same tissue for a better assessment of morphology and cell-type identification (C, F, I, L). Original magnification of each individual panel ×400.

ERβ is expressed in Leydig and germ cells of human testis but not in Sertoli cells

ERβ immunoreactivity (Figure 2.3, panels B, E, H, K) was localized to Leydig cells, spermatocytes, round spermatids and elongated spermatids/spermatozoa, with no staining being detected in Sertoli cells and spermatogonia. Negative controls obtained by pre-absorption or omission of the primary antibody showed the counterstain only (Figure 2.3, panels A, D, G, J).

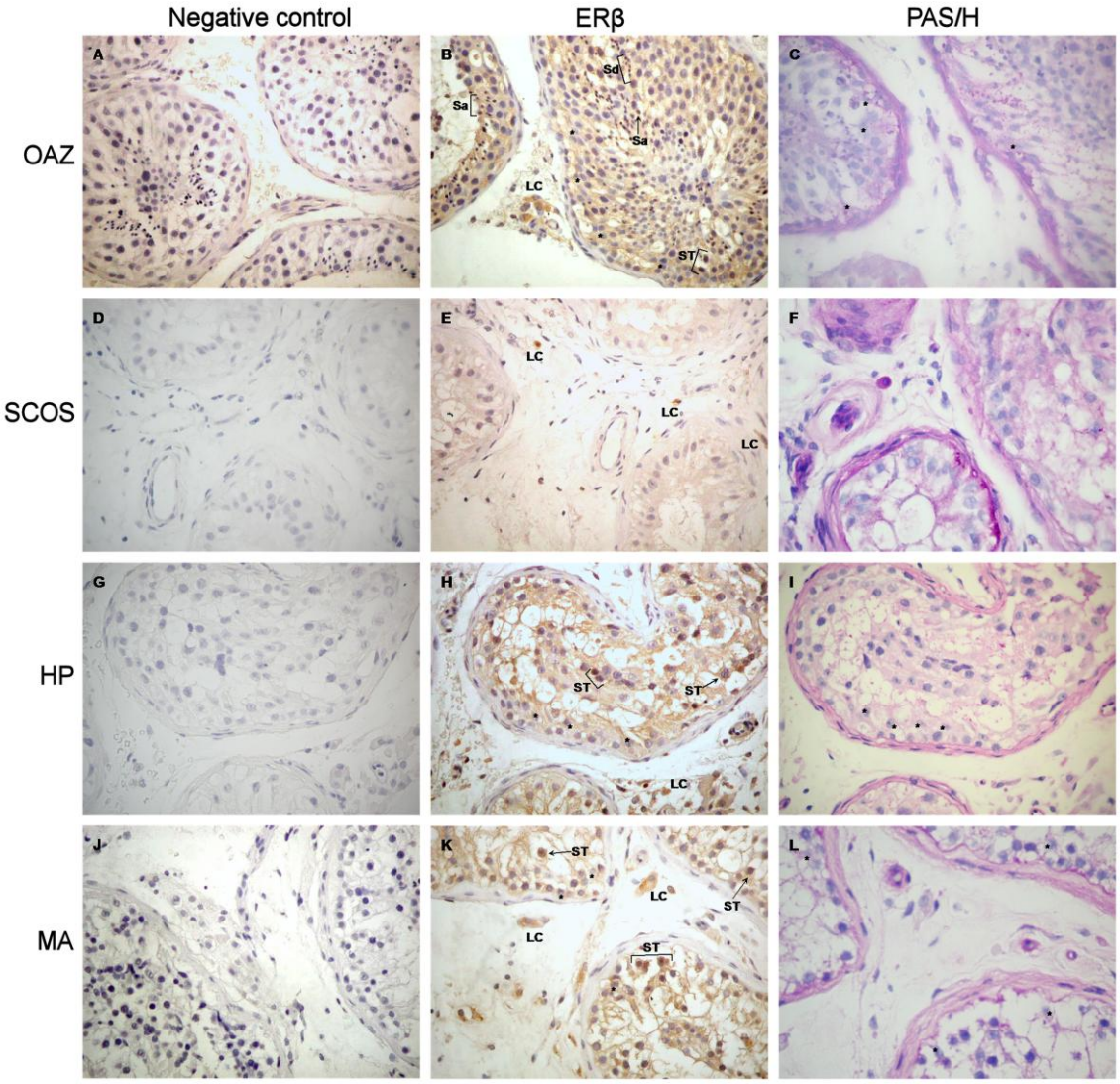

Figure 2.3 Immunohistochemical localization of ERß in adult human testis from patients with secondary obstructive azoospermia (OAZ), Sertoli cell-only syndrome (SCOS), hypospermatogenesis (HP) and meiotic arrest (MA). The immunohistochemical procedure was repeated at least twice for each sample and the representative results are shown. ERß was detected in Leydig cells, spermatocytes, round spermatids, and elongated spermatids/spermatozoa (B,E,H,K). Note the complete absence of intratubular ERß immunostaining in the section displaying Sertoli cell-only syndrome (E). Negative controls obtained by pre-absorption of the primary antibodies show complete absence of staining (A,D,G,J). Periodic-acid Schiff and hematoxylin staining was performed on the same tissue for a better assessment of morphology (C, F, I, L). Original magnification of each individual panel ×400.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
ERbeta          MDIKNSPSSL NSPSSYNCSQ SILPLEHGSI YIPSSYVDSH HEYPAMTFYS PAVMNYSIPS
ERbetacx        MDIKNSPSSL NSPSSYNCSQ SILPLEHGSI YIPSSYVDSH HEYPAMTFYS PAVMNYSIPS
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
ERbeta          NVTNLEGGPG RQTTSPNVLW PTPGHLSPLV VHRQLSHLYA EPQKSPWCEA RSLEHTLPVN
ERbetacx        NVTNLEGGPG RQTTSPNVLW PTPGHLSPLV VHRQLSHLYA EPQKSPWCEA RSLEHTLPVN
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
ERbeta          RETLKRKVSG NRCASPVTGP GSKRDAHFCA VCSDYASGYH YGVWSCEGCK AFFKRSIQGH
ERbetacx        RETLKRKVSG NRCASPVTGP GSKRDAHFCA VCSDYASGYH YGVWSCEGCK AFFKRSIQGH
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Figure 2.4 Alignment of human ERß and ERßcx proteins (amino acids 1-175) showing the conserved sequence used as immunogen to produce the anti-ERß antibody (shaded region).

Discussion

Some studies have already described the expression of ERα and ERβ in human testis although they have yielded divergent results. Whereas others were not able to detect ERα, ERβ has been more extensively detected and presented a wide distribution within somatic and germinal testicular cells. This suggested ERβ could be the major receptor involved in the estrogenic actions in human testis.

In the present study, the expression of both ERα and ERβ mRNAs and the localization of the corresponding proteins were studied in testicular biopsies of men with conserved spermatogenesis (secondary obstructive azoospermia) as well as in samples with hypospermatogenesis, meiotic arrest and Sertoli cell-only syndrome. All testicular samples analysed expressed ERs mRNA and protein regardless of the testicular phenotype.

The ERα protein was detected in Leydig cells, Sertoli cells, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa. Previous reports have found no ERα immunostaining in human testis[190,192], or staining confined to a few cell subtypes, including Leydig cells[183], Leydig and Sertoli cells[184], spermatocytes[185] or spermatids/spermatozoa[186,187]. Our immunohistochemistry results were paralleled by RT-PCR analysis, which allowed the detection of ERα mRNA in all types of testicular samples. Although also using a RT-PCR strategy others have failed to detect ERα mRNA in human testicular biopsies[191,192], in agreement with our findings similar results have been obtained by studying ERα mRNA expression in human testicular tissues[186,371]. In addition, the number of cycles necessary to reach the exponential phase of the amplification reaction for ERα was lower than that for ERβ, which suggests a higher expression of ERα in human testis. Thus, the present study demonstrates that ERα is present in somatic and germ cell subtypes of the human testicular tissue. To our knowledge this is the first report consistently demonstrating the expression of ERα mRNA and protein in human adult testicular biopsies of different phenotypes, which indicates that ERα also mediates estrogenic actions in the human testis. The report of a man with a disruptive mutation of ERα and reduced sperm viability[193], and the association found between ERα polymorphisms and oligozoospermia[364,365] further accentuates the role of this ER subtype in testicular physiology.

The ERβ protein was localized to the nuclei of Leydig cells, spermatocytes, round spermatids and elongated spermatids/spermatozoa, with use of different testicular phenotypes illustrating the absence of ERβ staining in spermatogonia and Sertoli cells. Other reports described ERβ immunostaining in spermatids/spermatozoa[187], spermatocytes and spermatids[185], spermatogonia, spermatocytes and spermatids[192], Sertoli cells, spermatogonia, spermatocytes and spermatids[191], Leydig cells, Sertoli cells and spermatogonia[190], Leydig cells and Sertoli cells[183], or Leydig cells, Sertoli cells, spermatogonia, spermatocytes and

spermatids[184]. The sequence of the synthetic peptide used to produce the anti-ERβ antibody is identical in the ERβcx variant[127], as shown in Figure 2.4. Thus the results presented herein may also reflect the ERβcx distribution. Saunder's group reported ERβcx expression in Sertoli cells, spermatogonia and spermatocytes[191]. The inability to identify ERβ mutations associated with human male infertility, and the fact that male ERβ knockout mice are fertile[202] does not clarify the role of ERβ in spermatogenesis.

The differences in the immunolocalization of ERs in human testis could be associated with the existence of several ERα and ERβ isoforms[372], and probably depends on different antibodies. Different patterns of ERs expression have been described in human testis by the use of different antibodies[183,184,191,192]. Discrepant results have also been described in rodents[176,179,180,373], and Selva et al[374] demonstrated that in the mouse testis the pattern of ERβ immunostaining differs depending on the antibodies used. Differences being associated with methodological aspects cannot be ruled out, as discussed by Saunders et al[190]. However, Lee et al.[375] compared the ERs immunohistochemistry results for 420 breast cancer patients from seven different hospitals, and concluded that differences in fication or processing regimens did not have a significant adverse effect on the sensitivity of ER assessment.

Our results describing the ER localization in human testis support the role of estrogens regulating Leydig, Sertoli and germ cells development and function. Androgens synthesized by Leydig cells are absolutely necessary for initiation and maintenance of spermatogenesis[285]. Estrogen was shown to block Leydig cell regeneration[376], and to control steroidogenesis in adult Leydig cells[210] inhibiting androgen synthesis, which could have relevant consequences for male fertility (see[366] for review). Sertoli cells support and nourish germ cells being essential for initiation of spermatogenesis and maintenance of the full spermatogenic potential of the adult (reviewed by[377]). Estrogen administration seems to alter Sertoli cells proliferation and activity, leading to permanent defects in reproductive function in adulthood (reviewed by [168]). It is also worth noting the effects of estrogens on germ cells. Estrogen administration stimulates gonocyte proliferation[378-380] and induces an increase of the number of spermatogonia type A in rat testis[381]. In human adult seminiferous tubules cultured *in vitro* 17β-estradiol (E_2) acts as a germ cell survival factor[185]. This observation is supported by a study in male monkeys treated with an aromatase inhibitor which displayed a decrease in germ cell number[382]. Very recently it was demonstrated that E stimulates DNA synthesis in rat spermatogonia, an effect also maintained by the dihydrotestosterone metabolite, 5α-androstane-3β,17β-diol via interaction with ERβ[383].

In conclusion, the present study demonstrates the presence of ERα and describes the subcellular localization of ERα and ERβ proteins in human adult testicular tissues. These findings suggest that both ERs are involved in the control of human spermatogenesis, highlighting the importance of estrogens for male fertility.

3. IDENTIFICATION AND CHARACTERIZATION OF ANDROGEN RECEPTOR VARIANTS: TISSUE AND VERTEBRATE EVOLUTIVE LINE EXPRESSION

Co-authors: S Laurentino, J Tomas, JEB Cavaco, AVM Canario, S Socorro

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Abstract

Alternative splicing, the process by which a single gene originates several messenger RNA molecules and therefore several proteins, has been shown to be particularly prevalent in the testis. Many sex steroid hormone receptors are known to originate variants by alternative splicing. Some of these variants were shown to have functional importance and to regulate the activity of the wild type/prototype receptors. Particularly, several variant forms of estrogen receptors α and ß have been described, and some were hypothesized to play a role in the regulation of spermatogenesis. However, little is known about the existence of splice variants of the androgen receptor (AR), a receptor that plays a pivotal role in the regulation of spermatogenesis. Although alternative splicing has been reported on the AR in pathological situations such as cancer and androgen insensitivity syndrome, only one AR splice variant, AR45, has been detected in normal tissues. The objective of this work is to search for novel AR forms originated by alternative splicing in human testis, to check for their presence in other human tissues, and also study their conservation along the vertebrate evolutive line (*Cannis lupus*, *Rattus norvegicus*, *Sparus aurata*, and *Xenopus laevis*). We have found four alternatively spliced ARs in human testis with full spermatogenesis, two of them (ARΔ2 and ARΔ3, with exon 2 or 3 deleted, respectively) were also expressed in other normal human tissues (liver, lung, kidney, and heart). In addition ARΔ2 was also expressed in *Rattus norvegicus* and *Sparus aurata* testis, while ARΔ3 was expressed in *Xenopus laevis* testis, indicating a potential functional importance for these two transcripts. In addition, another splice variant was detected in the testis of both *Cannis lupus* and *Rattus norvegicus*, ARΔ4, with a deletion of exon 4 of the AR gene. The results presented herein indicate that there are several AR splice variants expressed in testis, which may be implicated with the androgenic regulation of spermatogenesis.

Introduction

Alternative splicing is the process by which several distinct mRNAs can be originated from one single gene. Also, alternatively splicing events can be associated with pathological events, and can be originated by gene mutations near splice site junctions causing aberrant splicing[384-386]. Amongst steroid hormone receptors (SR), the existence of isoforms is very common, some of them originated by mechanisms of alternative splicing[372]. Estrogen receptors α and ß genes (ERα and ERß, respectively), for example, have been shown to originate alternatively spliced variants by several mechanisms including deletion or duplication of exons, and use of untranslated and/or intronic (*i.e.* exons located in regions thought to be introns) exons[122,124,126,127,387-390]. In contrast with the great volume of information regarding ER variants[123,126,127,191,391,392], very little is known about alternative androgen receptor (AR) forms in testis. Alternatively spliced variants of the AR have been

related to breast and prostate cancers, and to androgen insensitivity[257-259,261,393,394]. However, a naturally occurring variant, AR45, has been described in several tissues, including testis[256].

Testis have been shown to be one of the tissues where alternative splicing is most abundant[395,396]. This probably happens because the testes are the site of a developmental process involving the division and differentiation of a great number of cells, spermatogenesis[396]. One of the most impressive examples of the importance of alternative splicing for spermatogenesis is the conversion of transcription factor cAMP-responsive element modulator (CREM) from antagonist in pre-meiotic cells into transcription activator, through the insertion of two glutamine-rich domain[397]. Several ER splice variants have been identified in testis, and some have been shown to play a role in the modulation of estrogen action[123,124,126,127,191,389,392]. Albeit being one of the principal androgen target tissues, to this date only one AR variant, AR45, had been detected in human testis[256].

The first identified AR alternative mRNA transcript was described in breast cancer samples[257]. This transcript was named ARΔ3 because it had a complete deletion of exon 3, which codes for the second zinc (Zn)-finger of the AR protein. In prostate cancer there is a growing search for alternatively spliced ARs, especially those lacking the LBD but retaining full DBD, which might be linked to androgen independency. Several transcripts of this type have been detected in the past few years and the first one to be described was detected in a relapsed CWR22 cell line, AR^{Ex2dup} [394]. It was recently found that other two AR variants, $AR^{1/2/2b}$ and $AR^{1/2/3/2b}$, are expressed concomitantly with AR^{Ex2dup} in the same cell line, both presenting a novel exon 2b[259]. Seven transcripts lacking LBD have been described, using four novel intronic cryptic exons, in hormone-refractory prostate cancer and are also thought to be responsible for androgen independency[260]. Another three novel AR variants (AR3, AR4 and AR5) lacking the ligand-binding domain (LBD) were detected in androgen independent prostate cancer cell lines and tissues[261]. In fact, the authors have shown that one of the transcripts was upregulated during cancer progression and its level was correlated with tumour recurrence[261]. Still in prostate cancer another group has detected a rather unusual transcript in a sample from a patient with metastatic prostate cancer[258]. This transcript, AR23, retained part of intron 2 resulting in a new splice site located 69 nucleotides upstream of the classic site, resulting in an in-frame insertion of 23 amino acids between the two Zn-fingers of AR protein. This insertion seems to impair the entry of this variant protein into the nucleus after hormone stimulation and cause the formation of cytoplasmic aggregates[258].

Materials and Methods

Samples

Human testicular tissue was obtained from two infertile men with normal karyotypes, undergoing treatment testicular biopsy due to obstructive azoospermia with conserved spermatogenesis[368]. Patients gave their informed consent and the study was approved by the

local ethical committee. Tissue samples were immediately frozen in liquid nitrogen after collection and stored at -80°C for RNA extraction.

Commercial total RNA samples from human heart, kidney, liver, and lung were obtained from Clontech (Saint-Germain-en-Laye, France).

Dog (*Cannis lupus*) testes were obtained from sterilization surgery at a veterinary clinic (Clínica Veterinária da Covilhã), African clawed frog (*Xenopus laevis*) testes and gilthead seabream (Sparus aurata) testis polyA+ RNA were obtained from CCMAR – Center of Marine Sciences, University of Algarve (Faro, Portugal). Tissues were collected and immediately frozen in liquid nitrogen, and stored at -80ºC for RNA extraction. Rat (*Rattus norvegicus)* testis total RNA (3 months old) was obtained from our animal facilities.

RT-PCR

Total RNA was extracted using Tri Reagent (Sigma-Aldrich, St-Louis, Missouri, USA) according to the manufacturer's instructions. Quality of the extracted RNA was assessed by ethidium bromide stained agarose gel electrophoresis and 28S/18S ratio. One µg total RNA was reverse transcribed in a final volume of 20 µL with 200 U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) according to the protocol provided by the manufacturer, using 250 ng random hexamer primers (Invitrogen) and 0.5 mM each dNTP (GE Healthcare, Buckinghamshire, UK).

Previously published primer pairs suitable for the detection of human AR splice variants[257], spanning one or more exons, were used (Table 3.1 and Table 3.2). One µL of synthesized human testis cDNA was amplified in a final volume of 50 µL, containing 1xPCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM each dNTP (GE Healthcare), 0.5 µM each primer (Metabion, Munich, Germany) and 1 U Platinum Taq DNA polymerase High Fidelity (Promega). For each set of PCR reactions a template-free control was run in parallel. Products were run in 1.5% agarose gels, and bands with lower than expected size were cut, purified (NucleoSpin Extract II, Macherey-Nagel, Germany), cloned into pGEM-T Easy vector (Promega), and sequenced using the Sanger's Method (Stabvida, Oeiras, Portugal). The reactions were repeated using cDNA of human liver (L), lung (Lg), kidney (K), and heart (H).

Table 3.1 Sequences of oligonucleotides used as primers for the detection of human AR alternative transcripts (number at each primer name indicates exon localization)

Primer name	Sequence (5'-3')
hAREx1Fwd	GTCAAAAGCGAAATGGGCCCC
hAREx4Fwd	ACTGAGGAGACAACCCAGAAG
hAREx6Fwd	ACCCCCCAGGAATTCCTGTGC
hAREx4Rv	CTTCTGGGTTGTCTCCTCAGT
hAREx5Rv	GTCGTCCACGTGTAAGTTGCG
hAREx7Rv	GCGTCTTGAGCAGGATGTGGG
hAREx8Rv	CTGCAGAGGAGTAGTGCACAG

Table 3.2 Primer pairs used for amplification of human AR alternative transcripts

Primer pair	Amplicon size (bp)	AT (ºC)	C
hAREx1Fwd x hAREx4Rv	422		
hAREx1Fwd x hAREx5Rv	644		
hAREx4Fwd x hAREx7Rv	612	55	35
hAREx4Fwd x hAREx8Rv	895		
hAREx6Fwd x hAREx8Rv	451		

AT: Annealing temperature; C: Number of cycles

Primers spanning exons 2 to 4 of AR of different species were designed using Primer3 v0.4.0 (available online in http://frodo.wi.mit.edu/primer3/; Table 3.3), in order to analyse the conservation of alternatively spliced AR forms detected in human cDNAs. RT-PCR reactions were carried out in a similar way as previously described for human cDNAs. Products were purified, cloned, and sequenced in a similar way.

Table 3.3 Primers designed for the detection of AR splice variants in different species

Species	Primer name	Sequence (5'-3')	Amplicon size (bp)	AT (°C)	C
Cannis lupus	ClAREx1	CAGAGTGCCCTTTCCAAGTC	687	55	35
	ClAREx5	TGAATGACTGCCATCTGGTCC			
Rattus norvegicus	RnAREx1	GTGAAATGGGACCTTGGATG	655	55	38
	RnAREx5	TACTGAATGACCGCCATCTG			
Sparus aurata	SaAREx1	CGATGTCCCCTACAATGACC	599	55	40
	SaAREx5	GGTCGTCCACATGGAGATTT			
Xenopus laevis	XlAREx1	GCACCTTGGATGGAAGGATA	641	55	40
	SlAREx5	ACGGTCATTTGGTCGCTTAC			

Sequences were analysed using BioEdit v7.0.5.3 (available through http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared to AR sequences available in GeneBank (http://www.ncbi.nlm.nih.gov/genbank/) and Ensembl databases (http://www.ensembl.org/index.html; Table 3.4). Sequence of gilthead seabream AR has been provided by Prof. Sílvia Socorro (unpublished results).

Table 3.4 AR sequences used for analysis of the RT-PCR amplified transcripts

Species	GeneBank AR accession number	Ensembl AR accession number
Homo sapiens	NM_000044.2	ENSG00000169083
Cannis lupus	NM_001003053.1	ENSCAFG00000016656
Rattus norvegicus	NM_012502.1	ENSRNOG00000005639
Xenopus laevis	NM_001090884.1	ENSXETG00000005089

Results and Discussion

Several AR alternative transcripts are expressed in human testis

Analysis of human testis cDNA by RT-PCR, using primers pairs hAREx1Fwd and hAREx4Rv (Table 3.2), showed that samples expressed the prototype (wild-type) AR, as showed by the presence of the expected size band (Figure 3.1). However, there were also bands of smaller size (approximately 300bp) which could correspond to alternatively spliced AR variants (Figure 3.1). After sequencing and analysis of obtained results, the smaller bands were identified as corresponding to three AR alternative forms. One had a complete deletion of exon 2, the second had a complete deletion of exon 3, and the third transcript also had a deletion of

exon 2 while retaining 69 nucleotides of intron 2, similarly to AR23 previously described in prostate cancer[258], and was called AR55 due to the molecular weight of the predicted translated protein (Figure 3.1 and Figure 3.3).

Exon 3-deleted AR has been described in patients with androgen insensitivity syndrome (AIS)[398] and in breast cancer tissues and cells[257]. It lacks the second Zn-finger of AR, responsible for receptor orientation and interaction with DNA. It is also thought that the second Zn-finger has a role in the autoregulation of AR levels *in vivo*[398]. This Zn-finger includes the D-box, which is involved in DNA-dependent dimerization and its deletion may compromise receptor-DNA interaction and transactivation of target genes[89,398,399]. A variant with a similar deletion has been detected for ERα, which has a dominant negative activity on prototype ERα[400]. The deletion of exon 2 has also described previously in a patient with AIS, due to a mutation in a splice donor site[401].

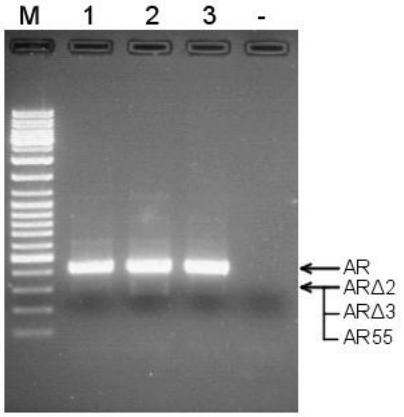

Figure 3.1 Electrophoresis of PCR (primers hAREx1Fwd and hAREx4Rv) products, showing the presence of prototype AR, and also three potential splice variants. M, molecular weight marker; ARΔ2, exon 2 deleted AR; AR3, exon 2 deleted AR; AR55, exon 2 deleted with partial retention of intron2; -, template-free negative control.

The same strategy was then used with primer pair hAREx1Fwd and hAREx5Rv in the same samples, which enabled the detection of not only prototype AR but also another potential splice variant, as shown by the lower-than-expected size band in electrophoresis (Figure 3.2). After sequencing, the variant was identified as containing an in-frame deletion of 120 nucleotides in exon 4 of the AR. The variant was named AR94 due to the predicted molecular weight of the potential protein.

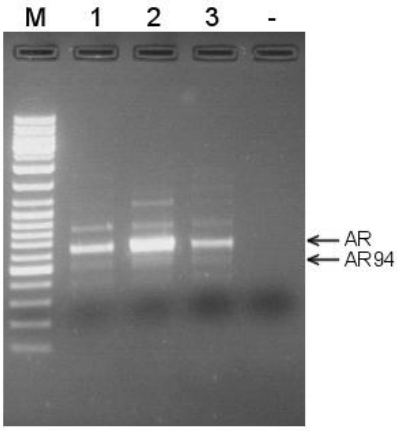

Figure 3.2 Electrophoresis of PCR (primers hAREx1Fwd and hAREx5Rv) products, showing the presence of prototype AR and a potential splice variants. M, molecular weight marker; ARΔ2, exon 2 deleted AR; AR3, exon 2 deleted AR; AR55, exon 2 deleted with partial retention of introns 2; -, template-free negative control.

The sequence alteration in AR94 would result in a protein lacking part of the LBD, but retaining complete DBD. This may indicate that ligand-binding is compromised or altered. Changes and even deletion of the full LBD does not compromise transactivation and have been shown to play a role in androgen independency in prostate cancer[402,403], and several naturally occurring alternatively spliced AR variants lacking LBD were detected in hormone-refractory prostate cancer[259,261,393]. It is predictable that AR94 might be implicated in the ligand-independent activation of AR or in the regulation of prototype AR activity. An ERα variant lacking part of LBD has been shown to be capable of ligand-independent activity[400,404-406] and has been identified both as dominant negative regulator and an enhancer of prototype ERα[404,405].

Further details of the transcripts characteristics can be found in Table 3.5. The scheme of the structure of the PCR fragments obtained can be seen in Figure 3.3.

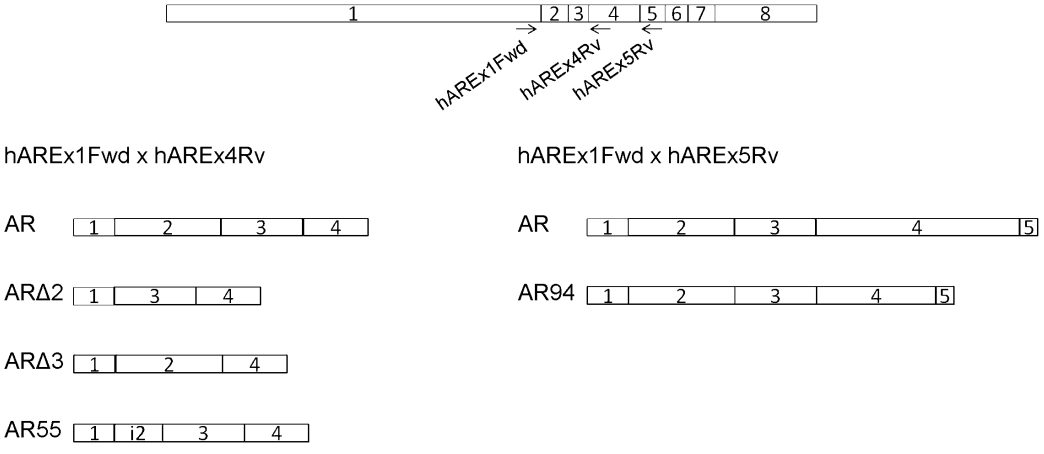

Figure 3.3 Schematic representation of the structure of PCR products obtained with each primer pair (details in text), representing potential human androgen receptor (AR) splice variants. A. Full-length AR cDNA with localization of primer binding sites. B. PCR products obtained using the hAREx1Fwd and hAREx4Rv primer pair. C. PCR products obtained using the hAREx1Fwd and hAREx5Rv primer pair. AR, prototype AR; ARΔ2, exon 2 deleted AR; ARΔ3, exon 3 deleted AR; AR55, exon 2 deleted with partial retention of intron2; AR55, exon 2 deleted with partial retention of introns 2;

AR alternative transcripts identified in the testis are expressed in several other human tissues

Applying the same strategy used for human testis, the search for potential AR alternatively spliced variants was conducted in other tissues, namely L, Lg, K, and H. The transcripts resulting from exon skipping, namely ARΔ2 and ARΔ3, were detected in all tissues analysed. Details regarding these transcripts can be found in Table 3.5.

Table 3.5 AR splice variants identified in several human tissues

Tissue	AR transcript	Splicing mechanism	Possible functional implications of translated protein
Liver Lung Kidney Heart Testis	ARΔ2	Exon deletion	Premature STOP codon that will lead to an incomplete *AR* protein with only the N-terminal domain (NTD).
	ARΔ3	Exon deletion	Lacks 2nd Zn^{2+} Finger. May be unable to orientate the receptor for DNA binding, stabilize DNA-protein interaction and provide the interface receptor dimerization.
Testis	AR55	Exon deletion; Alternative 3'-splice site.	Deletion of exon 2 and partial retention of intron 2, resulting in 23 amino acid insertion, a premature STOP codon and truncated protein with only NTD.
	AR94	Alternative 5'-splice site.	In-frame deletion of the last 120 nucleotides of exon 4 which will result in a protein with an incomplete LBD.

While ARΔ2 and ERΔ3 were detected in all tissues studied, AR94 and AR55 were only found in human testis. These may be testis-specific transcripts, although a wider study should be undertaken to ascertain that. However, mechanistic studies would be necessary to assess the activity of these altered AR forms. The fact that ARΔ2 and ARΔ3 were amplificated from different tissues may be indicative that these variants have a functional importance. However ARΔ2 sequence variation introduces a premature stop codon and this might indicate that this variant can be targeted for non-sense mediated mRNA decay[407]. Nevertheless, the existence of truncated forms for other receptors has been described, which dimerize with their respective prototype receptor and modulate their activity[408]. The presence or absence of ARΔ2 in other species may nevertheless indicate if it can have functional importance[407,409].

AR alternative transcripts identified in the testis are expressed in vertebrate evolutive line

The same strategy used for the detection of potential AR splice variants in testis and other tissues, was applied to different species throughout the vertebrate evolutive line, namely two mammals, a carnivore (dog) and a rodent (rat), fish (gilthead seabream), and frog (African clawed frog). Using this method, and species-specific primer pairs indicated in Table 3.4, the testicular prototype AR for each species was detected, as well as several potential AR splice variants (Figure 3.4).

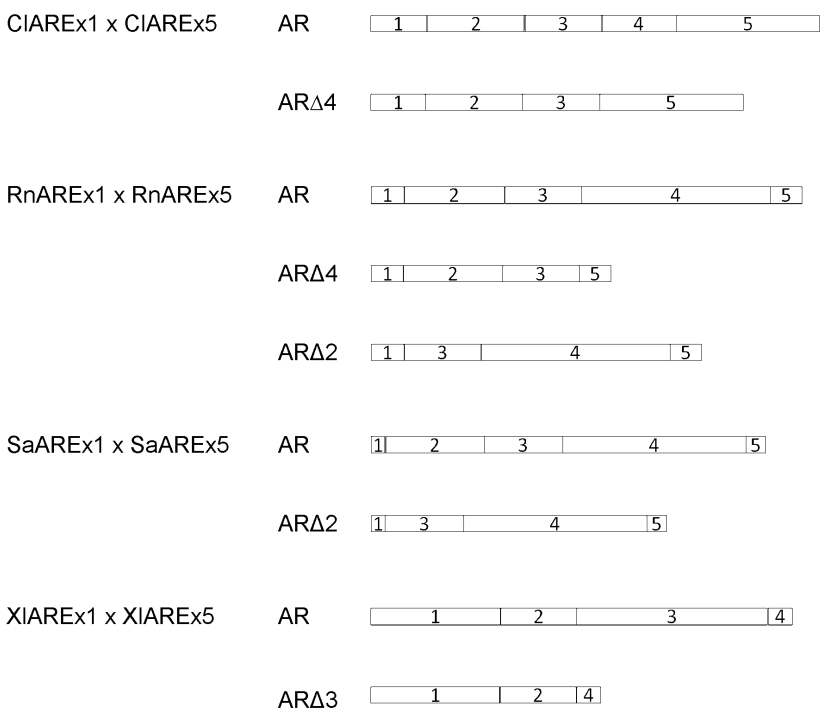

Figure 3.4 Schematic representation of the structure of PCR products obtained with each primer pair (details in Table 3.3), representing potential androgen receptor (AR) splice variants in testis of several vertebrate species. The left panel indicates the primer pair used to obtain the products depicted on the right panel. AR, prototype AR; ARΔ4, exon 4 deleted AR, ARΔ2, exon 2 deleted AR; ARΔ3, exon 3 deleted AR; Cl, *Cannis lupus*; Rn, *Rattus norvegicus*; Sa, *Sparus aurata*; Xl, *Xenopus laevis*.

Three different variants were identified, all of them resulting from exon skipping. An AR variant lacking exon 2, ARΔ2, was identified in rat and seabream testis. This variant was also identified in human testis, as well as other human tissues (Table 3.5). The deletion of exon 2 in all three species AR introduces a premature stop codon (Table 3.6), and if translated will result in a truncated protein consisting mainly of the NTD. This type of transcript generally is targeted to degradation by the mechanism of nonsense-mediated mRNA decay, however the fact that ARΔ2 is expressed in several human tissues and in more than one species is a consistent indication that it may skip the degradation pathway and have a functional role[407,409,410]. These truncated ARs may have a role in the regulation of AR action, although further studies are necessary in order to test this hypothesis.

A variant lacking exon 3, ARΔ3, was detected in frog testis (Figure 3.4). The deletion is in frame and is predicted to result in a protein without the hinge region (Table 3.6). In several human tissues including testis an ARΔ3 was found, that would result in a protein without the second Zn-finger of the DBD. The differences found between the two species ARΔ3 represent only the sequence variability due to phylogenetic distance. The hinge region of AR is responsible for the flexibility of protein, enabling the interaction between NTD and LBD[247].

Also, it contains the nuclear localization signal[121], which allows AR to translocate into the nucleus. Deletion of this domain does not mean that the resulting protein is not functional, as not all AR functions depend upon nuclear localization and NTD/LBD interaction. This variant, if translated, would be expected to be involved in non-genomic actions and as a regulator of AR activity. In ERα there are two splice variants without the hinge region that have been found in several tissues and shown to be important regulators of receptor function[411,412].

A variant lacking exon 4 of AR (ARΔ4) was found in dog and rat testis. Although lacking the same exon, these transcripts have different characteristics, as the deletion in dog AR is in frame, while the deletion in rat AR causes a frame-shift that introduces a premature stop codon (Table 3.6). Dog ARΔ4, if translated, will result in a protein lacking part of the hinge region and part of LBD, including the nuclear localization signal. What was previously said for seabream ARΔ3 applies to dog ARΔ4, and this transcript is expected to be involved in non-genomic actions as well as in the regulation of prototype AR, similarly to variants of ERα[411,412]. The rat ARΔ4 contains a stop codon that causes the protein to be truncated, while retaining the NTD and DBD. This might indicate that although this variant will be unable to bind androgens, it will still theoretically be able to bind DNA. However, it lacks the nuclear localization signal and therefore the mechanism by which it would be transported to the nucleus is unknown. Variants of AR lacking the LBD have been described and shown to have functional activity[259,261,393,402,403]. There is also the possibility that this variant might dimerize with the prototype AR and modulate its activity, as the DBD is also important for receptor dimerization[89,398,399].

Table 3.6 Potential AR alternative transcripts detected in several species and predicted protein features

Organism	Tissue	AR transcript	Splicing mechanism	Possible functional implications of translated protein
Dog	Testis	ARΔ4	Exon deletion	In-frame deletion of exon 4. Will result in the absence of almost the entire hinge region and the first 47 amino acids of the LBD: • Translocation to the nucleus may be compromised; • May not be able to have interactions between NTD and LBD, which may lead to an unstable structure; • Binding to androgens may be compromised or altered.
Rat	Testis	ARΔ2	Exon deletion	Premature STOP codon may lead to truncated protein with only NTD.
		ARΔ4	Exon deletion	Premature STOP codon may lead to truncated protein without hinge region and LBD. Retains complete DNA-binding domain.
Seabream	Testis	ARΔ2	Exon deletion	Premature STOP codon that will lead to an incomplete AR protein with only the NTD
Frog	testis	ARΔ3	Exon deletion	In-frame deletion of exon3, resulting in absence of hinge region.

Conclusion

Androgen receptor splice variants have been detected in several human tissues, especially in cases of prostate and breast cancer. Only one AR variant has been found in normal human tissue. Apart from that, there have been no studies characterizing AR alternative transcripts in normal tissues and along the vertebrate evolutive line. Through a RT-PCR based strategy with primer pairs spanning different exons, we have identified several potential AR alternative splice variants. In human testis we have identified four AR splice variants, two of them seem to be testis-specific, namely ARΔ2 and ARΔ3. Two other transcripts were found, AR55 and AR94, which were also detected in human L, Lg, K, and H. Some splice variants were conserved in testis tissue along the vertebrate evolutive line. ARΔ2 was expressed in rat and seabream testis, and ARΔ3 was present in frog testis. In addition, another exon-skipping AR variant, ARΔ4, was found in dog and rat testis.

Sequence analysis and protein translation prediction allowed extrapolating about the potential function of the hypothetical proteins. However, the translation of these variants into protein has to be confirmed, and transactivation and functional studies will be needed to clarify their functional role.

Nevertheless, the results presented herein are indicative of the existence of several alternatively spliced AR forms which may have a relevant role as regulators of androgenic

actions in testis. In addition, this study opens new lines of research to study the AR transcriptome in testis and other androgen-target tissues. Splice variants for the AR may play a role in the control of AR function, or have independent roles in androgenic signaling pathways. A deeper knowledge of these new ARs may open new lines of research and indicate provide novel targets in the treatment of idiopathic male infertility.

Acknowledgments

I would like to acknowledge Dr. Hugo Brancal (Clínica Veterinária da Covilhã, Covilhã, Portugal) for dog testis, Professor Sílvia Socorro for gilthead seabream AR sequence, and Professor Adelino Canário (CCMAR, University of Algarve, Faro, Portugal) for frog and seabream testis.

4. APOPTOSIS-INHIBITOR AVEN IS DOWN-REGULATED IN DEFECTIVE SPERMATOGENESIS AND A NOVEL ESTROGEN TARGET GENE IN MAMMALIAN TESTIS

Published in: Laurentino, SS, J Gonçalves, JE Cavaco, PF Oliveira, M Alves, M Sousa, A Barros, and S Socorro, *Apoptosis-inhibitor Aven is down-regulated in defective spermatogenesis and novel estrogen target gene in mammalian testis.* Fertil Steril. DOI: 10.1016/j.fertnstert.2011.06.009 (*in press*)

Structured Abstract

Objective: To study the expression and localization of Aven in rat and human testis from azoospermic patients with different etiologies, and its regulation by estrogens.

Design: Experimental study.

Setting: University research centre and private in vitro fertilization clinic.

Patient(s): Six men with obstructive azoospermia, five with hypospermatogenesis, and six with Sertoli cell-only syndrome. Male Wistar rats.

Intervention(s): Testicular biopsies. Rat seminiferous tubules (SeT) cultured in presence or absence of 17β-estradiol (E_2).

Main Outcome Mesure(s): Testicular cell localization of Aven protein was analysed by immunohistochemistry. Expression levels of Aven in testicular biopsies and cultured SeT, in presence or absence of 17β-estradiol (E_2), were determined by quantitative reverse transcription-polymerase chain reaction and Western blot.

Result(s): Aven is expressed in Sertoli cells, spermatocytes and spermatogonia of both rat and human testis. Aven is under-expressed in testis of men with non-obstructive azoospermia, and its expression levels correlate with severity of spermatogenic status. Aven expression is regulated by E_2 in rat SeT cultured *ex vivo*.

Conclusion(s): The results suggest that deregulation of the expression of apoptosis-inhibitor Aven may be related to male infertility. Moreover, Aven is an estrogen-target gene and may be involved in the mechanism of testicular apoptosis control by estrogens.

Key Words: Aven, apoptosis, caspase inhibitor, 17β-estradiol, testis, seminiferous tubules, Sertoli cells, spermatogenesis

Introduction

Spermatogenesis is a complex process requiring a fine tuning between germ cell proliferation and apoptosis [reviewed in [413]]. The importance of apoptosis in spermatogenic process was highlighted from studies in genetically modified animals, with loss- or gain-of-function of apoptosis regulator genes, showing impaired spermatogenesis and/or infertility[414-418]. In addition, apoptosis deregulation has been shown in the testis of infertile men with hypospermatogenesis or maturational arrest[419,420], and the expression of several apoptosis-related genes has been found to be altered in defective spermatogenesis[421-424].

Aven is an apoptosis inhibitor that acts by enhancing antiapoptotic Bcl-xL and inhibiting pro-apoptotic Apaf1 self-assembly, thus inhibiting caspase activation and apoptosis[425]. Recently,

Aven mRNA expression was identified in human[425] and mouse testis, but Vasa-homolog deficient mice, which display abnormal spermatogenesis, fail to express Aven[426]. However, the cellular localization of Aven protein in testis and the identification of the factors regulating its expression remain unknown. Using a suppressive subtractive hybridization strategy aimed at screening for novel estrogen targets in mammalian testis (data to be published elsewhere), we have identified Aven as an estrogen up-regulated gene in rat seminiferous tubules (SeT).

Estrogens, such as 17ß-estradiol (E_2), have been suggested to be involved in the control of apoptosis in mammalian testis. Mice with disruption of aromatase (*CYP19*) gene, and consequent absence of E_2, display impaired spermatogenesis due to increased germ cell apoptosis[198]. Moreover, several experiments have indicated that E_2 may act as a germ cell survival factor[185,213].

In this study we describe Aven localization in human and rat testis. Also, we identify an under-expression of Aven in non-obstructive azoospermia cases, which is correlated with the severity of spermatogenic disorder. Moreover, we report that Aven expression is under E_2 control in rat SeT cultured *ex vivo* suggesting its action as a mediator in the estrogenic regulation of testicular cells apoptosis.

The results indicate that Aven may have a relevant role in the control of testicular apoptosis as an estrogen-target gene, and changes in its mRNA levels appear to be associated with spermatogenic defect.

Materials and Methods

Reagents and chemicals

All reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA) and all antibodies supplied by AbCam (Cambridge, UK) unless stated otherwise.

Animals and tissues

Male rats (*Rattus Norvegicus*) were housed, maintained and handled in compliance with the NIH guidelines and the rules for the care and handling of laboratory animals (Directive 86/609/EEC). All rats were sacrificed under anesthesia (Clorketam1000, Vetoquinol, Lure, France).

Human testicular samples were obtained under informed consent from men undergoing treatment testicular biopsy[368] (aged 26-45 with normal kariotypes), according to the local ethical committee guidelines. Samples were histologicaly analysed and classified into the following groups according to spermatogenic status/modified Holstein's score (HS)[427]: obstructive azoospermia with conserved spermatogenesis (OAZ/HS=10, n=6), hypospermatogenesis (HP/HS=9-8, n=5), and Sertoli cell-only syndrome (SCOS/HS=2, n=6).

Ex vivo culture of rat SeT

Testes from 90 days old rats were removed, trimmed free of fat, washed in cold PBS and placed in DMEM-F12 culture medium at 33ºC. Tunica was cut and peeled back to expose tubules. Ten SeT fragments (~1cm each) and 2mL of culture medium were used per well (Nunclon D 12 well multidishes, Nunc, Denmark). Tubules were incubated with control medium (DMEM-F12 with 20mg/L gentamicin sulphate, 0.1mM 3-isobutyl-1-methylxanthine and 1mg/L BSA) alone or supplemented with 10^{-7}M E_2 and cultured for 6, 12, 24, and 48 hours. At the end of the experiment, tubules were recovered from medium, snap-frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated from human and rat testis, and rat cultured SeT with TRI reagent according to the manufacturer's instructions. Total RNAs were decontaminated from genomic DNA by digestion with deoxyribonuclease I (amplification grade DNase I, 1U/µg RNA). Complementary DNA (cDNA) was synthesized in a final volume of 20µL using 160U M-MLV reverse transcriptase (Promega, Madison, WI, USA), 0.5µg random primers (Invitrogen, Carlsbad, CA, USA), 10mM each dNTP (GE Healthcare, Buckinghamshire, UK) and 1µg of each RNA sample according to the manufacturer's protocol. cDNA was stored at -20ºC until further use.

Reverse transcription polymerase chain reaction (RT-PCR)

Specific intron spanning primer sets were designed for the amplification of human and rat Aven cDNA (Table 4.1). One µL of cDNA was amplified in a 25µL reaction containing 1x DreamTaq buffer (20mM of $MgCl_2$, Fermentas, Burlington, Ontario, Canada), 0.5U of DreamTaq DNA polymerase (Fermentas), 10mM each dNTP (GE Healthcare) and 0.2µM each primer (StabVida, Oeiras, Portugal).

Table 4.1 PCR primer sequences, amplicon size and conditions used

Gene and accession number	Primer sequences (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Concentration used (nM)
Human Aven NM_020371.2	S: CCA GCG CGC CGG TTG AAG AT AS: TGC CCA GCA ACA CAG GGC AG	513	60	200
Human Avenb	S: CTC TGC CTC CGA CTC AAC AS: CCT TGC CAT CAT CAG TTC TC	97	60	300
Rat Avena,b NM_001107757.1	S: CAG ATA GCC CAG GAG GAA ATA G AS: CTC ACG CAG ACA GCA ACC	185	60	200
Human GAPDH NM_002046.3	S: CGC CAG CCG AGC CAC ATC AS: CGC CCA ATA CGA CCA AAT CCG	76	60	300
Human ß2-microglobulin NM_004048.2	S: ATG AGT ATG CCT GCC GTG TG AS: CAA ACC TCC ATG ATG CTG CTT AC	93	60	300
Rat ß-Actin NM_031144.2	S: ATGGTGGGTATGGGTCAG AS: CAATGCCGTGTTCAATGG	97	60	200
Rat GAPDH NM_017008.3	S: GTTCAACGGCACAGTCAAG AS: CTCAGCACCAGCATCACC	115	60	200
Rat cyclophilin A NM_017101.1	S: CAAGACTGAGTGGCTGGATGG AS: GCCCGCAAGTCAAAGAAATTAGAG	163	60	200

[a] RT-PCR; [b] qPCR

Quantitative RT-PCR (qPCR)

Quantification of Aven expression in human testicular biopsies, rat testis at different post-natal ages (10, 30, 90, 120, 180, 240, and 365 post-natal days; n≥5 in all groups), and rat cultured SeT was performed by real time qPCR. Intron spanning primer sets (Table 4.1) were designed for the amplification of Aven and also internal reference genes ß-actin, GAPDH, cyclophilin A, and ß2-microglobulin . Reactions were carried out in an iQ5 system (Bio-Rad) and efficiency of the reactions was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:10, and 1:100). Primer concentration and annealing temperature were optimized and specificity of the amplicons was determined by melting curve analysis. Reaction mixtures consisted of SYBR Green master mix (Bio-Rad), sense and antisense primers (see Table 4.1 for concentrations) and 1µL of cDNA in a final volume of 20µL. All reactions were carried out in triplicate. Normalized expression values were calculated according to a published mathematical model proposed by the Vandesompele group[428].

Western blot (WB)

Total protein was isolated from rat and human testis, and rat cultured SeT using RIPA buffer supplemented with protease inhibitors (1mM PMSF; 5mM EDTA; 1x protease inhibitor cocktail). Protein concentration was determined by Bio-Rad protein assay (Hercules, CA, USA).

Proteins (50µg) were mixed with sample buffer, denatured for 10 minutes at 100ºC and resolved by SDS-PAGE. Proteins were blotted onto PVDF membranes (GE Healthcare) by wet transfer using Tris-glycine pH8.3 with 15% methanol. Membranes were blocked with tris-buffered saline with 0.05% Tween-20 and 5% dry skimmed milk for 1 hour at RT and then incubated overnight with primary mouse anti-Aven (1:750; ab77014) or rabbit anti-cleaved Caspase-9 (Asp353) antibodies (1:1000; #9507, Cell Signaling Technology, Danvers, MA, USA). Membranes were incubated for 1 hour at RT with the appropriate secondary antibody conjugated with alkaline phosphatase (1:10000, anti-mouse IgG-AP, ab7069; 1:5000, anti-rabbit IgG-AP, sc-2007, Santa Cruz Biotechnology, Santa Cruz, CA, USA), developed for 5 minutes with ECF substrate (GE Healthcare) and scanned with Molecular Imager FX (Bio-Rad). For normalization of protein expression, membranes were stripped [0.2M glycine, 1% (w/v) SDS, 1% (v/v) Tween-20, pH2.2], blocked, and reprobed using a mouse anti-ß-actin monoclonal primary antibody (1:20000; A1978, Sigma-Aldrich).

Immunohistochemistry (IHC)

Five µm sections of human (10% formalin-fixed) and rat testis (90 day old; 4% PFA-fixed) were used. Antigen retrieval was performed in 10mM citric acid pH6.0 for 30 min, at 80-85ºC. Endogenous peroxidase was blocked with 3% H_2O_2 (Panreac, Barcelona, Spain) for 10 minutes at RT and unspecific staining was blocked with normal goat serum (1:20) for 30 minutes at RT. Sections were incubated overnight at 4ºC with primary monoclonal anti-Aven antibody (1:200; ab77014) in phosphate buffered saline (PBS) with 1%BSA (PBA). Sections were then incubated with secondary goat anti-mouse biotinilated antibody (1:200; ab7067) in PBA for 1 hour at RT, followed by incubation with ExtrAvidin Peroxidase (1:20) in PBA. Staining was detected using HRP substrate solution (Dako, Glostrup, Denmark). Sections were slightly counterstained with Harris' haematoxylin (Merck) and specificity of the staining was assessed by the omission of primary antibody.

Statistical analysis

Statistical significance of differences in AVEN expression between groups was evaluated by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Aven expression in human testis), two-way ANOVA followed by Bonferroni's post test (Aven expression time-course analysis), or unpaired t-test using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Correlation between Aven average expression and spermatogenic status/modified HS was analysed by calculating a linear regression. Statistically significant differences were considered for $P<0.05$. Experimental data are shown as mean±standard error of the mean (SEM).

Results

Aven expression and localization in human and rat testis

The expression of Aven mRNA and protein was demonstrated in both human and rat testis by RT-PCR (Figure 4.1A) and WB (Figure 4.1B), respectively. In addition, qPCR analysis showed that Aven expression in rat testis increases until adulthood (90 post-natal days), after which levels are maintained constant (Figure 4.1C).

Localization of Aven protein in human and rat testis was assessed by IHC (Figure 4.1D). In both species Aven is localized mostly in Sertoli cells (SC) and spermatocytes (ST), although a weaker staining can also be observed in spermatogonia (SG).

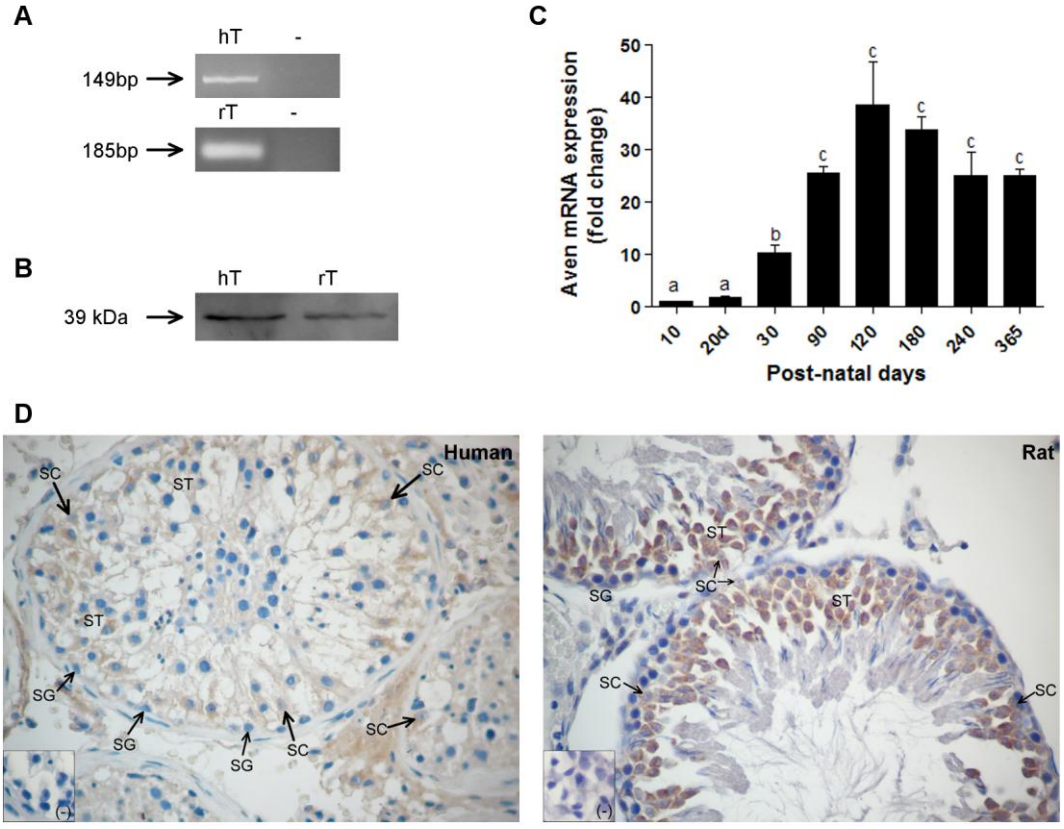

Figure 4.1 Aven expression and localization in human (hT) and rat (rT) testis. (A) RT-PCR detection of Aven in hT and rT; (-) negative control. (B) Western blot detection of Aven protein in hT and rT. (C) Aven expression during rat post-natal development determined by quantitative PCR (normalization made with cyclophilin A and GAPDH as internal reference genes); n≥5 in all groups; Different letters indicate statistically significant difference between groups (all $P<0.001$ with the following exceptions: $P<0.005$ for 30 vs. 90, 240, and 270; $P<0.05$ for 90 vs. 365). (D) Localization of Aven protein on hT and rT, left and right panels, respectively; (-) negative control, obtained by omission of primary antibody; SC – Sertoli cell; SG – spermatogonia; ST – spermatocytes. Magnification 400x

Aven expression in human testicular biopsies with different pathologies

Aven mRNA expression was quantified in human testicular biopsies with OAZ, HP, and SCOS by qPCR. Aven mRNA is lower in testis with HP and SCOS ($P<0.05$ and $P<0.001$ respectively; Figure 4.2) than in OAZ group which had normal conserved spermatogenesis, and its expression is positively correlated with spermatogenic status/HS ($r=0.79$; $P<0.001$).

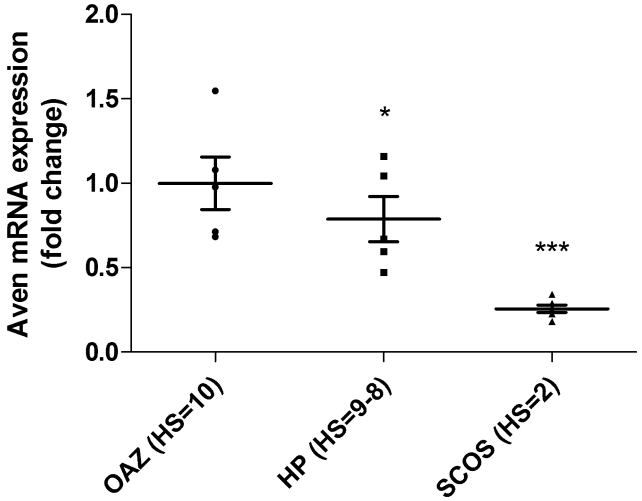

Figure 4.2 Expression levels of Aven in testicular biopsies from men with obstructive azoospermia with conserved spermatogenesis (OAZ), hypospermatogenesis (HP) and Sertoli cell-only syndrome (SCOS) determined by quantitative PCR; normalization was made using β2-microglobulin and GAPDH as internal reference genes; A trend can be observed (P<0.001) towards lower expression of Aven with increasing spermatogenic failure and decreasing modified Holstein score (HS); *P<0.05, ***P<0.001

Effect of 17β-estradiol on Aven expression in rat seminiferous tubules cultured *ex-vivo*

The effect of E_2 on Aven expression in cultured rat SeT was evaluated in a time-course experiment (Figure 4.3) by qPCR analysis. Hormonal treatment induced a significant increase in Aven mRNA expression levels (Figure 4.3A) at 12 (P<0.05) and 24 hours (P<0.001), compared to control at the same experimental times. At 48 hours a statistically significant increase of Aven protein expression (Figure 4.3B) can be observed (P<0.001) when compared to control group at the same time.

The rise in Aven expression in response to E_2 is accompanied by decreased Caspase-9 activation, a marker of apoptosis (Figure 4.3). The relative levels of cleaved Caspase-9, evaluated by quantification of its 38kDa cleavage product by WB analysis, were lower in SeT cultured in the presence of E_2 than in absence of hormone (P<0.01).

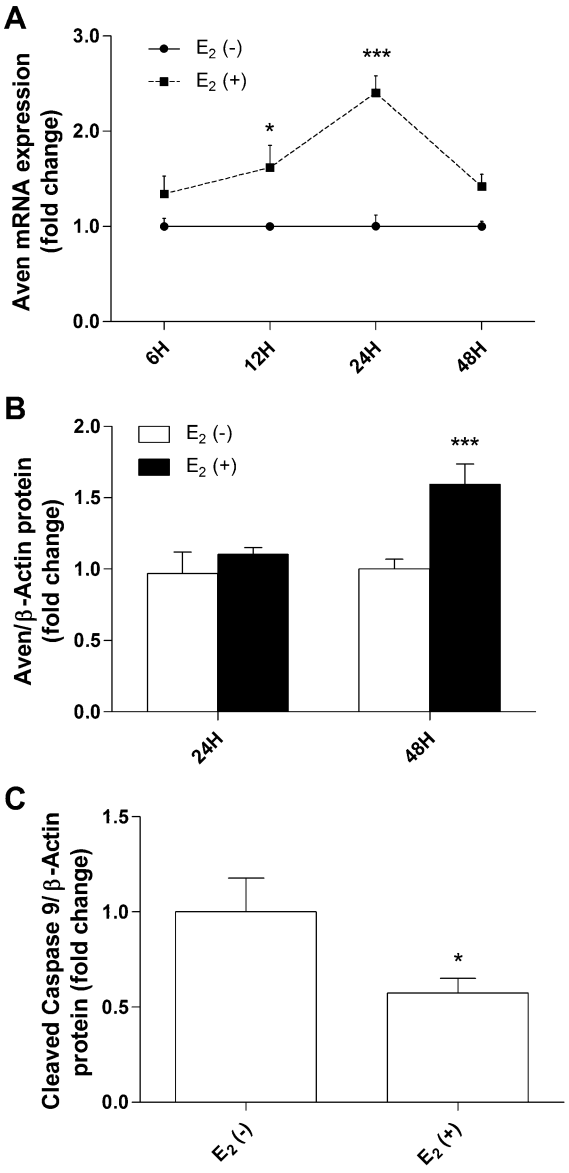

Figure 4.3 Effect of 17ß-estradiol (E_2) on Aven expression and Caspase-9 activation in rat seminiferous tubules (SeT) cultured *ex-vivo*. SeT were cultured in the absence [E_2 (-)] or presence [E_2 (+)] of 10^{-7}M of E_2 for 6, 12, 24, or 48 h. (A) Time-course expression of Aven mRNA determined by quantitative PCR; Expression levels were normalized using ß-actin and GAPDH as internal reference genes. (B) Expression of Aven protein at 24 and 48 h of E_2 stimulation determined by Western blot (WB). (C) Caspase-9 activation at 24 h of E_2 stimulation evaluated by WB quantification of the 38kDa cleavage product; * $P<0.05$; *** $P<0.001$

Discussion

Apoptosis is a physiological event necessary for the occurrence of normal spermatogenesis as it enables the control of the number of germ cells, according to the capacity of SC[429]. However, disturbance of this delicate equilibrium towards increased germ cell apoptosis can cause male infertility[413,430]. Aven has been shown to be an inhibitor of apoptosis in several cell types[425,431-434] by enhancing Bcl-xL antiapoptotic activity and inhibiting Apaf-1 self-assembly[425]. A possible role for Aven in the regulation of testicular apoptosis had not yet been

studied, although Vasa-homolog deficient mice displaying defective spermatogenesis fail to express Aven[426].

We report for the first time the expression and localization of Aven in rat and human testis (Figure 4.1) with Aven protein being localized to SC, SG and ST in both species (Figure 4.1D). One of the roles of testicular apoptosis is the quality-control of male gametes, preventing the transmission of genetic abnormalities to offspring[435]. Aven has been also shown to act as a transducer in DNA-damage response functioning as an ATM (ataraxia-telangiectasia) protein kinase activator, inhibiting G2/M cell cycle progression[436,437]. Apparently Aven is involved in a mechanism by which cells with low levels of DNA damage are saved while others, with higher levels of damage, are killed by apoptosis[436,437]. Therefore, it is likely that Aven might be involved in the regulation of germ cell progression and survival or death by apoptosis, due to DNA-damage or chromosomal abnormalities. Maintenance of SC survival may also depend of Aven's role. Bcl-W, another pro-survival protein essential for spermatogenesis[438], is expressed by SC and has been related to maintenance of these cells' integrity[417,439,440]. The SC expression of pro-survival factors such as Aven may be a mechanism by which these cells are more protected from apoptosis than germ cells[440]. However, only the development of Aven null animals may give more clues on the complete functions of this protein in spermatogenesis, both in somatic and germ cells.

Apoptosis is pivotal for the control of germ cell number in testis, which occurs by the way of extensive germ cell apoptosis during the first wave of spermatogenesis[441]. Developmentally, Aven mRNA expression in rat testis remains low until 20 post-natal days (Figure 4.1C), a period described as having the highest rate of apoptosis, during the first wave of spermatogenesis[442]. Aven expression starts increasing at 30 post-natal days (Figure 4.1C), which is also known to correspond to the start of a decrease in testicular apoptosis, remaining low throughout rat adulthood[442].

The results present herein show that Aven mRNA is under-expressed in human testis with non-obstructive azoospermia (Figure 4.2). The expression is high in OAZ with conserved spermatogenesis, decreasing in cases of HP, and reaching the lowest levels in SCOS. This shows a tendency towards lower Aven expression with increased disruption of spermatogenesis, and a positive correlation between Aven mRNA expression and HS was found. This pattern is common to other apoptosis-related genes[421-424]. For example, the expression of apoptosis inhibitor Survivin, has been shown to be correlated to spermatogenic status is a manner similar to the results presented here for Aven[423,424]. Although SCOS etiology is not related to increased apoptosis, a lower expression of Aven, as well as other apoptosis-related genes, in this phenotype may be due to the lack of several Aven-expressing cell types.

We showed that E_2 up-regulates Aven mRNA and protein expression in rat cultured SeT (Figure 4.3A and Figure 4.3B). This is the first time the control of Aven expression by a steroid hormone is described. *In silico* analysis of Aven promoter region enabled the identification of

several potential estrogen-response-elements consensus sequences, especially in region comprising nucleotides -855 to -730 (data not shown), which suggests that the E_2 observed effects on Aven expression may be driven by the intracellular estrogen receptors. Further studies will be needed to fully characterize the mechanism of estrogenic regulation of Aven expression.

The role of estrogens in the control of spermatogenesis has been given increasing importance in the past few years, namely from the evidence of aromatase knockout mice which display impaired spermatogenesis, caused by increased apoptosis of round spermatids[198]. Other studies have identified E_2 as a germ cell survival factor [185,213], but the link between estrogens' actions and the molecular targets of survival/apoptotic pathways was not evidenced in these reports.

We show that the rise in Aven expression in cultured SeT evoked by E_2 is accompanied by a decrease in apoptosis as indicated by the fragmentation of Caspase-9, a marker of apoptosis (Figure 4.3C). Therefore, the regulation of Aven expression by E_2 may be a mechanism involved in testicular apoptosis control by estrogens.

In conclusion, the results presented in this study suggest that apoptosis-inhibitor Aven may have an important role in testicular physiology, namely in the estrogenic control of apoptosis. Moreover, the changes in Aven expression seem to be associated with specific spermatogenic phenotypes.

Acknowledgments

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5. REGUCALCIN, A CALCIUM-BINDING PROTEIN, AS AN ANDROGEN TARGET GENE IN RAT TESTIS

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Abstract

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein which regulates intracellular Ca^{2+} homeostasis by modulating the activity of enzymes regulating Ca^{2+} concentration and enhancing Ca^{2+}-pumping activity. Several studies have described the pivotal role of proper Ca^{2+} homeostasis regulation to spermatogenesis and male fertility. Recently RGN was identified as a sex steroid regulated gene in prostate and breast however, a possible role of RGN in spermatogenesis has not been examined. In the present study the expression and localization of RGN in rat and human testis, and other rat reproductive tissues was analysed. Moreover, we studied whether RGN protein was present in seminiferous tubule fluid. Finally, we examined the effect of 5α-dihydrotestosterone on the expression of *Rgn* mRNA in rat seminiferous tubules cultured *ex vivo*. The results presented herein show that RGN is expressed in Leydig and Sertoli cells, as well as in all types of germ cells of both rat and human testis. Regucalcin is also expressed in rat prostate, epididymis, and seminal vesicles. Moreover RGN protein is present in rat seminiferous tubule fluid. The results also demonstrate that RGN expression is age dependent in rat testis, and is up-regulated by the non-aromatizable androgen 5α-dihydrotestosterone in rat seminiferous tubules cultured *ex vivo*. Taken together, these findings indicate that RGN is a novel androgen-target gene in rat testis and that it may have a role in male reproductive function, particularly in the control of spermatogenesis.

Introduction

Regucalcin (RGN) was first identified in the late 1970's by the Yamaguchi group in Japan as a calcium (Ca^{2+}) binding protein that does not contain EF-hand motif[443]. Independently, another research group identified and named it Senescence Marker Protein-30 (SMP30), based on its characteristic down-regulated expression with aging in rat liver[444]. Regucalcin plays an important role in intracellular Ca^{2+} homeostasis by modulating the activity of enzymes regulating Ca^{2+} concentration, and enhancing Ca^{2+}-pumping activity through the plasma membrane, endoplasmic reticulum and mitochondria of several cell types[445]. In turn, *Rgn* expression is up-regulated by increased Ca^{2+} concentration in liver and kidney cells[446-448].

Although there are no studies characterizing expression of RGN in testis, several evidences have highlighted the importance of Ca^{2+} to spermatogenesis. Calcium is essential for the maintenance of Sertoli cell (SC) tight junctions forming the blood-testis barrier[67] and modulates the activity of enzymes interfering in SC architecture[68]. The tight regulation of Ca^{2+} influx and outflux maintaining intracellular Ca^{2+} homeostasis also seems to be essential for Leydig cells (LC) steroidogenesis, for example by controlling the expression of steroidogenic acute regulatory protein[70,71]. Moreover, it has been shown that administration of Ca^{2+} channel blockers has deleterious effects on mammalian spermatogenesis, being associated with reversible infertility [*e.g.* [72-74,77,78]].

Recently, we have identified *Rgn* as a sex steroid regulated gene in rat reproductive organs such as breast and prostate[449,450]. Also we have shown that 5α-dihydrotestosterone (DHT) treatment decreases *RGN* expression in human prostate cancer cells [LnCaP; [449]]. However, the effects of sex steroids controling RGN testicular expression are unknown.

The first aim of the present work was to study the expression and cellular localization of RGN in rat and human testis and other male reproductive tissues, such as prostate, epididymis and seminal vesicles. Regucalcin has been shown to be secreted to biological fluids, namely saliva[451] and plasma[452-455]. Thus, secondly we investigated whether RGN is present in seminiferous tubule fluid (STF). Our third aim was to determine the effect of DHT on *Rgn* expression in rat seminiferous tubules (SeT) cultured *ex vivo*.

Material and Methods

Animals and tissues

Wistar male rats (*Rattus Norvegicus*) were obtained from Charles River (Barcelona, Spain) and housed under a 12-h light, 12-h dark cycle, with food and water available *ad libitum* during the course of all experiments. Housing, maintenance and handling of animals was in compliance with the NIH guidelines and the rules for the care and handling of laboratory animals (Directive 86/609/EEC). All rats were sacrificed under anesthesia (Clorketam1000, Vetoquinol, Lure, France).

Human testicular samples with conserved spermatogenesis were obtained by TESE (testicular sperm extraction) from men undergoing treatment testicular biopsy due to obstructive azoospermia (aged 35-38, with normal kariotypes). Samples were obtained under informed consent according to the local ethical committee guidelines, and treated as previously described[368].

Reagents

All chemical reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and all antibodies were obtained from AbCam (Cambridge, UK) unless stated otherwise.

Primary SC culture

Testes were removed from 22 days old rats and washed in ice cold Hanks balanced salt solution without Ca^{2+} and Mg^{2+} (HBSSf). Testes were decapsulated and washed in HBSSf. Rat SC were then isolated by a method adapted by Oliveira and collaborators[456]. Briefly decapsulated testicular tissue was placed in a glycine-containing medium [HBSSf, 1M glycine (Merck, Darmstadt, Germany), 0.005%(w/v) DNase, 2mM EDTA and 0.002%(w/v) soybean trypsin inhibitor, pH 7.2] for 10 min at room temperature (RT) in order to remove peritubular cells. The dispersed tubules were forced through a large-bore Pasteur pipette and digested with 0.015%(w/v) type I collagenase and 0.005%(w/v) DNase in HBSSf for 20 min at RT. The SC

suspension was collected by centrifugation at 300xg for 3 min, washed 3 times in HBSSf and ressuspended in SC culture medium [1:1 mix of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 15mM HEPES, 50IU/mL penicillin, 50mg/mL streptomycin sulphate, 0.5mg/mL fungizone, 50µg/mL gentamicin and 5%(v/v) heat-inactivated FBS (Biochrom, Berlin, Germany)]. The cell suspension was forced through a 20G needle, plated in culture flasks (Cell+; Sarsted, Nümbrecht, Germany) at a concentration of 5000 clusters/mL and incubated a 37ºC in an atmosphere of 5% CO_2: 95% O_2.

To obtain a culture of human SC, testicular biopsies were treated according to a method described by[457]. Isolation of SC was made by a method described elsewhere[458] with some modifications. The tubules were centrifuged at 500xg for 5 min and washed in HBSSf followed by another centrifugation. The pellet was redissolved in SC culture medium with a composition similar to the one used for rat SC culture except for the addition of 10%(v/v) heat-inactivated FBS, and cultured in a similar way.

STF collection

Seminiferous tubule fluid was collected from 90 day old rats following a method described by[32] with some modifications. Briefly, testis were removed and trimmed free of fat and connective tissue. A small incision was made at the caudal end of each testis, which was placed inside a syringe barrel within a centrifuge tube. The apparatus was centrifuged at 54xg for 15 min at 0ºC to remove interstitial fluid. The testis was removed from the barrel, the tunica was cut and peeled back, and tubules were washed 4 times in saline to remove remaining interstitial fluid and blotted onto gauze. Tubules were extruded through the hub of a syringe into a centrifuge tube and centrifuged for 30 min at 0ºC. Supernatant containing STF was collected into a fresh tube.

Ex vivo culture of rat SeT

Rat SeT were used for culture instead of individual cell types, since this model has been shown to be suitable to mimic the testicular cellular environment *ex vivo* by several groups[459-461]. Testes (90 days old rats) were removed, trimmed free of fat, washed in cold PBS and placed in DMEM-F12 culture medium at 32ºC. Tunica was cut and peeled back to expose tubules. Ten SeT fragments of about 1cm and 2mL of culture medium were used per well (Nunclon D 12 well multidishes, Nunc, Denmark). Experimental groups according to the medium composition were (n=5 in each group): control, DHT (10^{-7}M), Flu (10^{-7}M), DHT+Flu (10^{-7}M DHT and 10^{-7}M Flu), CHX(10µg/mL), DHT+CHX (10^{-7}M DHT and 10µg/mL of CHX). Control medium, to which DHT/Flu/CHX were added was DMEM-F12 supplemented with 20mg/L gentamicin sulphate, 0.1mM 3-isobutyl-1-methylxanthine and 1mg/L BSA. In groups with DHT plus Flu/CHX, the hormone was added 30 min after Flu/CHX. Tubules were incubated for 6, 12, 24 and 48 h in time-course experiments, and for 24 h in experiments with Flu and CHX. Tubules were also cultured in control medium supplemented with survival factors [10%(v/v)

heat-inactivated FBS, 1mM Na pyruvate, 4 mM glutamine, 100ng/mL vitamin A, 200ng/mL vitamin E, 50ng/mL vitamin C, 12µg/mL insulin] for 24 h. Seminiferous tubules remain viable during the course of the experiment as assessed by morphological analysis of haematoxylin and eosin stained tissue sections. At the end of each experiment tubules were recovered from medium, snap-frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated from rat and human testis, rat SeT, rat prostate, rat epididymis, rat seminal vesicles, and rat and human SC with TRI reagent according to the manufacturer's instructions. RNA concentration was measured in a spectrophotometer (NanoPhotometer, Implen, Munich, Germany) and integrity was assessed by agarose gel electrophoresis. Total RNAs were decontaminated from genomic DNA by digestion with deoxyribonuclease I (amplification grade DNase I) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized in a final volume of 20µL using 160IU M-MLV reverse transcriptase (Promega, Madison, WI, USA), 0.5µg random primers (Invitrogen, Carlsbad, CA, USA), 10mM each dNTP (GE Healthcare, Buckinghamshire, UK) and 1µg each RNA sample according to the protocol supplied by the manufacturer. Synthesized cDNA was stored at -20ºC until further use.

RT-PCR

For the amplification of human and rat RGN, rat Vim and rat AMH specific intron spanning primer sets were designed (Table 5.1). One µL of cDNA was amplified in a final volume of 25µL containing 1x DreamTaq buffer with 20mM of $MgCl_2$ (Fermentas, Burlington, Ontario, Canada), 0.5IU of DreamTaq DNA polymerase (Fermentas), 10mM each dNTP (GE Healthcare) and 0.2µM each primer (StabVida, Oeiras, Portugal). Every set of PCR reactions included a no-template control.

Table 5.1 PCR primers sequences, amplicon size and cycling conditions

Gene and accession number	Primer sequences (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
Rat Vimentin NM_031140.1	S: AGATCGATGTGGACGTTTCC AS: TCCGGTATTCGTTTGACTCC	198	50
Rat anti-Mullerian hormone NM_012902.1	S: GGCTGTGTTACAGGCTGACA AS: GACTCTTGGACAGCCTCCAG	210	54
Human RGN NM_152869.2	S: GCCTGTCCTACTCCGTGGATGC AS: GGCCACCCAGAGCTTCCCCT	143	55
Rat RGN NM_031546.1	S: TCAAAGACTGTCTGCCGATG AS: GACTGTCGAAGTGCCACTGA	93	56
Rat RGN[a] NM_031546.1	S: GGAGGAGGCATCAAAGTG AS: CAATGGTGGCAACATAGC	155	60
B-actin[a] NM_031144.2	S: ATGGTGGGTATGGGTCAG AS: CAATGCCGTGTTCAATGG	97	60
GAPDH[a] NM_017008.3	S: GTTCAACGGCACAGTCAAG AS: CTCAGCACCAGCATCACC	115	60
Cyclophilin A[a] NM_017101.1	S: CAAGACTGAGTGGCTGGATGG AS: GCCCGCAAGTCAAAGAAATTAGAG	163	60
ß2-microglobulin[a] NM_012512.1	S: CCGTGATCTTTCTGGTGCTTGTC AS: CTATCTGAGGTGGGTGGAACTGAG	150	60

[a] primer pairs used in quantitative PCR. Legend: S – sense primer, AS – anti-sense primer.

In situ hybridization

Detection of RGN mRNA in rat testis (90 days old) 4%PFA-fixed, paraffin-embeded sections was performed by hybridization with digoxigenin-labeled probes according to a protocol previously described[450].

Western blot

Total protein was isolated from rat SC, testis, prostate, epididymis, and seminal vesicles using RIPA buffer supplemented with protease inhibitors (1mM PMSF; 5mM EDTA; 1x protease inhibitor cocktail). Protein content in STF was concentrated by acetone precipitation. Protein

pellet was dissolved in RIPA buffer with inhibitors. Protein concentration was determined by Bio-Rad protein assay (Hercules, CA, USA).

Proteins (100µg) were mixed with sample buffer, denatured for 10 min at 100ºC and resolved by SDS-PAGE on 12.5% gels. Proteins were blotted onto PVDF membranes (GE Healthcare) by wet transfer using 10mM pH11 CAPS with 20%(v/v) methanol. Blotted membranes were blocked with tris-buffered saline with 0.05%(v/v) Tween-20 and 5%(w/v) dry skimmed milk for 1 h at RT and then incubated overnight with primary monoclonal anti-regucalcin antibody (1:250; ab81721). Membranes were incubated with secondary antibody conjugated with alkaline phosphatase (1:15000; anti-mouse IgG-AP, ab7069), developed for 5 min with ECF substrate (GE Healthcare) and scanned with Molecular Imager FX (Bio-Rad).

Immunohistochemistry

Five µm sections of rat (90 day old) testis, prostate, epididymis, seminal vesicles (4% PFA-fixed) and human testis tissue sections (10% formalin-fixed) were deparaffinized in xylene and rehydrated in graded alcohols. Heat-induced antigen retrieval was performed in 10mM citric acid pH6.0 for 30 min, at 80-85ºC. Endogenous peroxidase was blocked by incubating samples in 3%(v/v) H_2O_2 (Panreac, Barcelona, Spain) for 10 min at RT and unspecific staining was blocked by incubation with 1:20 normal goat serum for 30 min at RT. Sections were incubated overnight at 4ºC with primary monoclonal anti-RGN antibody (ab81721) diluted 1:50 in phosphate buffered saline (PBS) with 1%(w/v) BSA (PBA). Sections were then incubated with secondary goat anti-mouse biotinilated antibody (ab7067) diluted 1:200 in PBA for 1 h at RT, followed by incubation with ExtrAvidin Peroxidase diluted 1:20 in PBA. Antibody binding was detected using HRP substrate solution (Dako, Glostrup, Denmark). Sections were slightly counterstained with Harris' haematoxylin (Merck), dehydrated, cleared and mounted. Specificity of the staining was assessed by the omission of primary antibody.

Immunocytochemistry

Human and rat cultured SC were washed with PBS and fixed with cold 4%PFA. Permeabilization was performed by incubation with 0.01%(w/v) digitonin for 10 min at RT. Endogenous peroxidase was blocked by incubation with 0.1%(v/v) H_2O_2 for 10 min at RT and unspecific staining was blocked with PBA for 30 min at RT. Cells were incubated overnight at 4ºC with primary monoclonal anti-RGN antibody (1:50 in PBA, ab81721) or ready-to-use polyclonal anti-Vim antibody (V9 clone, Invitrogen). Cells were then incubated with secondary horse anti-mouse biotinylated antibody (BA-200, Vector labs, Burlingame, CA, USA) diluted 1:400 in PBA for 1 h at RT, followed by incubation with ExtrAvidin peroxidase diluted 1:20 in PBA. Antibody binding was detected using HRP substrate solution. After colour development sections were slightly counterstained with Harris' haematoxylin. Specificity of the staining was assessed by omission of primary antibody.

qPCR

Quantification of *Rgn* expression in rat cultured SeT and rat testis at different post-natal ages (10, 30, 60, 90, 120, 180, 240, 270 and 365 days, n≥5 in each group) was performed by qPCR. An intron spanning primer set was designed for the quantification of rat *Rgn* expression (Table 5.1). In addition two internal reference genes (ß-actin and GAPDH for cultured SeT; ß2-microglobuline and cyclophilin A for rat testis at different post-natal ages) were selected from a panel for the normalization of the expression based on their stability in the experimental conditions used according to two methods described elsewhere [[462,463]; data not shown]. Reactions were carried out in an iQ5 system (Bio-Rad) and efficiency of the reactions was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:10 and 1:100). Primer concentration and annealing temperature were optimized prior to the assay and specificity of the amplicons was determined by melting curve analysis. Reaction mixtures consisted of SYBR Green master mix (Bio-Rad), sense and antisense primers (500nM for RGN and 200nM for all other primer pairs) and 1µL of cDNA in a final volume of 20µL. Also, a no-template control was included for each reaction and all reactions were carried out in triplicate. Normalized expression values of *Rgn* were calculated according to a published mathematical model proposed by the Vandesompele group[428].

Statistical analysis

Statistical significance of differences in *Rgn* expression between groups was evaluated by one way analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison test or unpaired t test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistically significant differences were considered for $P<0.05$. Experimental data are shown as mean±standard error of the mean (SEM).

Results

Regucalcin expression and localization in rat and human cell types of the testis

Regucalcin mRNA and protein expression were analysed in rat and human whole testis and isolated SC by reverse transcription polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH), Western blot (WB), immunohistochemistry (IHC) and/or immunocytochemistry (ICC).

Analysis by RT-PCR demonstrated RGN mRNA expression in rat and human testis (Figure 5.1A). Through real time quantitative PCR (qPCR) analysis at different post-natal ages it was shown that RGN mRNA expression in rat testis reaches a maximum at 120 days, and decreases during aging process (Figure 5.1C).

The localization of RGN mRNA in rat testis was assessed using a specific digoxigenin-labelled probe (Figure 5.1D). We were able to localize RGN hybridization signal in SC and several germ cells, namely spermatogonia (SG), spermatocytes (ST) and round spermatids (RS). Specificity

of ISH staining was evaluated by hybridization with sense probe, which resulted in absence of signal (Figure 5.1D insert).

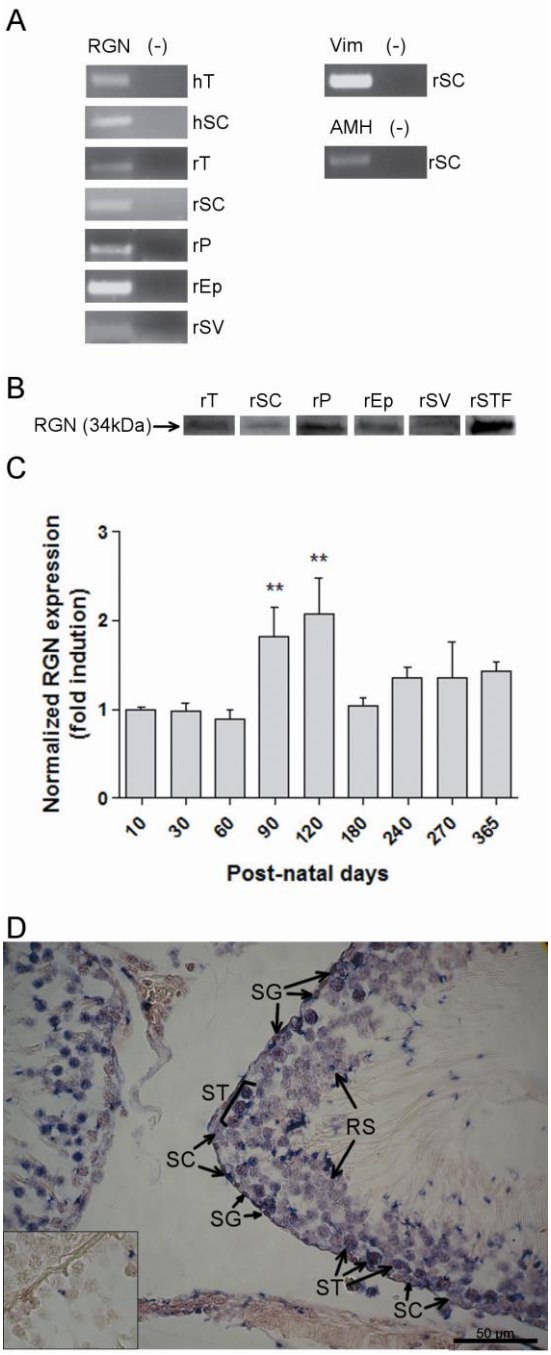

Figure 5.1 Expression of regucalcin (RGN) in reproductive tract tissues and seminiferous tubule fluid (STF). (A) RT-PCR of RGN, and Sertoli cells (SC) specific markers vimentin (Vim) and anti-mullerian hormone (AMH). (-) negative control (no cDNA template). (B) Western blot detection of RGN in protein extracts. (hT) human testis. (rT) rat testis. (hSC) human Sertoli cells. (rSC) rat Sertoli cells. (rP) rat prostate. (rEp) rat epididymis. (rSV) rat seminal vesicles. (rSTF) rat seminiferous tubule fluid. (C) Expression of *Rgn* in rT at different post-natal ages, determined by quantitative PCR, normalized with cyclophilin A and ß2-microglobulin as internal reference genes. n≥5 in each group. ***P*<0,005 relative to 10 post-natal days. Error bars represent SEM. (D) Localization of RGN mRNA transcript in adult rT by *in situ* hybridization using a digoxigenin-labelled antisense probe. Insert - hybridization with sense probe resulting in no staining. SC – Sertoli cell, SG – spermatogonia, ST – spermatocyte, RS – round spermatid. Magnification indicated as scalebar.

In rat testis RGN protein is localized to the cytoplasm and nucleus of LC and SC and also in a variety of germ cells, more specifically SG, ST, RS, elongating spermatids (ES) and spermatozoa (Sz; Figure 5.2A and Figure 5.2C). Immunohistochemistry in human testicular tissue showed a similar expression pattern for RGN, as observed by the staining of the same cell types as in rat testis (Figure 5.2B and Figure 5.2D). Although cell localization was essentially cytoplasmic, some nuclear staining is visible particularly in rat sections (Figure 5.2A and Figure 5.2C). Specificity of the IHC staining was assessed by omission of primary antibody, resulting in complete absence of immunological staining (inserts in corresponding panels, Figure 5.2).

The expression of RGN mRNA and protein in SC was confirmed using rat and human primary SC cultures. PCR amplification of SC specific markers Vimentin (Vim) and anti-mullerian hormone (AMH) (Figure 5.1A), and ICC detection of Vim (Figure 5.2G and Figure 5.2H) were used to confirm the isolation of SC. After 96 h of culture contaminant cells were fewer than 5% for both cultures. By RT-PCR, RGN mRNA expression was detected in both rat and human SC (Figure 5.1A). These cells also express RGN protein, which was detected mainly to the cytoplasm (Figure 5.2E and Figure 5.2F). Specificity of the immunostaining was assessed by the omission of the primary antibody, which resulted in complete absence of immunological staining (inserts in corresponding panels, Figure 5.2). The presence of RGN in rat SC was further confirmed by WB analysis (Figure 5.1B).

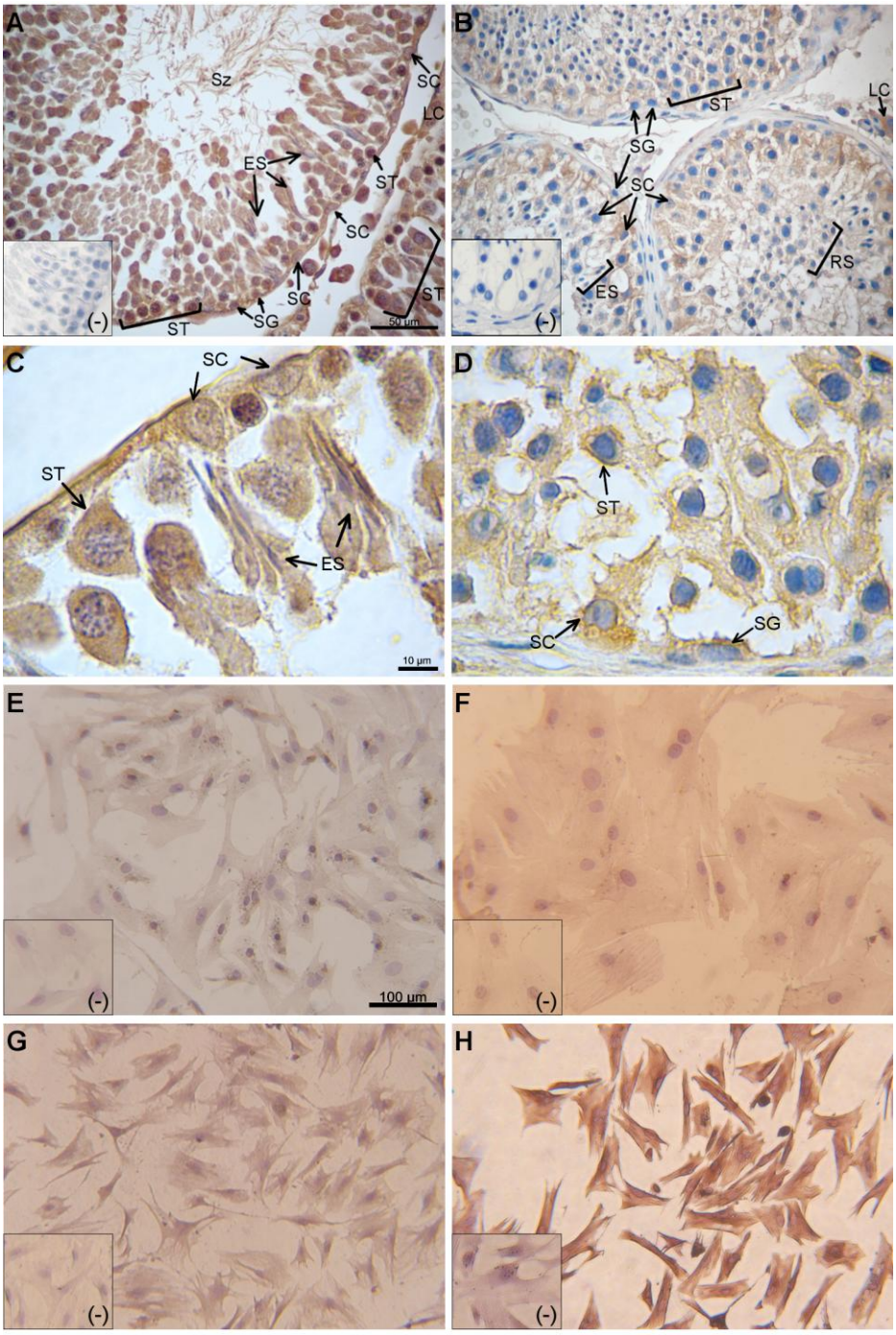

Figure 5.2 Immunochemical localization of regucalcin in testis paraffin-sections and Sertoli cells (SC) cultures. (A) rat testis. (B) human testis. (C) rat SC. (D) human SC. Purity of the rat and human SC cultures was assessed by staining with anti-vimentin antibody (E and F respectively). (-) – negative control obtained by omission of the primary antibody (inserts in corresponding panels). SC – Sertoli cell, LC – Leydig cell, SG – spermatogonia, ST – spermatocyte, RS – round spermatid, ES – elongated spermatid, Sz – spermatozoa. Magnification indicated as scalebar (B similar to A; D, E, and F similar to C)

Regucalcin expression in rat reproductive tissues and STF

The expression of RGN in other rat reproductive tissues besides testis was investigated. We have detected RGN mRNA expression in rat prostate, epididymis and seminal vesicles (Figure 5.1A). Subsequently, we confirmed the expression of RGN protein in these tissues by

detecting an immune-reactive band of expected size in WB (Figure 5.1B). Moreover, we detected the same immunoreactive band in rat STF (Figure 5.1B).

The cellular localization of RGN in rat prostate, epididymis and seminal vesicles was determined by IHC. In all three tissues RGN is localized mainly in the epithelial cells (Figure 5.3A/B/C); in epididymis it is also localized on smooth muscle cells and connective tissue (Figure 5.3B). Specificity of staining was assessed by omission of the primary antibody, which resulted in complete absence of staining (inserts in corresponding panels, Figure 5.3).

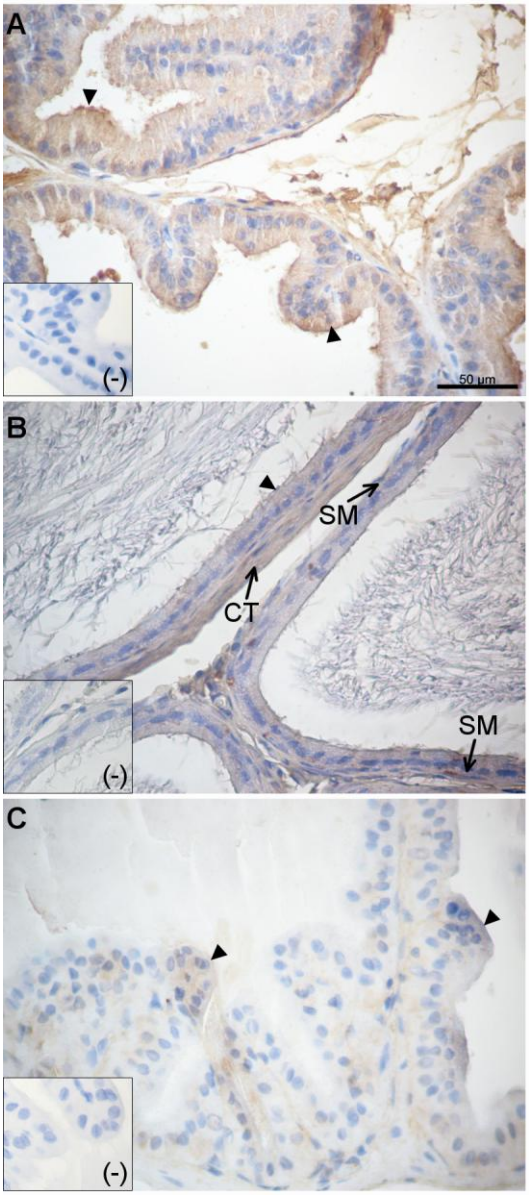

Figure 5.3 Immunohistochemical localization of regucalcin in rat prostate (A), epididymis (B) and seminal vesicles (C). (-) – negative control obtained by omission of the primary antibody (inserts in corresponding panels). Arrowhead – epithelium, CT – connective tissue, SM – smooth muscle cells. Magnification indicated as scalebar (similar in all panels)

DHT regulation of regucalcin expression in rat SeT

The effect of DHT (10^{-7}M) on the expression of RGN mRNA in cultured rat SeT was evaluated by qPCR. Firstly, a time-course experiment was performed showing that DHT induced a sharp increase in the expression levels of *Rgn* at 24 h (4.37±0.64; $P<0.001$) when compared to control (1.00±0.26), while at all other experimental times *Rgn* expression levels remained similar to control group (Figure 5.4A). The 24 h experimental time was therefore selected to explore the mechanisms involved in DHT regulation of *Rgn* expression by incubating rat SeT with 10^{-7}M DHT, and with anti-androgen Flutamide (Flu) and protein synthesis inhibitor cycloheximide (CHX), alone or in presence of DHT (Figure 5.4B). Treatment with DHT produced an increase in RGN mRNA expression (2.20±0.27; $P<0.01$) when compared to the control (1.00±0.08). Administration of Flu neutralized the hormone's effect (1.04±0.06 *vs.* 1.00±0.08 control), while incubation with CHX (2.34±0.15 *vs.* 1.00±0.08 control; $P<0.001$) did not change the up-regulating effect of DHT on *Rgn* expression. Incubation with Flu or CHX alone had no significant effect on the expression of *Rgn*.

Regucalcin expression is up-regulated in rat SeT cultured in presence of survival factors

Rat SeT were cultured in a hormone-free medium with or without survival factors, and the expression of *Rgn* quantified by qPCR (Figure 5.4C). Under survival-promoting conditions there was an up-regulation of *Rgn* expression (2.23±0.22 *vs.* 1,00±0.23 without survival factors; $P<0.005$).

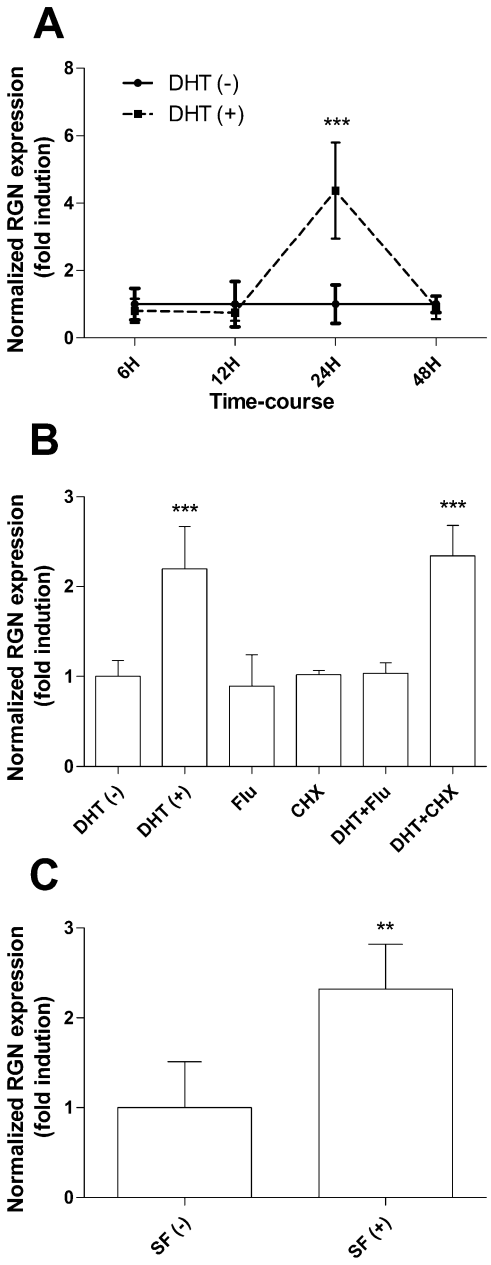

Figure 5.4 Effect of DHT and survival factors on regucalcin (RGN) expression in rat seminiferous tubules (SeT) cultured *ex vivo* determined by quantitative PCR. *Rgn* expression was normalized with ß-actin and GAPDH as internal reference genes. n=5 in each experimental condition. Error bars represent SEM (A) time-course experiment in which rat SeT were culture in the absence [DHT (-)] or presence of 10^{-7}M DHT [DHT (+)] for 6, 12, 24 or 48 h. (B) rat SeT cultured for 24 h with 10^{-7}M DHT, 10^{-7}M Flutamide (Flu), 10µg/mL cycloheximide (CHX), 10^{-7}M DHT plus 10^{-7}M Flu and 10^{-7}M DHT plus 10µg/mL CHX . ***P*<0.005 and ****P*<0.001 compared to DHT (-). (C) rat SeT cultured for 24 h in the absence [SF (-)] or presence [SF (+)] of survival factors. ***P*<0.005

Discussion

Although several evidences have highlighted the importance of Ca^{2+} homeostasis and signaling for normal spermatogenic process, a possible role of RGN in testicular physiology had not been explored. In the present study we report the expression and localization of RGN in rat and human testis and the effect of DHT on its expression.

Expression of RGN mRNA was detected in rat and human testicular tissue (Figure 5.1A) and transcripts were localized both to somatic and germ cells in adult rat testis (Figure 5.1D). A developmentally regulated expression pattern, where a peak is reached after which levels decrease with aging, has been described for *Rgn* in rat kidney and liver tissues. In rat kidney the expression of RGN mRNA starts to increase at 21 post-natal days and reaches a peak at 35 days, levels are maintained high until 3 months when it starts to decrease, returning to the low levels observed prior to 21 days[464]. In a comparable manner, *Rgn* expression in liver increases until 10 days old, reaching a plateau that is maintained until 6.5 months, decreasing in senescent rats[464]. The authors hypothesized that the age-dependent increase of *Rgn* expression in liver and kidney was coupled with periods of maturation and differentiation for both tissues and suggested it as a senescence marker[464]. Developmental analysis shows that in rat testis the characteristic down-regulation of *Rgn* expression during aging is also observed. The expression of RGN mRNA increases until it reaches a maximum at 120 days of age, a period which corresponds to rat adulthood, decreasing afterwards with rat aging (Figure 5.1C).

The results presented herein demonstrate that RGN protein is broadly expressed in rat (Figure 5.2A) and human (Figure 5.2B) testis, being localized to all cell types of the SeT epithelium, somatic as well as germ cells. Relative intensity of RGN staining appears to be weaker in human sections, a pattern we have consistently observed when analysing the localization of other proteins in human and rat testis sections (data not shown). We think that the use of different fixation protocols may be causing this histological artefact. Regucalcin immunostaining is visible in cytoplasm as well as in nucleus, which is in accordance with reports showing that RGN is able to translocate to the nucleus regulating DNA synthesis and gene expression[449,465,466]. This is the first report describing RGN expression and localization in testis of any vertebrate.

Tight control of intracellular Ca^{2+} homeostasis has been shown to be of uttermost importance to LC steroidogenesis[70,71] and maintenance of SC function[67,68,467]. The deleterious effects of Ca^{2+} channel blockers on male fertility emphasize even more the importance of tight Ca^{2+} regulation to spermatogenesis[72-74,77,78]. The common cellular localization pattern observed in rat and human testis, together with the wide cellular distribution of RGN indicate a relevant role in testicular physiology suggesting that RGN may play a role in spermatogenesis as a Ca^{2+} homeostasis regulator in both somatic and germ cells.

Knowing that RGN was identified as a secreted protein in a pea aphid saliva[451], and murine[452,453], rat[454,455], and human plasma[452] we decided to investigate its presence in STF, which could be confirmed by WB analysis (Figure 5.1B). The STF is produced essentially due to the secretory activity of SC[468], which we demonstrated, by several approaches, to express RGN. Therefore, it is highly expected that RGN present in STF may be a secretion product of SC. Exogenous RGN has been shown to translocate to the nucleus being capable of altering

gene expression and modulating enzyme activity in osteoblasts[469] and liver cells[470]. Nevertheless, to this point, the role of SeT secreted RGN remains to be determined.

In addition to testis we also analysed the expression and localization of RGN in other rat reproductive tissues: prostate, epididymis and seminal vesicles (Figure 5.3). This is the first time RGN expression and immunolocalization are reported in rat epididymis and seminal vesicles. In seminal vesicles, RGN immunostaining is confined to epithelial cells, while in epididymis it is also present in connective tissue and smooth muscle cells. In rat prostate RGN protein was detected in epithelial cells, which is in accordance with published results in rat and human prostatic tissue[449,450]. Regucalcin has been proposed to have a physiological function in prostate, as its expression is downregulated in prostate cancer tissues, and RGN immunoreactivity correlates with the grade of adenocarcinoma cellular differentiation[449]. However further studies are required to detail RGN function in these tissues.

Administration of 17β-estradiol (E_2) to rats causes an increase in the expression of RGN mRNA in liver[471]. The same effect is observed in cultured rat hepatoma cells[472]. Also, in osteoblastic cells incubation with E_2 causes an up-regulation of *Rgn* expression, while treatment with 1,25-dihydroxyvitamin D3 causes down-regulation of *Rgn* expression[473]. Contrarily, administration of E_2 to rats decreases the expression of *Rgn* in renal cortex[474]. The hormonal regulation of *Rgn* expression has also been described in sex-hormone target organs. Our group has described the down-regulation of *Rgn* expression in rat prostate and mammary gland by E_2[450]. Moreover, *RGN* is under-expressed in breast and prostate cancer cases and E_2 up-regulated while DHT down-regulated RGN mRNA expression in MCF-7 and LNCaP cell lines, respectively[449]. Dihydrotestosterone, a non-aromatizable androgen, has been shown to stimulate spermatogenesis in a similar way to testosterone[285,475,476], therefore it was used to analyse the effect of androgens on *Rgn* expression in rat SeT cultured *ex vivo*. Quantitative PCR analysis showed that DHT up-regulates RGN mRNA expression in rat cultured SeT at 24h of exposure (Figure 5.4A). Dihydrotestosterone up-regulation of *Rgn* expression is completely reversed by incubation with anti-androgen Flu, but not with CHX, an inhibitor of protein synthesis (Figure 5.4B). These data suggest the involvement of a classical genomic mechanism of gene expression regulation through androgen receptor, which seems not to depend of *de novo* protein synthesis. *In silico* analysis of the *Rgn* promoter region has in fact enabled the identification of androgen response elements upstream from transcription initiation site at positions -906, -915, -4126, and -5822 bp[449]. Nevertheless, androgens are known to increase intracellular [Ca^{2+}] in a wide array of cells, namely SC[279], human prostatic stromal cells[477], rat thoracic aorta[478] and human lymphocytes[479]. It is also known that *Rgn* expression is up-regulated by increased [Ca^{2+}][446,447]. Therefore, we do not exclude that the DHT-induced rise in *Rgn* expression may be partly due to a secondary increase of intracellular Ca^{2+}.

Maintenance of spermatogenic epithelium homeostasis requires a fine tuning between germ cell proliferation and death. Apoptosis is an essential mechanism that enables the elimination

of abnormal and exceeding germ cells and therefore its regulation is vital for normal spermatogenesis[413]. Up to 75% of germ cells undergo death by apoptosis in testis during the pubertal maturation process[480]. On the other hand, androgens are known to inhibit apoptosis of male germ cells[185,305] and testosterone withdrawal stimulates their apoptosis[481,482]. Germ cell apoptosis induced by androgen deprivation seems to be associated with caspases activation[483,484]. The role of RGN controlling apoptotic cell death has been established. Regucalcin over-expression inhibits apoptosis induced by several factors, namely, tumour necrosis factor- α and thapsigargin, dibucaine and Bay K[485,486], and it was suggested that it may regulate Akt activity[487]. In addition, RGN knockout mice hepatocytes are more susceptible to apoptotic cell death than their wild-type counterparts[488]. We hypothesize that the up-regulated expression of *Rgn* in SeT in response to DHT might be a mechanism by which androgens regulate apoptosis in testis. This is further supported by the observation that culture of SeT under hormone-free conditions although in presence of survival factors induces an up-regulation of *Rgn* expression similar to the one caused by treatment with DHT (Figure 5.4C).

In conclusion, we demonstrate that RGN is expressed in rat and human testis and other tissues of male reproductive tract, namely, prostate, epididymis and seminal vesicles. In addition, the presence of RGN in STF was identified. Our results also indicate RGN as a novel androgen-target gene in rat testis which may have an important role in the control of spermatogenesis. This opens new lines of research to explore the role of RGN and Ca^{2+} homeostasis in male spermatogenic process and fertility.

Acknowledgments

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6. REGUCALCIN, A CALCIUM-BINDING PROTEIN WITH A ROLE IN MALE REPRODUCTION?

Co-authors: S Laurentino, S Correia, JEB Cavaco, S Socorro

In Preparation for submission to Reproduction

Introduction

Regucalcin was first identified in 1978 as a calcium (Ca^{2+}) binding protein[443]. It differs from common Ca^{2+}-binding proteins such as calmodulin because it does not contain the typical ER-hand Ca^{2+}-binding motif[489]. Later it was independently identified by another group and named Senescence Marker Protein-30 (SMP-30), for its characteristic down regulation with aging in rat liver[444]. The expression of RGN is stimulated by Ca^{2+} [446,448,490] and can be regulated by several factors which include AP-1[491], ß-catenin[492], and nuclear factor I-A1 (NF1-A1)[493]. In turn, NF1-A1 binding to promoter is stimulated by phosphatidylinositol-3-kinase which can be activated by the action of numerous hormones[494].

The functions of RGN include the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which is achieved by the regulation of the activity of Ca^{2+} channels, Ca^{2+}-ATPase in the membrane of mitochondria and endoplasmic reticulum[495,496] and (Ca^{2+}-Mg^{2+})-ATPase in the plasma membrane[497,498]. Regucalcin also plays an important role in the regulation of Ca^{2+}-dependent enzymes[499]. One of these enzymes is AMP phosphodiesterase, which degrades cyclic AMP (cAMP), providing a way by which RGN regulates cAMP levels in cells[500,501]. However, most enzymes in which can be regulated this way are protein kinases and phosphatases, which in turn will regulate other proteins[502-505]. Another important enzyme whose activity is regulated, in this case inhibited, by RGN is nitric oxide synthase[506-508]. Also, overexpression of RGN can regulate the expression of a number of genes. For example, RGN overexpression up-regulates the expression of Akt-1 and Bcl-2 while down-regulating the expression of Caspase-3, resulting in inhibition of apoptosis[509]. In addition RGN also inhibits Ca^{2+}-dependent endonuclease activity, another anti-apoptotic RGN activity[510].

Regucalcin Expression in Male Reproductive Tract

For a long time the study of RGN expression was focused mainly on non-reproductive tissues. It was shown to be expressed mainly in liver and kidney cortex[446,511], but also in brain[512], heart[513], bone[514], lung[515], and submandibular gland[516]. However, RGN was also shown to be expressed in reproductive tissues such as the ovary[517], breast and prostate[449,450].

More recently, RGN expression was studied in male rat reproductive tract[518]. In prostate, RGN mRNA and protein are localized to epithelial cells[449,450,518]. In rat seminal vesicles RGN immunoreactivity was also confined to epithelial cells while on epididymis, besides epithelium, RGN was also localized to smooth muscle and connective tissue[518]. In prostate RGN's function may be related to the regulation of cell proliferation and apoptosis, since loss of RGN expression is detected in prostate cancer cases and it seems to be associated to cancer development[449].

In the testis, RGN mRNA was localized by *in situ* hybridization in all rat testicular cell types[518]. By immunohistochemistry, the broad expression of RGN protein in both rat and

human testis was confirmed, showing that in both species all testicular cell types, somatic and germ line, express RGN (Table 6.1). This common interspecies testicular localization of RGN indicates that it may play an important role in testicular physiology, an idea that is further reinforced by its presence in seminiferous tubule fluid. The SC-secreted complex fluid creates the perfect environment for germ cell maturation[468,519]. It is known that RGN protein can enter cells and modulate the activity of several enzymes, including protein kinases and phosphatases. Regucalcin also regulates Ca^{2+}-ATPases, which are known to play an important role in the mechanisms of sperm capacitation and motility[520,521]. Whether or not the presence of RGN in seminiferous tubule fluid is related to the control of testicular sperm maturation and function through the regulation of enzyme activities and intracellular $[Ca^{2+}]_i$ concentration is unknown at this point, however it would be interesting to explore RGN possible actions in sperm physiology.

Table 6.1 Localization of Regucalcin in male reproductive organs

Tissue	Cell type	Reference
Epididymis	Epithelial, smooth muscle, and connective tissue	522
Prostate	Epithelial	450,522
Seminal vesicles	Epithelial	522
Testis	Leydig, Sertoli, Spermatogonia, Spermatocytes, spermatids	522

Regucalcin Expression in Distinc Spermatogenic Phenotypes

Human testis samples were obtained by testicular biopsy from men with obstructive azoospermia and conserved spermatogenesis (OAZ; n=6), hypospermatogenesis HP (n=5), and Sertoli cell-only syndrome (SCOS; n=6). Upon RNA extraction and cDNA synthesis RGN mRNA expression was determined in different groups. This was made by quantitative PCR, using SYBR Green mastermix (Bio-Rad, Hercules, Ca, USA) and specific primers for amplification of RGN (sense: gcaagtacagcgagtgacc; antisense: ttcccatcattgaagcgattg) and internal reference genes ß2-microglobulin (sense: atgagtatgcctgccgtgtg; antisense: caaacctccatgatgctgcttac) and GAPDH (sense: cgccagccgagccacatc; antisense: cgcccaatacgaccaaatccg). Another interesting data suggesting the importance of RGN to spermatogenesis is that testis of men with HP show higher expression of RGN mRNA in (1.6 fold; $P<0.001$) relative to testis with conserved spermatogenesis (Figure 6.1).

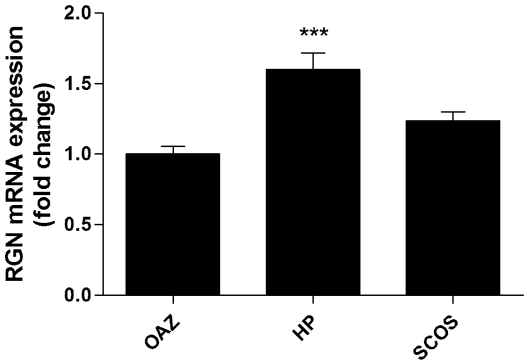

Figure 6.1 Expression levels of Regucalcin in testicular biopsies from men with obstructive azoospermia with conserved spermatogenesis (OAZ), hypospermatogenesis (HP) and Sertoli cell-only syndrome (SCOS) determined by quantitative PCR (qPCR); normalization was made using ß2-microglobulin and GAPDH as internal reference genes; ***P<0.001.

Hypospermatogenesis is thought to be caused by a deregulation of cell proliferation or apoptosis[419] and RGN has been shown to suppress cellular proliferation[523,524]. This raises the question wether the higher expression of RGN in testis of patients with HP may be causing a blockage in cell proliferation in this phenotype.

Effects of Sex Steroids on Regucalcin Expression

Regucalcin expression is regulated by numerous factors, including Ca^{2+} [446], insulin[525], aldosterone[474], sex steroid hormones[449,450,471,474,522], amongst others (Table 6.2). The regulation of RGN expression by sex steroid hormones was first described in 1995[471]. Subcutaneous administration of 17ß-estradiol (E_2) to rats leads to a sharp increase in the expression of RGN mRNA in liver, which was suggested to be related to estrogen regulation of liver metabolism[471]. However, this estrogenic control of RGN expression is not limited to the liver, as shortly after it was reported the regulation of RGN expression by E_2 in rat kidney cortex[474]. It was shown that administration of E_2 to rats caused a reduction of RGN expression in the rat kidney cortex, an effect opposite to the one observed in liver[474]. More recently, the expression of RGN in breast and prostate has been reported, as well as its regulation by sex steroid hormones[449,450]. Administration of E_2 to rats induces a down-regulation of RGN expression in prostate and mammary gland[450]. On the other hand stimulation of MCF-7 breast cancer cells with E_2 causes an up-regulation of RGN expression by a mechanism that is likely to involve a membrane estrogen receptor[449]. In LNCaP prostate cancer cells DHT down-regulates RGN expression by a mechanism that seems to involve the androgen receptor and *de novo* protein synthesis[449].

The differences in sex steroid regulation of RGN expression among different tissues may be explained by several factors[474] including tissue and cell-specific expression of different steroid hormone receptors and the interplay between them. However, the sex steroid regulated gene expression can also be modulated by several different mechanisms. For instance, the gene expression pattern in each tissue/cell also depends on the presence and activity of

coregulators (coactivators and corepressors) of the receptors transcriptional activity (for reviews on the subject read [272,526,527]). Also, sex hormone receptor activity can be modulated by the co-expression of different receptors on the same cell as some of them can form heterodimers, for example those between AR and ERs and between different ERs[528,529].

Table 6.2 Hormonal factors regulating Regucalcin expression in reproductive and non-reproductive tissues

Hormone	Tissue	Reference
Aldosterone	Kidney	474
17ß-estradiol	Liver Kidney breast, prostate	449,450,471,474
Calcitonin	Liver	530
5α-dihydrotestosterone	Testis	522
Dexamethasone	Kidney	474
Insulin	Liver	531

Regucalcin Actions in Testis Physiology

Although RGN has been shown to be an important regulator of cellular calcium homeostasis in several tissues, its role in spermatogenesis is only beginning to be studied. In mammalian spermatogenesis $[Ca^{2+}]_i$ variations have been shown to be pivotal in the control of spermatozoa motility, regulation of flagellar shape, capacitation and acrosomal reaction[532-534]. Tight control of intracellular Ca^{2+} homeostasis has also been shown to be of critical importance for the maintenance of SC function[67-69,467] and to LC steroidogenesis[70,71]. On the other hand Ca^{2+} channel blockers are known for causing negative effects on mammalian spermatogenesis, being associated with reversible infertility[72-78]. A study has also shown that spermatozoa of infertile men have altered dihydropyridine-sensitive Ca^{2+} channel activity[535]. On the other hand, the testicular localization of Ca^{2+} indicates that this ion is quite abundant in all testicular cell types, which indicates that it may play a role in cell growth and differentiation[536 537].

Calcium serves several important biological functions, such as acting as a second messenger in several transduction pathways, regulation of apoptotic cell death, among others[538,539]. Apoptosis is vital for the occurrence of normal spermatogenesis[413] since about 75% of testicular germ cells undergo apoptosis[480]. Deregulation in the cell death/survival balance is

thought to cause disruption of spermatogenesis[430] and altered expression of apoptosis-related genes has been shown to be related to male infertility[421-424]. Regucalcin has been demonstrated to regulate apoptosis both *in vivo* and *in vitro*[485,488]. Also, it has been localized on all testicular cell types in both rat and human, and is also present in seminiferous tubule fluid, indicating a potentially important role for this protein in testicular physiology, probably through the control of proliferation and apoptosis[522].

Conclusion

Regucalcin is a Ca^{2+} binding protein that plays important roles not only in the regulation of $[Ca^{2+}]_i$ but also in the regulation of cell apoptosis and proliferation. Its expression is controlled by several factors which include sex steroid hormones. Regucalcin is widely expressed along the male reproductive tract, most notably in several cells in the testis, and therefore it is predicted that it might play an important role in the regulation of spermatogenesis. A schematic model hypothesis for the role of RGN in spermatogenesis can be seen in Figure 6.2. The figure shows the hypothetical RGN gene regulation pathways, which includes the classical action through the AR as well as other pathways triggered by androgens which might also be involved. It is known that PI3K, AP-1 and ß-catenin transcription factors can regulate RGN gene expression and these factors can be activated by AR, therefore regulating RGN expression by a non-genomic to genomic pathway. Regucalcin action in the control of intracellular Ca^{2+} concentration by regulating the activity of Ca^{2+} pumps and channels in the plasma membrane and in the membrane of the endoplasmic reticulum and mitochondria. Also, it can regulate the activity of protein kinases and phosphatases, which in turn can regulate the activity of numerous phospho-proteins. Last but not least, RGN can regulate the expression of genes and the activity of proteins involved in apoptosis, favouring cell survival, is also depicted.

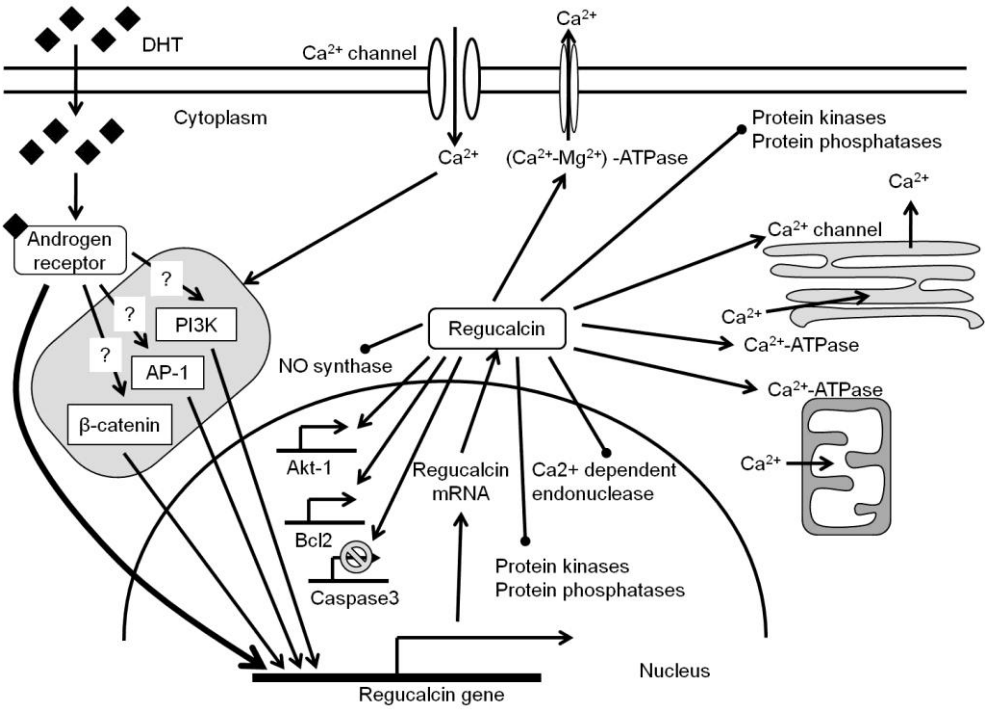

Figure 6.2 Schematic representation of the potential signalling pathways involved in the control of regucalcin (RGN) expression in testis, and the possible roles of RGN protein in testicular cells. Ball-headed arrows represent inhibition. Regucalcin expression can be regulated by an increase in the intracellular concentration of Calcium (Ca^{2+}) which activates multiple transcription factors, including PI3K, AP-1 and ß-catenin. These transcription factors are also known to be activated by androgen (such as 5α-dihidrotestosterone, DHT)-bound androgen receptor, which can also activate RGN transcription independently. In turn, RGN can increase the expression of apoptosis inhibitors Akt-1 and Bcl2, while repressing the expression of Caspase 3. It can also inhibit the activity of nitric oxide (NO) synthase and Ca2+ dependent endonucleases, thereby inhibiting apoptosis. RGN regulates intracellular Ca2+ concentration by regulating the activity of Ca^{2+} channels, Ca^{2+}-ATPase in mitochondria and endoplasmic reticulum and (Ca^{2+}-Mg^{2+})-ATPase in the plasma membrane. It can also control the activity of numerous proteins by inhibiting the activity of protein kinases and phosphatases.

In conclusion, RGN is a protein with potential importance in the regulation of mammalian spermatogenesis and the study of its precise functions can improve the knowledge of the androgenic regulation of spermatogenesis, as well as that of the control of cell survival and proliferation in testis and regulation of spermatozoa maturation.

7. SEX STEROID HORMONES AND APOPTOSIS REGULATORS AVEN AND REGUCALCIN: AN INTEGRATIVE VIEW IN SPERMATOGENESIS

Introduction

Spermatogenesis is a complex cellular process culminating with the release of the male gamets, spermatozoa, which needs to be tightly regulated in order to proceed normally. One of the main mechanisms of control is performed by the hypothalamus-pituitary-testicular axis[40]. Gonadotrophin-releasing hormone acts on the pituitary stimulating the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These two later hormones act on different cell types of the testis, FSH acts on Sertoli cells (SC) and LH acts on Leydig cells (LC). The principal function of LC is to produce sex steroid hormones from cholesterol, by a series of enzymatic reactions collectively called steroidogenic pathway, most precisely androgens like testosterone (T) and to a lesser extent 5α-dihydrotestosterone (DHT) and estrogens like 17β-estradiol (E_2)[27,29]. These sex steroid hormones play pivotal roles in the initiation and maintenance of spermatogenesis. The action of sex steroid hormones is mediated by members of the nuclear receptor superfamily, which act as ligand-activated transcription factors[80]. Androgens act through a single receptor, the androgen receptor (AR), while estrogens act through two receptors, estrogen receptor α (ERα) and β (ERβ). Male mice with a disruption of AR gene display a feminized external appearance and have severe disruption of spermatogenesis[13,323,324]. Male mice with deletion of ERα also display disrupted spermatogenesis[201,205], in contrast with animals with disrupted ERβ which seem to have normal reproductive phenotype[202,203].

Estrogens and androgens have both been linked to the regulation of male germ cell survival. The effect of androgens, mainly T, is better characterized and it is known that the survival of germ cells is controlled by the action of androgens[301-305]. On the other hand, absence of endogenous E_2 in aromatase deficient mice induces male infertility through progressive germ cell apoptosis[198], which can be prevented by treatment with exogenous estrogens[199]. Also, several studies have identified E_2 as a germ cell survival factor[185,213].

This integrative view aims at discussing the various results presented throughout the thesis and providing conclusions that integrate the different pieces of information.

Estrogen Receptors in Spermatogenesis: Monologue or Double Act?

Estrogens were generally considered female hormones, even though the first evidence that estrogens played some role in male reproduction came from a study published in the 1930's[166]. Testosterone can be converted to E_2 by the action of enzyme P450 aromatase, which is expressed by several cell types of the testis, including LC[169-172]. However there is controversy surrounding the expression of ERs in testis. Expression patterns for ERα and ERβ in rodent testis have reached divergent results (Table 7.1).

Table 7.1 Expression of ERα and ERβ in rodent testicular cells

Cell type	Expression	Reference
Leydig cells	ERα	176-178
	ERβ	179,180
Sertoli cells	ERβ	176,179,181,182
Peritubular myoid cells	ERα	179
	ERβ	179,180
Germ cells	ERα	176
	ERβ	179-181

Still, it is in human testis that the controversy is greater, as some studies have shown that both ERα and ERβ are expressed while others state that ERβ is the only receptor for estrogens in this tissue (Table 7.2). The divergent patterns in the detection of ERα and ERβ may result from the use of different antibodies and methodologies[374]. However, the expression of ERα and ERβ in human testis, both in mRNA and protein forms, has been unequivocally proven by a recent paper employing testicular biopsies of patients with distinct spermatogenic phenotypes[518] (see Chapter 2 Estrogen Receptors A and B in Human Testis: both isoforms are expressed, page 25). The use of samples from men with Sertoli cell-only syndrome (*i.e.* absence of germ cells within tubules) has also proven that SC only express ERα and not ERβ[518].

Table 7.2 Expression of ERα and ERβ in human testicular cells

Cell type	Expression	Reference
Leydig cells	ERα	183,184,518
	ERβ	183,190,518
Sertoli cells	ERα	184,518
	ERβ	183,190
Peritubular myoid cells	ERβ	190
Germ cells	ERα	185-189,518
	ERβ	185,187-189,191,192,518

Although some studies failed to detect ERα in human testis, there are indications of the importance of this receptor to spermatogenesis in humans. There are no reports describing ERβ mutations in humans, but there is one case of a disruptive mutation of ERα in a man which presented with reduced sperm viability[193]. Moreover, in humans there is an association between polymorphisms in the ERα gene and oligozoospermia[364,365]. This information adds to the fact that male knockout mice for ERα but not for ERβ are infertile[201-203,205] to show that both ERs are expressed and may influence estrogenic action in testis. This information is valuable for the study of estrogenic actions in testis because the expression of both ERs on the same cell type has consequences. Each receptor has its own effects on the transactivation of target genes[138-140]. Moreover ERα and ERβ can form heterodimers[141,142], modulating each others activity and regulating the transcription of different target genes[142,143]. Therefore, in

tissues where both receptors are present there is interplay between them and the final estrogenic effect will be determined by the balance of each receptor's activity[540].

Androgen Receptor in Spermatogenesis: the Many Faces of a Single Gene

Alternative splicing is the process which enables that one gene can give rise to a variety of different proteins. Events of alternative splicing are much more prevalent in tissues undergoing extensive developmental processes, such as the testis[395,396]. Within the nuclear receptor superfamily the existence of alternatively spliced isoforms is very common[372]. In the testis several alternative forms of ERα and ERß have been detected[123,124,126,127,191,389,391,392], and the regulation of ER action by these splice variants has been described[126,127,191]. In contrast, only one AR splice variant had been described so far in testis, AR45, which has been shown to modulate the activity of prototype AR[256]. Earlier in this thesis (Chapter 3 Identification and characterization of androgen receptor variants: tissue and vertebrate evolutive line expression, page 35) the existence of several potential AR splice variants was described. Some of them are conserved in the testis of other vertebrates being possible that they have an important functional role to play in the regulation of spermatogenesis and male fertility and to androgenic action in general. In addition, and considering the data obtained with the same type of ER transcripts, it is probable that these novel AR splice variants can be regulators of AR function in testis and other tissues. Although the role of prototype AR in spermatogensis in unquestionable, the precise function of these novel AR variants remains to be determined.

Estrogen and Androgen Regulated Genes in Testis: a Matter of Life or Death?

Estrogens and androgens act through their cognate receptors, AR and ERα/ERß, by controling the expression of target genes. In the present thesis two new sex steroid target genes were identified in testis, Aven and Regucalcin (RGN), respectively, estrogen and androgen regulated[522,541]. Both Aven and RGN play a role in cellular physiology regulating apoptosis and cell proliferation (Figure 7.1).

Aven is an inhibitor of apoptosis that acts by enhancing the activity of antiapoptotic B-cell lymphoma-extra large (Bcl-xL) and inhibiting the self-assembly of apoptosis-activating factor 1 (Apaf1) units[425]. Bcl-xL is a member of the Bcl-2 family of apoptosis regulators which includes inhibitors and promoters of cell death[542]. Bcl-xL is able to inhibit Bax and Bak induced mitochondrial membrane permeabilization (MMP), an event which leads to cytochrome c (CytC) release and activation of Apaf1 self-assembly[543]. The assembly and activation of Apaf1 converts pro-caspase 9 into active caspase-9, leading to apoptosis. Therefore, Aven inhibits apoptosis by inhibiting activation of Caspase-9 by two simultaneous

ways, inhibiting MMP and release of CytC and Apaf1 self-assembly (Figure 7.1). Aven also plays a different role in cell physiology, by modulating DNA-damage response. Aven acts as an ATM (ataraxia-telangiectasia) protein kinase activator, inhibiting G2/M cell cycle progression[436,437]. It seems that a small fraction of Aven protein is localized to the nucleus, where it regulates cell-cycle progression[544]. It seems that Aven is involved in a mechanism which enables cells with low levels of DNA damage to survive while letting cells with higher levels of damage to be eliminated by apoptosis[436,437], which might be a potentially important in preventing that DNA damage can be passed on to the offspring by way of DNA-damaged gametes.

Regucalcin has also been shown to suppress cell death and apoptosis (Figure 7.1) caused by various factors when overexpressed in numerous cell types[485,486,509,524,545,546]. The mechanism by which RGN inhibits cell death involves the increase in the activity of Ca^{2+}-pumps[545], decreasing the cytosolic concentration of Ca^{2+}, but not only. Regucalcin inhibits the activity of Ca^{2+}-dependent endonucleases, enzymes responsible for DNA fragmentation, a typical step in the events that lead to apoptosis[510]. Moreover, overexpression of RGN protein represses the expression of Caspase 3 and increases the expression of anti-apoptotic genes Akt-1 and Bcl-2, therefore inhibiting apoptosis[509].

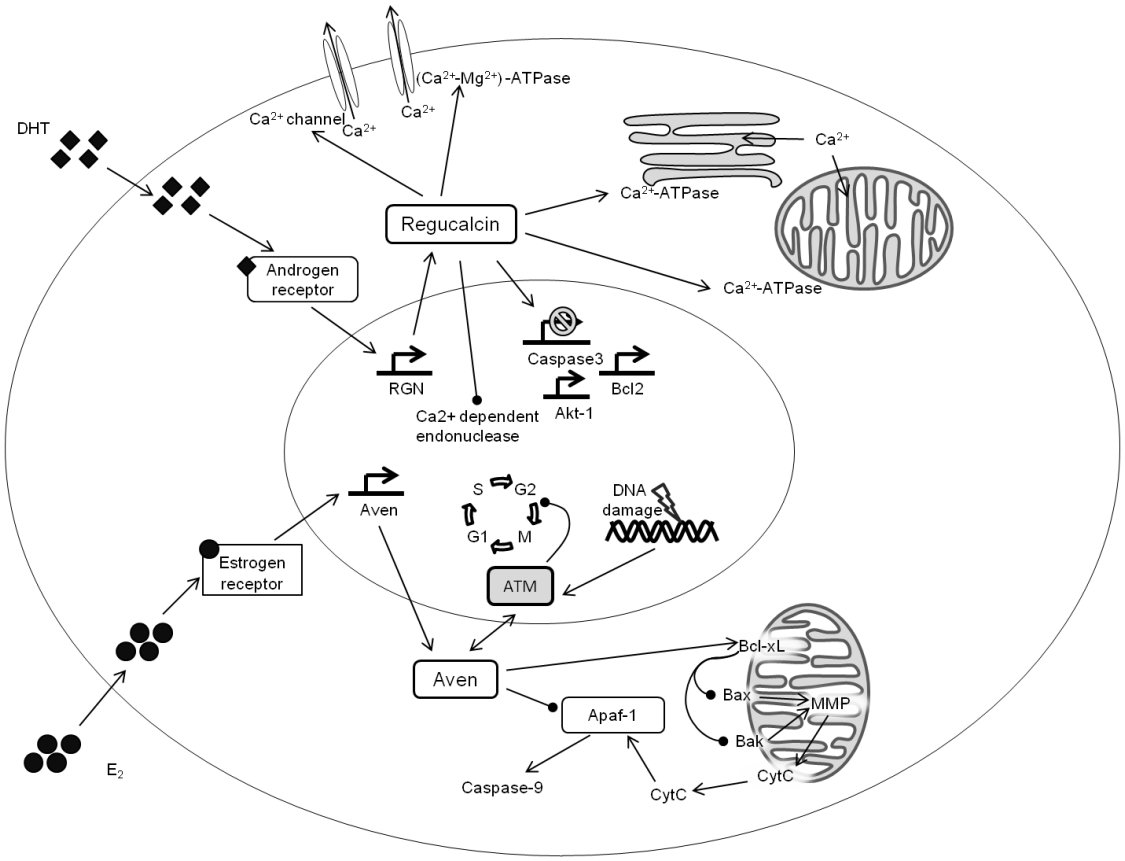

Figure 7.1 Integrative view of the potential actions of Aven and Regucalcin (RGN) in testicular apoptosis. Ball-headed arrows represent inhibition. 5α-Dihydrotestosterone (DHT) up-regulates the expression of RGN. In turn, RGN decreases intracellular calcium (Ca^{2+}) concentration by increasing the activity of several Ca^{2+} pumps in the plasma membrane, endoplasmic reticulum and mitochondria. On the other hand, RGN increases the expression of anti-apoptotic genes Akt-1 and Bcl2, while decreasing the expression of Caspase 3. Finally RGN inhibits the activity of Ca^{2+} dependent endonucleases. 17β-Estradiol (E_2) up-regulates the expression of Aven. Most Aven stays in the cytosol but a small portion will localize to the nucleus. In the cytosol Aven activates Bcl-xL, which will inhibit Bax and Bak induced mitochondrial membrane permeability (MMP) and therefore release of cytochrome c (CytC). CytC release would induce Apaf-1 self-assembly, leading to the activation of Caspase-9, stimulating apoptosis. Aven binds and inhibits Apaf-1 self-assembly thus inhibiting Caspase-9 induced apoptosis by two different pathways. In the nucleus Aven can also activate and be phosphorylated/activated by ataraxia-telangiectasia protein kinase activator (ATM) in case of DNA damage, inducing G2/M cell cycle arrest and potentially targeting cells for apoptosis.

Conclusion

Androgens and estrogens play a pivotal role in the regulation of spermatogenesis. Acting through their nuclear receptors, sex hormones act in target cells in the testis, giving rise to classical (genomic) and rapid (non-genomic) actions. Therefore understanding the structure and cellular distribution of AR, ERα, and ERß is a key step to decipher the action of androgens and estrogens in the testis. Action of estrogens in testis is now known to occur through both ERα and ERß. This has consequences where it comes to the control of gene expression as these receptors have distinct roles in the transactivation of target genes. Although the nature of androgenic action seems to be simpler at first because there is only one receptor, this is not the case. The description of splice variants for AR adds complexity to the testicular

androgenic actions. At this point the role of these variants remains to be deciphred; however the conservation along the evolutive line indicates that when it comes to AR, there is more than meets the eye. Spermatogenesis requires a fine tuning between germ cell proliferation and apoptosis [reviewed in[413]] and the importance of apoptosis in spermatogenic process has been confirmed by studies using genetically modified animals, with loss- or gain-of-function of apoptosis regulator genes, showing impaired spermatogenesis and/or infertility[414-418]. Both androgens[302-305] and estrogens[185,198,213] have been shown to inhibit germ cell apoptosis. Aven and RGN are, therefore, strong candidates as effectors of estrogenic and androgenic control of cell survival in testis (Figure 7.1).

8. CONCLUDING REMARKS

Hormonal regulation of spermatogenesis is a complex and multivariate process. Although it is clear that both androgens and estrogens are necessary for the normal occurrence of spermatogenesis, the precise mechanism by which these hormones exert their actions has gaps which are continually being filled.

This thesis has contributed to the overall picture of sexual steroid hormonal action in testis and spermatogenesis by clarifying the expression of estrogen receptors (ERs) and by improving the current knowledge on androgen receptor (AR) alternatively spliced variants. Moreover the characterization of novel estrogen and androgen target genes in the testis sheds light on the molecular mediators orchestrating estrogenic and androgenic actions in spermatogenesis and consequently in male fertility.

The identification of these "new actors" in testicular hormonal action has much potential. The fact that both ERs are expressed in testis has consequences in the study of regulation of target genes in testicular cells. On the other hand, the study of AR splice variants may improve the comprehension of androgen regulation of gene expression. The study of these variants' function as well as its expression and distribution may be an interesting target for the study of androgenic action especially in cases of idiopathic male infertility and disrupted spermatogenesis. Moreover, the newly identified target genes are connected with mechanisms of inhibition of apoptosis and control of cell proliferation, which indeed is coherent with some of the well recognized estrogenic and androgenic actions in spermatogenesis. Also, these genes were shown to be differentially expressed in testis of men with defective and disrupted spermatogenesis. The description of the precise functions of these genes in testicular physiology, as well as their possibly defective action in cases of infertility, has a strong potential in the study of male reproductive biology.

We believe that the data presented herein has given a substantial contribution towards the full-understanding of estrogenic and androgenic actions in testis and in the regulation of spermatogenesis opening new lines of research which can be further explored in the study of the ethiology, diagnostics and perhaps even treatment of male idiopathic infertility.

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SUPPLEMENTS

The ballad of Calcium and Regucalcin

3rd prize of the 2009 EMBL PhD Symposium Writing Prize.

Calcium, a word you recall
You hear it every single day.
But do you know anything at all,
Except you need it to grow tall?

Imagine a castle in the mist,
Walls and people built to resist.
Along the road a stranger nears,
Hidden in the dark a sentinel fears.

Reached is the time for a signalling arrow
To warn the people and make them ready.
The castle is a cell, with receptors steady,
Only difference is that Calcium is the arrow.

As Calcium rises and Calcium lowers,
Great things happen at the micron level.
Cells will divide, others will fade.
Muscles contract, proteins are made.

"But how" do you ask "is that arrow fired,
Is Calcium raised to later be lowered?"
That, my friends, is a difficult matter,
And fool would I be to promise an answer.

Many have looked for answers,
Many tried to solve the puzzle,
And many have dreamt of the glory.
Do you want to hear a part of the story?

In the land of the rising sun,
Dreaming was Yamaguchi-san.
He looked near and far, he searched high and low,
For a protein that could Calcium control.

In liver and kidney he found,
A new one that could be Calcium-bound.
From others he knew this was so dissimilar
'Cause the structure she had was so singular.

Proteins of her family all seemed to have,
A finger pointing up in the air,
Telling Calcium where to move
So the response could be nothing but flair.

But this little girl was so very nice
She answered all questions, from humans to mice.
"Tell me now, how do you behave?"
Asked Yamaguchi his personal fave.

And to his surprise the answer was swift
“I regulate Calcium with simplicity.
And since both of us enjoy a good shift
He regulates me with velocity.”

“I shall give you a name to remember”
Said Yamaguchi with a smile.
“Regucalcin is the newest member
Of my Calcium-binding protein family file”.

She grew up with Yamaguchi and fellows
Asking her all sorts of questions.
“Tell me do you have any good mates
To help you in your vital fate?”

“In liver” she said “where I’m happy
I like to sit down and relax.
If estrogens decide to throw a party
They come invite me to dance.”

“If in the dark side liver falls
And hepatoma starts to emerge,
No longer I wish to walz
Of disappering I feel the urge.”

In the land of the rising sun
Dreaming was Fujita-san.
He looked near and far, he searched high and low
For a protein that with aging would un-grow.

In liver of rats he found
A protein that behaved well.
In a minute his heart was bound
He couldn’t wait for its name to spell.

“Senescence-marker protein be thy name,
For your friends SMP30 you’ll be.
To our lab you shall give fame
Our work finally set free!”

She stayed with Fujita and colleagues,
Who asked her all sorts of queries.
“Besides marking aging in rats
In what else do you have expertise?”

She said “My life is boring
But one friend never misses tea
Of intracellular Calcium I regulate storing
And he, in turn, regulates me!”

“Amazing” they laughed out loud,
“You are one funny girl.
And powerful, you make us so proud.”
And with these words they began to swirl.

In the land of the rising sun
Yamaguchi- and Fujita-san
Both tried, with success, to describe
Exactly how their loved proteins were like.

“Amazing” the world laughed out loud
When in journals the girls appeared!
“Twins, they must be so proud”
Yamaguchi and Fujita were cheered.

But one and the same was the protein,
And multiple functions she had.
“How to call you” her fans asked in vain
“As you like it, as both make me glad.”

Years and years have passed
The girl grew up very strong.
The whole world had she travelled
But her journey was yet to be long.

In Portugal she arrived one day
To watch the scenery and lay.
She was looking for a lab to rest
And in the mountains she found a new nest.

To Dr. Maia she would proudly state
“Estrogens and I are great friends”
He asked “Do you like breast and prostate,
Do you think they are good trends?”

She said “In both tissues I’m very happy
I like to sit down and relax.
Androgens and estrogens throw big parties,
They always invite me for a dance”

The rest of the group joined in prayer
Asking her each one a matter
“Do you still live there when it’s greyer
And they start developing cancer?”

“No” she said rapidly,
“It gets so lonely out there,
And I tell you all honestly
That sort of things is all but fair.”

All the group showed happy faces
Delighted with taking a glance.
“Tell us, are you happy in other places
Where androgens and estrogens have a dance?”

“There is a place I recall,
A bit cold but hormones adore it!
Hardly anyone would think at all

That estrogens inside of it fit!”

“People think that of males
Only androgens take care.
Foolish thought, as all beings have
Of masculine and feminine sides a share!”

“Eureka” the team set loose
“We know to what you refer!
For males sperm to produce
To androgens and estrogens they must defer.”

“Tell us, do you know, by chance
What steps you and hormones dance?”
She smiled and answered with flair
“They pick me up and raise me in air!”

The next time you pick up a glass
And fill it with milk so bright.
Inside you a protein is waiting
Helping to make Calcium do it right!

In science, one has to be bold
For small quests for knowledge to win.
Ladies and gentlemen you’ve just been told
The Ballad of Calcium and Regucalcin.