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Estrogens regulate the survival and death communication between Sertoli and germ cells: a clue for male infertility?

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Resumo

Nas últimas décadas os estrogénios têm sido considerados hormonas masculinas, desempenhando um papel importante no controlo das funções reprodutivas masculinas. Contudo, o efeito dos estrogénios na regulação das funções testiculares ainda não está completamente abordado. As ações estrogénicas nos tecidos alvo, entre eles o testículo, são mediadas por interações hormonais com os recetores de estrogénios (ERα e ERβ) clássicos e também através do recetor membranar associado à proteína-G (GPR30/GPER). Por fim, os estrogénios alteram a rede de expressão dos genes nas células e nos tecidos, modulando o seu funcionamento. A fertilidade masculina assenta numa espermatogénese bem sucedida, a qual é dependente do suporte das células de Sertoli (SCs), as células somáticas presentes nos túbulos seminíferos (SeT). A apoptose é o evento chave que mantem o ratio apropriado entre as células germinativas e as SCs e desta forma é crucial para manter a qualidade e quantidade do processo espermatogénico. Tem sido sugerido que as SCs desempenham um papel crucial no controlo do destino das células germinativas através da secreção de fatores de sobrevivência/morte, que atuam nos recetores nas células germinativas. Esses incluem o fator de sobrevivência Desert Hedgehog (Dhh), o Stem Cell Factor (SCF) e o seu receptor (c-kit), assim como os fatores de morte Fas Ligando (FasL) e o seu recetor (FasR). Tem sido demonstrado que os estrogénios regulam a expressão do Dhh, SCF, c-kit, FasL e FasR em diversos tecidos. Portanto colocou-se a hipótese de que os estrogénios podem influenciar a sobrevivência ou morte das células testiculares através do controlo da expressão dos referidos genes. Neste trabalho, SeT e SCs de rato foram colocados em cultura na presença ou ausência de 100nM de 17β-estradiol (E_2), e a expressão dos fatores acima-citados foi estuda através das técnicas de Real-Time PCR e Western Blot. Além disso, para elucidar qual o mecanismo molecular pelo qual o efeito dos estrogénios é conseguido, SCs foram colocadas em cultura na presença de 100 nM E_2 ou 100nM de agonistas específicos para cada um dos recetores: G1, DPN e PPT, respetivamente, agonistas para o GPER, ERα e ERβ. O E_2 diminuiu a expressão do c-kit enquanto por sua vez expressão do seu ligando SCF aumentou. Não houve diferenças na expressão do Dhh entre os diferentes grupos experimentais. A expressão do SCF e do FasL nas SCs foi muito aumentada pela estimulação com G1 indicando o envolvimento do GPER. Os nossos resultados demonstram que a estimulação com estrogénios pode moldar a apoptose das células germinativas tanto de uma forma directa como através da alteração da comunicação entre as SCs e as células germinativas, podendo isto ter um profundo impacto na fertilidade masculina em especial nos casos de hiperesteroidismo.

Palavras- Chave:

Espermatogénese, apoptose, estrogénios, células de Sertoli, túbulos seminíferos, SCF, Dhh, c-kit, FasL, FasR

Resumo Alargado

Nas últimas décadas os estrogénios têm deixado de ter uma conotação tipicamente feminina para serem igualmente considerados hormonas masculinas, desempenhando um papel importante no controlo das funções reprodutivas no homem. Uma das bases para esta ligação à fisiologia masculina conta é a presença dos recetores de estrogénios em vários tecidos e células do aparelho reprodutor masculino. No testículo, para além da presença dos recetores, há ainda a referir a sua capacidade de síntese de estrogénios, uma vez que a enzima aromatase, que converte o percursor testosterona a estradiol, está igualmente presente nos diferentes tipos de células no testículo. Contudo, o efeito dos estrogénios na regulação das funções testiculares ainda não está completamente abordado nem conhecido. As ações estrogénicas nos tecidos alvo, incluindo o testículo, são mediadas por interações hormonais com os recetores de estrogénios clássicos (ERα e ERβ) e também através do recetor associado à proteína-G (GPR30/GPER) alterando a rede de expressão dos genes nas células e nos tecidos, modulando o seu funcionamento e as suas funções no controlo dos processos para os quais estão destinados. A fertilidade masculina assenta numa espermatogénese bem sucedida, a qual é dependente do suporte das células de Sertoli (SCs), as células somáticas presentes nos túbulos seminíferos (SeT), que têm funções de suporte físico e bioquímico das células da linha germinativa. A regulação da espermatogénese é estreitamente assegurada por hormonas libertadas pela pituitária anterior, a FSH e a LH, que atuam, respetivamente, estimulando a função das SCs e a produção de androgénios pelas células de Leydig (LCs). Os níveis de FSH e LH são, por sua vez, regulados por feedbacks negativos pela inibina e testosterona presentes na circulação sanguínea, e também pela libertação de GnRH pelo hipotálamo. O desenvolvimento das células germinativas ocorre numa ligação intima com as SCs, apresentando uma distribuição dos diferentes estágios da diferenciação de uma forma não aleatória no epitélio seminífero. Esta distribuição é dependente da manutenção do número de células germinativas que cada SC pode suportar. A apoptose é o evento chave que mantem o ratio apropriado entre as células germinativas e as SCs e desta forma é crucial para manter a qualidade e quantidade do processo espermatogénico. A apoptose é ainda um processo natural usado para eliminar células germinativas deficientes ou em excesso. Tem sido sugerido que as SCs desempenham um papel crucial no controlo do destino das células germinativas pela secreção de fatores de sobrevivência/morte, que atuam nos recetores nas células germinativas, mas também por conferirem um suporte físico para o seu desenvolvimento. Os fatores de sobrevivência secretados pelas SCs incluem o Desert Hedgehog (Dhh) e o Stem Cell Factor (SCF). O Dhh está envolvido no controlo nas divisões meióticas e no funcionamento das LCs, ao passo que o SCF parece ser essencial para o desenvolvimento das células germinativas através da estimulação da migração das células germinativas primordiais, da proliferação e da sobrevivência das espermatogónias. O SCF secretado pelas SCs tem o seu recetor, o c-kit nas células germinativas. O c-kit tem um papel na regulação da espermatogénese no desenvolvimento das células germinativas antes e depois

do nascimento, desempenhando um papel importante na manutenção de um ratio saudável entre espermatogónias diferenciadas e em auto-renovação. Na ausência do c-kit tende a haver uma depleção do número de células germinativas no testículo. O sistema Fas, incluindo o Fas Ligando (FasL) e o seu recetor (FasR) são dois mediadores da morte celular programada, cuja ação determina a ativação das proteínas efetoras da apoptose. O FasL e o FasR têm sido apontados como estando envolvidos no controlo da apoptose das células germinativas por ação das SCs. Tem sido demonstrado que os estrogénios regulam a expressão do Dhh, SCF, c-kit, FasL e FasR em alguns outros tecidos onde eles desempenham a sua função. Deste modo, colocou-se a hipótese de que os estrogénios podem influenciar a sobrevivência ou morte das células testiculares através do controlo da expressão dos referidos genes. Assim neste trabalho, SeT e SCs de rato foram colocados em cultura na presença ou ausência de 17β-estradiol (E_2), e a expressão dos fatores acima-citados foi estudada através das técnicas de Real-Time PCR e Western Blot. Numa primeira fase, SeT foram postos em cultura com (100nM) ou sem E_2 e o efeito na expressão de Dhh, SCF, c-kit, FasL e FasR foi determinado. Além disso, para elucidar qual o mecanismo molecular pelo qual o efeito dos estrogénios é conseguido, SCs foram colocadas em cultura na presença (100nM) de agonistas específicos para cada um dos recetores: G1, DPN e PPT, respetivamente, agonistas para o GPER, ERα e ERβ. O E_2 diminuiu a expressão do c-kit nos SeT, enquanto a expressão do seu ligando SCF aumentou tanto em SeT como em SCs. Por sua vez, não houve diferenças na expressão do Dhh entre as condições com ou sem estimulação hormonal. A expressão do SCF e do FasL nas SCs foi muito aumentada pela estimulação por G1, o que indica o envolvimento do GPER. Os nossos resultados demonstram que a estimulação com estrogénios pode modular a apoptose das células germinativas tanto de uma forma direta, aumentando a expressão do FasR e diminuição da expressão do c-kit, assim como através da alteração da comunicação entre as SCs e as células germinativas através do aumento da expressão do FasL, podendo isto ter um profundo impacto na fertilidade masculina em especial nos casos de hiperesteroidismo.

Abstract

In the last decades estrogens have been regarded as “male hormones” playing an important role controlling male reproductive function. However, the effect of estrogens regulating testicular function and the spermatogenic process it is not fully addressed. Estrogenic actions in target tissues, including testis, are mediated by hormone interaction with the classical estrogens receptors (ERα and ERβ) and also via the membrane G-protein coupled receptor (GPR30/GPER). Ultimately, the estrogens alter the gene expression network in cells and tissues modulating its functioning. Male fertility relies on a successful spermatogenesis, which is dependent from the support of Sertoli cells (SCs), the somatic cells within seminiferous tubules (SeT). Apoptosis is a key event strictly maintaining the appropriate ratio between germ cells and SCs and, thus, it is crucial to maintain the spermatogenic output. It has been suggested that SCs play a crucial role controlling germ cells fate, by secretion of survival and death factors, which act on receptors in germ cells. These include the survival factor desert hedgehog (Dhh), the stem cell factor (SCF) and its receptor the c-kit, as well as the death factors Fas-Ligand (FasL) and Fas-receptor (FasR). It has been shown that estrogens regulate Dhh, SCF, c-kit, FasL and FasR expression in several other tissues. Therefore, we hypothesize that estrogens may influence germ cell survival or death in testicular cells by governing the expression of SCF, c-kit, FasL, FasR. In the present work rat SeT and SCs were cultured in presence or absence of 100nM of 17ß-estradiol (E_2), and the expression of the aforementioned factors was studied through real-time PCR and Western blot techniques. In addition, in order to start elucidating the molecular mechanisms by which the estrogenic effects are attained, SCs were cultured with E_2 0,1nM and with 100nM of each ER specific agonist: G1, DPN and PPT, respectively, agonists for GPER, ERβ and ERα. E_2 down-regulated the c-kit expression while increasing expression of its ligand, SCF, both in SeT and SCs. The expression of Fas system, FasR and FasL was also increased in response to E_2. No differences were found in Dhh expression between experimental groups. The expression of SCF and FasL in SCs was strongly increased by G1 stimulation indicating the involvement of GPRER. Our results demonstrated that the estrogenic stimulation may modulate germ cell apoptosis in a direct way or through altering germ cell:SCs communication, which could have a profound impact in male fertility, particularly in cases of hyperestrogenism.

Keywords

Spermatogenesis, apoptosis, estrogens, Sertoli cells, seminiferous tubules, SCF, Dhh, c-kit, FasL, FasR

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Abbreviations

Apaf-1 – Apoptotic peptidase activating factor 1

BSA- Bovine serum albumin,

cDNA - Complementary Deoxyribonucleic Acid

CytoC – Cytochrome C

DMEM: Ham's F12 - Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12

DEPC - dietilpirocarbonato

E2 – 17β-Estradiol

EDTA - Ethylene Diamine Tetra Acetic acid

ERs – Estrogen receptors

ERα – Estrogen receptor α

ERαKO - Estrogen receptor α knockout

ERβ – Estrogen receptor β

ERβKO - Estrogen Receptor β Knock-out

EtOH – Ethanol

FSH – Follicle-stimulating hormone

GnRH - Gonadotropin releasing hormone

HBSS - Hank's Balanced Salts Solution

HEPES- (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

LCs – Leydig cells

LH – Luteinizing Hormone

mRNA - Messenger Ribonucleic Acid

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PI3-K - Phosphatidylinositide 3-kinases

RIPA - Radio-Immunoprecipitation Assay

RNA - Ribonucleic Acid

RNAt - Total Ribonucleic Acid

SeT- Seminiferous tubules

SCs – Sertoli cells

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

T - Testosterone

TBS - Tris-Buffered Saline Solution

Introduction

1. Brief Overview of Testicular Anatomy

Testes are the central elements of the male reproductive tract having two main functions, steroid hormone synthesis and spermatozoa production [2]. Spermatogenesis is a multi-step process responsible by germ cell expansion and development, finishing with the release of spermatozoa [3]. Anatomically, testes are ovoid organs, suspended outside abdominal tissue, in the scrotum, which is internally divided into two sacs, one for each testis [4]. Testes are covered by a fibrous capsule called tunica albuginea, which emits extensions into the testis dividing it in lobules (Fig. I.1). Each lobule has between 3 and 10 convulated seminiferous tubules (SeT). Mammalian testes are composed of two main compartments (Fig.I.1): the interstitial space with androgens producing cells (Leydig cells, LCs) and SeT that contain germ cells in different stages of development and somatic Sertoli cells (SCs) [2]. This structural division also reflects the dual function of testes, with spermatogenesis occurring in the SeT and steroidogenesis happening essentially in the interstitium [5]. SeT are surrounded by mesenchymal cells and represent the majority of testicular mass, being considered the functional unit of the testis. Since they are the place where spermatogenesis occurs. The seminiferous epithelium (Fig. I.1) is composed by germ cells in various stages of development (spermatogonia, spermatocytes, spermatids and spermatozoa) and also SCs [6]. Besides LCs, the interstitium also contains blood and lymphatic vessels, connective tissue and other cell types, such as, fibroblasts, macrophages and leukocytes [7].

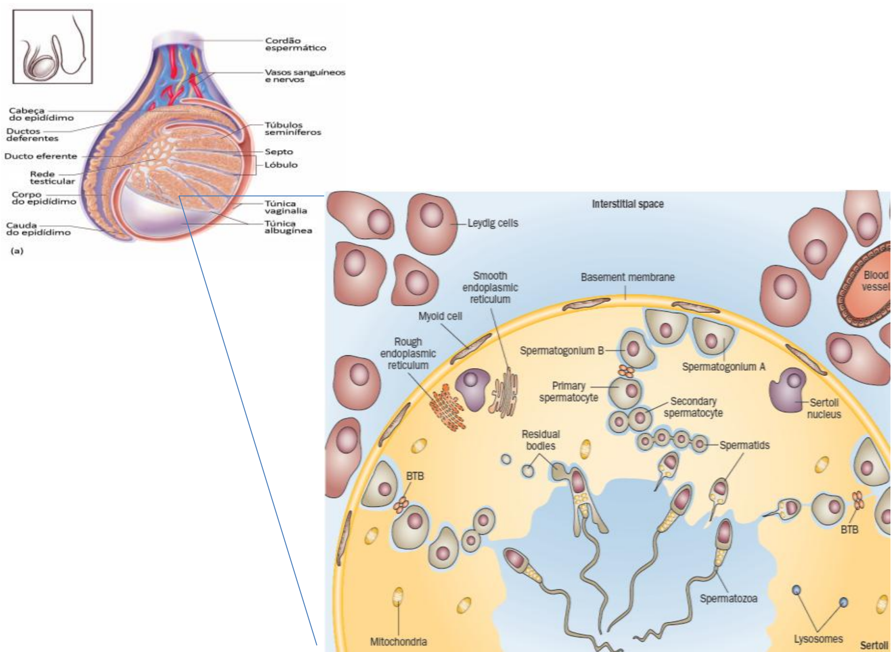

Figure I.1 Schematic representation of the organization of mammalian testis and seminiferous tubule (SeT). The testis is encased by two tissue layers, from the inside to the outside, tunica albuginea and tunica vaginalis. Various septum extending from the tunica albuginea divides testis in lobules where SeT are located. The seminiferous epithelium is composed of Sertoli and developing germ cells at different stages. Leydig cells and blood vessels are in the interstitium. Spermatogenesis produces male haploid germ cells from diploid spermatogonial stem cells. Spermatogonia type A divide and develop into spermatogonia type B, which differentiate into primary spermatocytes that undergo meiosis I to separate the homologous pairs of chromosomes and form the haploid secondary spermatocytes. Meiosis II yields four equalized spermatids that migrate towards the lumen where fully formed spermatozoa are finally released. Abbreviations: BTB, Blood-testis Barrier. Adapted from Rato [8] and Saladin [9].

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2. The Spermatogenic Process

2.1. Cellular and Hormonal Factors

Mammalian male fertility depends on successful generation of motile spermatozoa carrying an intact paternal genome and capable of fertilizing the egg [2]. Spermatogenesis is a cellular complex process controlled by a network of endocrine and other regulatory factors [10], where immature germ cells undergo division, differentiation, and meiosis giving rise to haploid spermatozoa [11]. Spermatogonial stem cells are maintained in a specialized microenvironment that is composed by germ cells, somatic support cells (SCs) and the extracellular matrix [12]. SCs play essential roles via paracrine pathways to control all aspects of development of germ cells in the testis, thereby regulating spermatogenesis [13]. In humans Spermatogenesis produce an average of $200{\times}10^{6}$ spermatozoa per day [14], which begins by mitotic proliferation of type A and type B spermatogonia. Then type B spermatogonia divide by mitosis and differentiate forming spermatocytes. After prophase of

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the first meiotic division, cell division process is completed and two subsequent divisions produce haploid round spermatids. Spermiogenesis is the last differentiation phase of spermatogenesis, where spermatids experience chromatin condensation and nuclear shaping, removal of excess cytoplasm, and the acrosome and sperm tail formation. Finally, fully developed spermatozoa are released into the lumen of SeT [15]. Although the spermatogonial differentiation starts in fetal life in humans, spermatogenesis only begins at puberty [16].

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The classical male sex steroid hormone testosterone and follicle-stimulating hormone (FSH) are the principal hormones controlling spermatogenesis. LCs, derived from interstitial mesenchymal tissue between the tubules, are the main source of testosterone playing a crucial role in masculinization events, descent of the testes into the scrotum and initiation and maintenance of spermatogenesis [17, 18]. Hormonal secretion by somatic cells of the testis is under the master control of hypothalamus-pituitary, encompassing the so-called hyphotalamus-pituitary-testis axis (Fig. I.2). Gonadotrophin releasing hormone (GnRH) is an hypothalamic hormone that induces production and secretion of gonadotrophins, FSH and luteinizing hormone (LH), by gonadotroph cells in the anterior pituitary [19].

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FSH acts in SCs through G-coupled receptors and controls the proliferation of SC during perinatal and pubertal periods [20]. FSH also established SCs functionality in adult life, being a major determinant of adult spermatogenic capacity [20]. In turn, LH regulates testosterone production by the LCs (Fig. I.2), which diffuses into the SeT where, together with FSH, stimulates SCs activity, germ cells maturation and sperm production [21].

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The fine accurate regulation of spermatogenesis is achieved by negative feedback mechanisms exerted by testicular hormones at pituitary and hypothalamus (Fig. I.2). Testosterone inhibits LH secretion in two ways. It acts on the hypothalamus to decrease the frequency of GnRH bursts, resulting in a decreased amount of GnRH reaching the pituitary and less secretion of the gonadotropins, or acts directly on the anterior pituitary leading to a decreased LH secretion, in response to any given level of GnRH. Inhibin is a hormonal product of SCs secretion acting on the anterior pituitary decreasing the FSH levels, or on the hypothalamus, decreasing GnRH levels [22].

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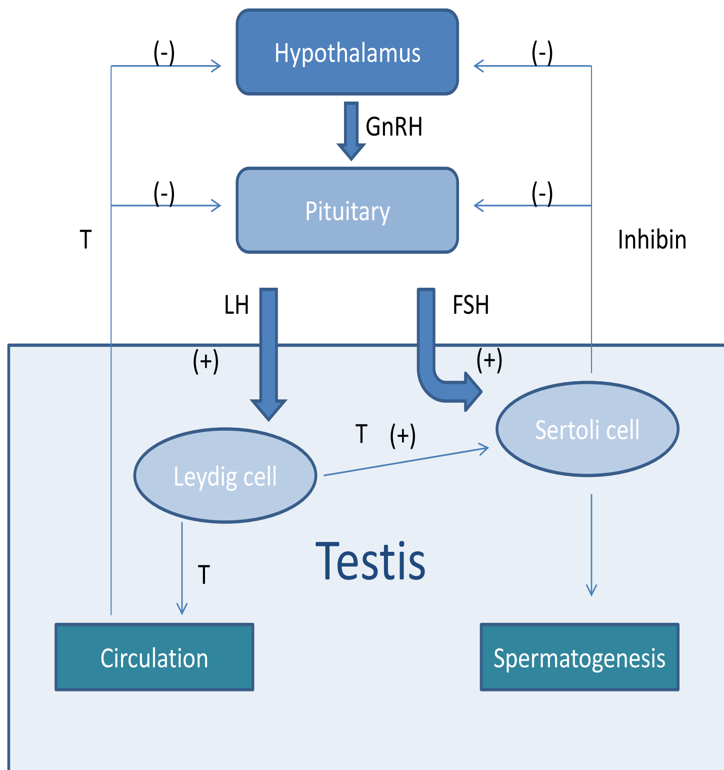

Figure I.2 Schematic representation of the hormonal regulation of spermatogenesis. Hypothalamus synthesizes the gonadotropin releasing hormone (GnRH), which in turn stimulates the anterior pituitary to produce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH acts only on the Sertoli cells (SCs) leading to the stimulation of spermatogenesis, whereas LH acts only on the Leydig cells that produce testosterone. The secretion of FSH is inhibited mainly by Inhibin, a proteic hormone secreted by the SCs, and the secretion of LH is inhibited mainly by testosterone. (-) negative feedback; (+) positive feedback

2.2. Importance of Sertoli cells in Spermatogenesis

Sertoli cells are located within the SeT along the basement membrane in close contact with germ cells in different stages of germ cell development [23]. SCs have a complex structure, with numerous cupshaped processes surrounding and conferring physical support to all germ cell types. The various generations of germ cells, are not randomly distributed within the seminiferous epithelium, but are arranged in strictly defined cellular associations [24]. SCs establish Sertoli-Sertoli and Sertoli-germ cell interactions by means of tight, anchoring, and gap junctions which are continuously being re-structured allowing germ cell movement from basal to luminal side, with elongated spermatids close to the lumen, while, spermatogonia are restricted to the basal compartment (Fig. I.1) [25].

Sertoli cells also confer biochemical support to germ cells at different stages of development, secreting a number of locally produced autocrine and paracrine factors, namely growth factors, such as glial cell line-derived neurotrophic factors [26], basic fibroblast growth factor, and epidermal growth factor [27]. SCs sustain the proliferation and differentiation of

spermatogonial stem cells, and by this reason, are also known as “nurse cells”. In fact, they secrete metal ions, nutrients and several metabolic substrates ensuring the nutritional support of germ cells, their appropriate development and, thus, male fertility [28-30].

In addition, since germ cells do not contain receptors for testosterone and FSH, the endocrine factors regulating germ cells development, exert their biological effects on spermatogenesis via the receptors located in or on the plasma membrane of SCs [31]. Therefore, SCs are the major cellular target for the testosterone signaling and are required to support male germ cell development and survival [29]. Within the SeT, germ cell development is maintained by signals originated from SCs in absolutely dependence of androgens and FSH, as well as on their receptors signaling pathways [19]. Not surprisingly, suppression of FSH and androgen in rats, primates, and men disrupts spermatogenesis and release of mature sperm from SCs (the process of spermiation) [32]. Spermiation failure is characterized by the retention and subsequent phagocytosis of spermatozoa by SCs, involving functional changes in the adhesion junction present between germ cells and SCs [33].

Sertoli cells have a limited capacity for the number of cells they can support, providing an appropriate environment only to a certain amount of germ cells. For this reason, waves of massive germ cell death by apoptosis occur in the testis during early stages of spermatogenesis, serving as a mechanism to remove excessive cells that cannot be supported by SCs [34]. Spermatogenesis is maintained by the strict control of the fine balance between cell proliferation and apoptosis, which ensures the appropriate ratio of germ cell:SCs [35]. Therefore, germ cell apoptosis has been shown to play an important role in controlling sperm output in many species and has been linked to infertility in humans [36].

In the last years, it has been assumed that SCs play a crucial role controlling germ cells fate, by secretion of a number of locally produced autocrine and paracrine factors, that can control germ cells death or survival, or through direct cell-to-cell membrane contacts[31].

2.2.1. Survival and Death Factors Secreted by Sertoli Cells

Survival or death factors are released by SCs or could be present at their plasma membranes [37]. In any case, germ cells have the receptors for SCs proteins being able to respond to SCs signals, surviving or undergoing apoptosis. SCs secrete the paracrine survival factor, the Desert hedgehog (Dhh) [38], and seem also to control germ cell survival by the expression of a membrane-bound germ cell survival factor, the Stem cell factor (SCF), which has tyrosine kinase receptors (c-kit) on the surface of adjacent germ cells [39]. SCs cells may also employ the Fas system and determine germ cell death, since they express the Fas-Ligand (FasL), while Fas-receptor (FasR) has been described on the surface of adjacent spermatogonia [40].

Desert hedgehog, the testis-specific member of mammalian hedgehog (Hh) protein family, is one of the first genes expressed in the developing of male gonad [38]. It is expressed by SCs and a secreted factor to seminiferous tubules fluid. Blocking Hh signaling in the genetically or pharmacologically testis development drive to severe disruption in testicular histology resulting in a defective spermatogenesis and infertility [41]. In addition, low androgen levels leads to the disorganized seminiferous epithelium and disrupted spermatogenesis since Hh signaling is needed for the differentiation of fetal LCs [42] and to maintain the expression of steroidogenic enzymes [43]. The role of Hh signaling in spermatogenesis has remained unknown until recently. In the mouse, SC-derived Dhh binds patched (PTCH) receptor on the surface of primary spermatocytes decreasing smoothened (SMO) repression and activating glioma-associated oncogene homologue (GLI) transcription factors [38, 44]. Gli1 and Ptch1 are two of the first genes that are transcript in response to the activation of Hh signalling pathway [45]. Late primary spermatocytes, secondary spermatocytes, round spermatids and LCs express PTCH1 in the adult mouse testis. This would suggest that Dhh/PTCH1 signalling takes part in control of meiotic divisions and adult-type LC function [44].

c-kit receptor belongs to a growth factor receptors family with intrinsic tyrosine kinase activity that transduces growth regulatory signals [46]. c-kit has functions during spermatogenesis and throughout male germ cell development before and after birth [1], playing a important role in maintaining the self-renewal and differentiation of spermatogonia ratio [47]. In normal seminiferous epithelium, this ratio is maintained at 1.0, and changes up entail greater spermatogonia self-renewal leading to the appearance of c-kit positive tumor cells in the seminiferous epithelium [48]. On the contrary, changes down entail stem cell depletion resulting in SCs only syndrome, with absence of germ cell in the epithelium [49].

The “undifferentiated” and “differentiating” spermatogonia display different expression of c-kit [50]. Moreover, the passage of undifferentiated spermatogonia to differentiating stages coincides with the gain of c-kit expression. In fact, the presence of c-kit has been routinely used to identify differentiated spermatogonia [51]. c-kit continues to be expressed until meiotic phases and play essential roles in the survival of the c-kit-expressing germ cells [52].

SCF, the c-kit ligand, is expressed at SCs plasma membranes in contact with the germ cells, which express the c-kit receptor. c-kit is activated only after binding with SCF and this pathway is considered to be crucial for the proliferation, migration, survival and maturation of the germ cells [52, 53]. SCF is a cytokine essential for haematopoiesis, melanogenesis and development of germ cells. Actions of c-kit/SCF system include stimulation of primordial germ cell migration, enhancement of proliferation and anti-apoptosis of primordial germ cells/spermatogonia [54]. However, the down-stream signaling pathways involved on spermatogonia proliferation and anti-apoptosis seem to be different. SCF might contribute to construct the potential niche which stimulates stem cell divisions [55] and is able to increase the percentage of sperm undergoing acrosome reaction when cultured *in vitro*. *In vitro*

addition of SCF leads to spermatogonia proliferation [56]. SCF was also detected in human seminal plasma, with levels significantly correlated with sperm counts [57]. Moreover, mutations in human SCF had a significant association with idiopathic male infertility [58], highlighting its relevance in spermatogenesis.

Fas receptor is a type I transmembrane protein belonging to the tumor necrosis factor receptor superfamily [59] and FasL is a member of the tumor necrosis factor family of proteins [60]. Both death mediators (FasR and FasL) are involved in apoptotic programmed cell death together with the effectors of apoptosis (caspases) as detailed in section 3.2.1. FasL is produced in SCs as a type II membrane protein and may be cleaved by a metalloproteinase to produce a soluble ligand [61]. FasR contain a cytoplasmic death domain that, upon activation, recruits other death domain-containing proteins, such as FADD/MORT1 [62] [62][62][62][61][61][61][61][61][61][61][61][61]and caspase 8 [63]. Self-activation of caspase 8 initiates activation of a cascade of other effector caspases, which results in rapid cell death [64]. In the testis, the Fas system has been implicated in maintaining the immune-privileged nature of this organ [65]. SCs expressing FasL eliminate Fas-positive activated T cells, thus protecting the germ cells against the immune system and preventing rejection reactions in the testicular environment. Recent studies in the rat and mouse testes have suggested another important function for the Fas system in the testis linking FasL and FasR with the regulation of testicular germ cell apoptosis [40].

3. Estrogens as Apoptosis Regulators in the Testis

3.1. Estrogen Receptors in Testicular Cells

Sex steroid hormones, androgens and estrogens, exert their effects mainly by the interaction with specific intracellular receptor proteins, which act regulating the expression of target genes and, thus, contribute to determine the cell protein network at any given time (reviewed by [66]). The steroid receptors (SRs) belong to the nuclear receptor superfamily that includes, among others, estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR) [67]. SRs are hormone-activated nuclear transcription factors with distinct specificities for endogenous steroid hormones and exogenous substances [68]. The ER shares with other members of the nuclear receptor superfamily a common arrangement of five structural-functional domains, denoted A to F [69]. A key domain is the DNA-binding domain (DBD or C region) composed of two highly conserved zinc-fingers, which coordinate receptor interaction with specific DNA sequences known as hormone-response-elements present in the promoter region of target genes [70]. The ligand-binding domain (LBD or E region) is located in the C-terminal region and possesses the ability of hormone recognition ensuring both specificity and selectivity of the physiologic response [67]. The A/B domain

with a variable transactivation function encompasses the N-terminal region [67]. The F domain at the C-terminus is characteristic of ERs and its functions are not totally known yet (reviewed by [66]).

Two subtypes of ER receptors, ER alpha (ERα) and ER beta (ERβ), encoded by separate genes located on different chromosomes have been found along the evolutionary line of vertebrates [71-75]

In humans, ERα and ERβ genes are located, respectively, on chromosomes 6 [76] and 14 [77]. Even so, and depending on the species considered, ERα and ERβ proteins share 41%-65% of overall amino acid identity (Fig. I.3) [72]. The least conserved region is the hypervariable amino-terminal A/B domain and in contrast, the DBD is the most conserved with 96% of amino-acid identity. The LBD also is highly conserved (57%-60%) and both receptors bind the natural ligand 17β-estradiol [78], phytoestrogens and other natural and synthetic ligands [79]. However, selective agonists and antagonists for ERα or ERβ have been identified helping to disclose their specific separate actions in a broad range of tissues [80].

The biological function assigned to the ERs in a tissue or organ is not only dependent of ligand nature itself but also of the cellular localization of receptors, as well as the balance between ERα and ERβ proteins levels [81-83]. ERs localization on the different cell types of the testis is species-specific and has been associated with the developmental stage of germ cells [11]. In rodents, ERα expression is more closely associated to LCs and peritubular cells whereas ERβ is found in multiple cell types like LCs, peritubular cells, SCs and spermatogonia, spermatocytes and spermatids [84]. In humans, the absence of consensus on the detection of ERα by distinct independent studies [85-92], together with the reports of the wide expression of ERβ in testicular cells [85, 87-91], lead some authors to assume ERβ as the main mediator of estrogenic actions in human testis. Recently, using human testicular biopsies with distinct phenotypes of spermatogenic development, our research group demonstrated that both receptor subtypes are expressed in human testis [93], with ERα being detected in LCs, SCs, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa, which highlighted for a relevant role of ERα in spermatogenesis. The importance of ERα in testicular physiology is also supported by studies reporting associations of ERα mutations and polymorphisms with male infertility [94-96]. Table I.1 summarizes the available information on the expression pattern of ERα and ERβ in human testicular cells ([93], see also a review by [97]).

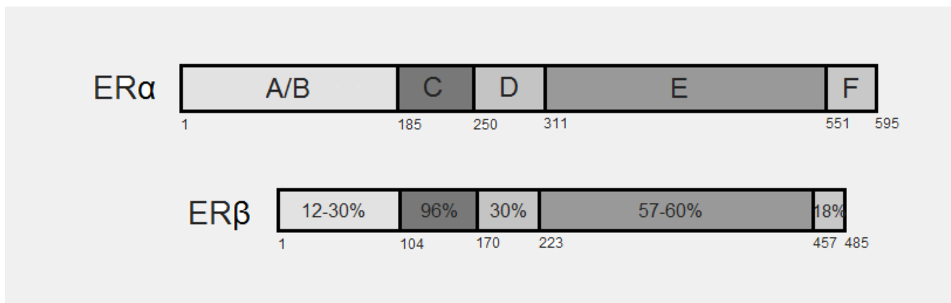

Figure I.3 Structure of ERα and ERβ proteins showing percentages of homology in each functional domain. N-terminal region (A/B domain), the DNA binding domain (C domain), the hinge region (D domain), the ligand-binding domain (E domain), and the C-terminal region (F domain) are indicated. Grey intensity scale highlights for domain conservation, with the most conserved domain C in dark grey.

In addition to the α and β receptors, estrogens induce rapid non-genomic responses via membrane-associated receptors, such as growth factor receptors and G protein-coupled receptors [98]. A member of the 7-transmembrane G protein-coupled receptor family, GPR30, promotes estrogen actions in cells [99]. It has been shown that this receptor show has estrogen-binding affinity and mediates some estrogen-signal transduction events, as for example calcium mobilization, kinase activation [100] and rapid transcriptional activation of early genes [101]. GPR30 is now recognized as an estrogen receptor, known as G-protein coupled estrogen receptor GPER. In humans, GPER has been detected in heart, lung, liver, intestine, ovary, prostate, kidney, brain and testis [102, 103]. However, GPER signaling in human testes is poorly known [103].

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Table I.1 Expression of ERs and Aromatase in Human Testicular Cells.

Cell type	Aromatase	ERα	ERβ	References
Sertoli	+	+	+	[90, 93, 104-107]
Leydig	+	+	+	[90, 92, 93, 104, 105]
Spermatogonia	-	+	+	[87, 88, 90, 93, 107-109]
Spermatocytes	+	+	+	[87-89, 93, 107, 108]
Spermatids	+	+	+	[86-89, 93, 107, 108]
Spermatozoa	+	+	+	[85-87, 108, 110, 111]

+ - positive, - negative.

3.2. Role of Estrogens in Spermatogenesis

Estrogen biosynthesis is catalyzed by a microsomal member of the cytochrome P450 superfamily, namely aromatase cytochrome P450. Aromatase is responsible for binding of the

C19 androgenic steroid substrate and catalyzing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens [112]. Besides being capable to respond to endocrine estrogenic stimuli, mammalian testis can also synthesize E_2, since the presence of a functional aromatase (Table I.1), converting testosterone to its estrogenic metabolite, E_2, has been detected in several cell types of the testis [105]. Curiously, E_2 concentrations in semen, rete testis fluid and spermatic vein [113-116] are in average 50 times higher than in peripheral plasma [117], which supports their physiological relevance in testicular physiology. The report of a man with a disruptive mutation of ERα and reduced sperm viability [94, 118] and the association found between ERα polymorphisms and oligozoospermia further accentuates the role of this ER subtype and estrogens in testicular physiology [118, 119].

Experimental data have been demonstrating that estrogens exert a feedback control over the hypothalamus-pituitary-gonadal axis, inhibiting testosterone production and balancing LH and testosterone levels [11]. Other reports have described the role of E_2 on capacitation, loss of acrosome and increased motility of spermatozoa [85].

However, the only definitive accepted role for E_2 in male reproductive tract is the regulation of fluid reabsorption in efferent ductules and rete testis [120], as demonstrated by the generation of knockout (KO) mice models. In ERα KO (αERKO) mice, testes are smaller than in wild-type and both sperm morphology and function are affected due to epididymal hypo-osmolality [121]. The lack of ability to absorb fluids in this mice model results in a generation of backpressure, which in turn affects the SeT architecture and consequently the testis function [122]. On the other hand, ERß knockout mice (ßERKO) were fertile, producing a sufficient number of sperm [123]. The double αßERKO mice appear to have a similar phenotype to αERKO mice with no major differences in spermatogenesis [123]. The role of estrogens in spermatogenesis was also highlighted by the observations of impaired fertility in aromatase KO mice, which displayed a progressive decrease in fertility with age in consequence of increased rate of germ cells apoptosis [124]. Thus, estrogens may be implicated in the regulation of testicular apoptosis.

3.2.1 Implication of Estrogens on Apoptosis

Apoptosis is one of the biological processes of programmed cell death, which is characterized by several hallmarks, such as: internucleosomal DNA fragmentation, caspase activation and externalization of phophatidyl serine on the plasma membrane [125]. There are two major pathways involved in the apoptotic process (Fig. I.4): receptor-mediated (or extrinsic pathway) and the mitochondrial (or intrinsic pathway). Both pathways converge at the level of the specific serine-proteases proteases, the caspases, that are synthesized as inactive zymogens and become active upon death stimuli [125]. The extrinsic pathway is initiated by activation of death receptors at plasma membrane, such as Fas (CD95/Apo-1) or tumor

necrosis factor receptor 1 (TNFR1). Trimerization of death receptors in response to ligand biding induces the activation of procaspase 8 in mice and caspase 10 in humans [125]. A cascade of effector caspases is then activated with cell commitment to apoptosis (Fig. I.4).

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The intrinsic pathway of apoptosis can be activated by different stimuli, such as radiation, DNA fragmentation, starvation, oxidative stress and autophagy [126]. This pathway is characterized by a decrease in mitochondria membrane potential by action of apoptotic members of the Bcl2 family proteins (BAX and Bid), with opening of the mitochondrial permeability pore and release of cytochrome C (CytoC) from the mitochondria. CytoC along with dATP, the cytosolic protein Apaf-1 and procaspase-9 assemble a complex termed apoptosome [127, 128], which is a catalytic multiprotein platform that activates caspase 9. Activated Caspase 9 then cleaves caspase 3, resulting in downstream events involved in cell death (Fig. I.4).

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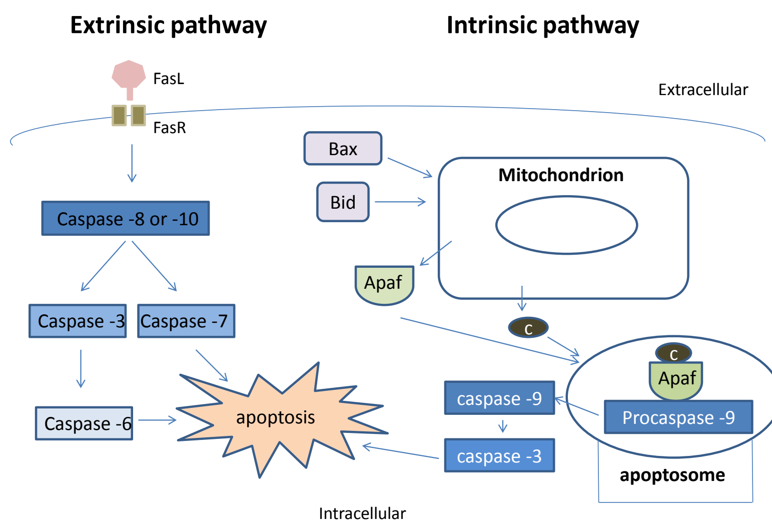

Figure I.4 A Schematic representation of molecular events leading to apoptosis. The two (extrinsic and intrinsic) pathways of apoptosis are indicated. Each pathway activates its own initiator caspase, which in turn will activate the executioner caspases. In extrinsic pathway death signals from extracellular space activate procaspases 8 and 10, which in turn activate, among others, caspase 3 promoting apoptosis. The intrinsic pathway, activated in response to cell stress, includes opening of mitochondrial pore, by actions of Bax and Bid proteins, with release of cytochrome C (c) and formation of the apoptosome. Apoptosome formation induces activation of caspases 9 and 3 and apoptosis. The execution of apoptosis results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

Estrogens have been widely considered as a hormone that can both stimulate and inhibit cell apoptosis, which has been particularly evident in breast cancer cells, where E_2 is a potent

inhibitor of apoptosis regulating expression of several apoptotic proteins, but is also capable of inducing apoptosis [129]. Indeed, *in vivo* and *in vitro* studies using rat models produced many conflicting reports about the estrogenic control of apoptosis in the testis (Table I.2).

E_2 was described as a survival agent promoting germ cell survival and inhibiting apoptosis during spermatogenesis. *In vitro* administration of E_2 (10^{-10} M and 10^{-9} M) effectively inhibited germ cell apoptosis [130]. Also, *in vivo* experiments found a decrease of apoptosis in germ cells following E_2 treatment [131]. Moreover, i*n vitro* studies with rat cultured SCs demonstrated that E_2 (10^{-7}M) inhibits apoptosis of these somatic cells [132]. Studies from our research group and others started elucidating the mechanisms underlying E_2 inhibition of testicular apoptosis. E_2 at 100 nM dosage up-regulated the expression of the apoptosis-inhibitor Aven being this response accompanied by a decrease in cleaved caspase 9 expression [133]. In rat cultured SCs, E_2-treatment down-regulated the mRNA expression of p53, Bax, caspase 9 and caspase 3 pro-apoptotic proteins [132]. Similar results were obtained by Royer [134], also using immature rat SCs cultures, with up-regulation of anti-apoptotic (BCL2 and BCL2L2) and down-regulation of pro-apoptotic (BAX) proteins.

However, it has also been reported that E_2 leads to increased rates of apoptosis in the testis and decreased sperm counts, based essentially on studies performed with E_2 analogues, namely, diethylstilbestrol (DES) (Table I.2) [135]. DES can mimic estrogen action by interfering with the functioning of the pituitary-gonadal axis, leading to the suppression of testosterone levels, which in turn results in increased spermatogenic cells apoptosis. *In vivo* exposure to 1 mg/kg body weight for 7 days induces apoptotic death in spermatogenic cells, which may be triggered by increased expression of Fas system. This process occurs by inducing the translocation of Bax from the cytosol to the mitochondria, followed by release of CytoC, which is accompanied by a loss of mitochondrial membrane potential, leading finally to the activation of caspases 9 and 3 [135]. In addition, increased intratesticular E_2 levels culminated in arrested spermatids at stages VII and VIII and consequent failure of spermiation [131]. On the other hand, when estrogen production is low, spermatogenesis was arrested primarily at early spermiogenic stages, being characterized by an increase in apoptosis, the appearance of multinucleated cells, and a significant reduction in round and elongated spermatids [124].

Also *in vitro*, a dose of 10^{-8} M of E_2 induced germ cells apoptosis with implication of Fas system through mitochondrial pathway as a consequence of nitric oxid and superoxide generation [136].

Table I.2 Role of Estrogens Controlling Apoptosis of Rat Testicular Cells.

Cell type	Type of study	Agent/dose	Apoptosis	References
Germ cells	*In vivo*	$4x10^{-6}$M until $4x10^{-10}$ M of E_2	↑	[137]
		20 and 100μg E_2/day/kg of body weight	↓	[131]
		75μg Estradiol benzoate /animal/day	↑	[138]
		75μg Estradiol benzoate /animal/day	↑	[139]
		50μg Estradiol benzoate /animal/day	↑	[140, 141]
		12.,5μg Estradiol benzoate /animal	↑	[142]
		$4x10^{-6}$M - $4x10^{-10}$ M of Diethylstilbestrol	↑	[137]
		0.01, 0.1 and 1mg Diethylstilbestrol /kg of body weight	↑	[135]
	In vitro	10^{-10} M and 10^{-9} M of E_2	↓	[89]
		10^{-8} M of E_2	↑	[136]
Sertoli cells	*In vivo*	12.,5μg Estradiol benzoate /animal	↑	[142]
	In vitro	10^{-7} M of E_2	↓	[143]

↑, induced; ↓, inhibited

I. Aim

Over the years, it has been described that SCs are able to control the fate of germ cells, determining its survival or death and, thus, establishing a proper ratio between germ cells and somatic support cells. This is achieved by the production and release of survival or apoptotic factors by SCs, which have their corresponding receptors in germ cells. Survival factors include the Dhh and the SCF, for which the c-kit receptor is present in germ cells. Also the apoptotic Fas system (FasL and FasR) has been implicated in the survival and death communication between SCs and germ cells.

Although several reports have pointed estrogens as important regulators of testicular apoptosis and demonstrated the E_2 regulation of Dhh, SCF, c-kit, FasL and FasR expression in several other tissues [38, 40, 50, 52, 65], the effect of these steroid hormones on the testicular expression of the mentioned factors remains poorly known.

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We hypothesized that E_2 disturbs SCs:germ cells communication by altering the expression of apoptotic or survival controller genes. Therefore, the aim of the present study is to determine the effect of E_2 on the expression of survival (Dhh and SCF/c-kit) and death factors (Fas System) in SeT cultured *ex vivo* and in SCs primary cultures. In addition, the use of ERα, ERβ and GPER specific agonists will allow starting elucidating the mechanism by which the estrogenic effects are attained.

II. Material and Methods

1. *Ex vivo* Culture of Rat Seminiferous Tubules and Hormonal Treatment

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 [144, 145]. 90-day-old Wistar rats (*Rattus norvegicus*) were anesthetized with 100µL of a mixture containing 30% xilazine:70% ketamine per 100g of animal weight and euthanized by cervical dislocation. Testes were removed, trimmed free of fat, washed in cold PBS and placed in DMEM-F12 culture medium at 32°C. DMEM-F12 medium was supplemented with 20 mg/l gentamicin sulfate, 0.1 mM 3-isobutyl-1- methylxanthine, and 1 mg/l BSA. Tunica was cut and peeled back to expose tubules. Ten SeT fragments of about 1 cm in 2 ml of culture medium were used per well (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark). Experimental groups (n=5) were: Control (culture medium only) and 100 nM E_2 (culture medium to which E_2 was added). Under physiological conditions, intratesticular concentrations of estrogens are considerably high relatively to plasma [113-116], in the range of 0,1-1 nM [89]. Moreover, increased E_2 levels have been detected in the seminal plasma of infertile men [114, 124]. Thus, we selected a pharmacological dose of 100 nM to study the estrogenic effects in SeT. Tubules were incubated under control and hormonal stimulation conditions for 24 h. SeT remain viable during the course of the experiment as assessed by morphological analysis of hematoxylin-and-eosin (H&E) stained tissue sections [146]. At the end of experiment, SeT were recovered from medium, snap-frozen in liquid nitrogen and stored at -80°C until RNA or protein isolation.

2. Sertoli Cells Primary Culture and Hormonal Treatment

SCs were isolated by a method previously described by Oliveira et al [147] and Laurentino et al [148] with some modifications. Briefly, 20 days-old male Wistar rats were anesthetized with 100 µL of a mixture containing 30% xilazine:70% ketamine per 100g of animal weight and euthanized by cervical dislocation. Testes were excised in aseptic conditions and washed two times in a 50 mL conical tube with 30 mL of ice cold HBSS (potassium chloride 0,4 g/L, potassium phosphate monobasic anhydrous 0,06 g/L, sodium chloride 8 g/L, sodium phosphate dibasic 0,045 g/L, D-Glucose 1 g/L, Sodium bicarbonate 0,35 g/L) containing 10000 U/mL of penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B (pH 7,4). Testes were decapsulated in HBSS, and the exposed SeT were washed three times in HBSS. To remove residual peritubular cells, the tubules were dispersed in a Petri dish in HBSS with

glycine (1M), EDTA (2 mM) , Soybean Trypsin Inhibitor (pH 7,2; 0,002% (w/v) and DNAse (0,5 mg/ml) during 10 minutes at room temperature. To disperse the tubules and release the interstitial cells, the dispersed tubules were forced to pass several times through a large-pore Pasteur pipette. Then, the pellet was digested during 15-20 minutes at room temperature with 0,225 mg/mL collagenase type I and 0,05 mg/mL DNase in HBSS. After the digestion step, disaggregated SeT were washed three times in HBSS and centrifuged 3 minutes at 300 *g*. The SC suspension was collected and resuspended in Sertoli culture medium which consisted of a 1:1 mixture of DMEM-F12 Ham, supplemented with 15 mM HEPES, 50 U/mL penicillin and 50 mg/mL streptomycin sulfate, 0,5 mg/mL fungizone, 50 µg/mL gentamicin and 10% heat inactivated FBS. In order to disaggregate large SC clusters, the cellular suspension was forced though a 20G needle. For cell culture, the concentration of the clusters on the cellular suspension obtained was adjusted to 5000 clusters/mL, plated on 25 cm^2 culture flasks (Cell+; Sarstedt), and incubated at 33ºC in an atmosphere of 5% CO_2, 95% O_2. The cultures were left undisturbed until day 2, considering the day of plating day 0 of culture.

When the SCs cultures were 90-95% confluent, culture medium was replaced by serum-free medium supplemented ITS (DMEM-F12 plus ITS, pH 7,4) containing 0,1 nM or 100 nM of E_2, or 100 nM of each ER specific agonist. Agonists were: G1 (Santa Cruz Biotechnology, California, USA), an agonist for GPER (GPR30); DPN (Santa Cruz Biotechnology), an agonist for ERα; PPT (Santa Cruz Biotechnology), an agonist for ERß. E_2 and receptor agonists were prepared in 0,025% ethanol (EtOH) and hormonal stimulation proceeded for 24hs. 0,1 nM E_2 has been indicated as the normal testicular E_2 concentration in physiological conditions [89], while 100 nM represents a pharmacological dose as stated before.

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3.RNA Extraction

Sertoli cells were removed from culture flasks using a trypsin-EDTA solution. To remove residual trypsin, detached cells were washed with 3 mL of phosphate buffered saline (PBS), by consecutive centrifugations of 5 minutes at 3000 *g*. SeT were collected from culture flasks and placed in centrifuge tubes. After centrifugation, SCs and SeT pellets were homogenized in 500 µL of TRI reagent (Sigma-Aldrich,St. Louis, MO, USA) using an Ultra-turax homogenizer (T25 basic, IKA). The samples were left to stand for 5 minutes at room temperature and then 100 µL of chloroform were added for phase separation, to ensure a complete dissociation of nucleoproteins complexes. The samples were shaken vigorously for 15 seconds, left to stand for 5 minutes at room temperature and centrifuged at 12000 *g* for 15 minutes at 4ºC, to separate the mixture into 3 phases: a colorless upper aqueous phase (containing total RNA, RNAt), an interphase (containing deoxyribonucleic acid (DNA)) and a red organic phase (containing proteins). To isolate RNAt, the aqueous phase was transferred to a fresh tube, 250 µL of 2-propanol were added to precipitate RNA, and the mixture was centrifuged at 12000 *g* for 10 minutes at 4ºC. The supernatant was discarded and the RNA pellet was washed with 500 µL of 75% cold-EtOH. This mixture was centrifuged at 7500 *g* for 5 minutes at 4ºC. This

washing step was repeated once more. The supernatant was discarded and the RNA pellet was air-dried for 5-10 minutes. The RNA pellet was dissolved in 10 µL of diethylpirocarbonate treated-water (DEPC-H_2O). RNAt concentration and the absorbance ratio (A260/A280) were spectrophotometrically determined (NanophotometerTM, Implen, Germany).

4.cDNA Synthesis

cDNA synthesis from SCs and SeT RNAt was performed using the NZY First strand cDNA synthesis kit (nzytech, Lisbon, Portugal) in a reaction mixture prepared as described in Table III.1. To the appropriate volume of RNA (1µg) was added 12µL of reaction mix and water to a final volume of 20 µL. RNA was reverse-transcribed by incubating samples at 25°C during 10 minutes, then 30 minutes at 50°C. Reaction was stopped by enzyme denaturation with an incubation at 85°C during 5 minutes,. At the end, 1µL of Nzy RNase H (*E. Coli*) was added to each sample of reverse-transcribed RNAt, which were incubated at 37°C for at least 20 minutes. One sample without RNA was reverse transcribed to be used as negative control in PCR reactions. cDNA was stored at -20°C until use.

Table III.1. Volumes and reagents used in cDNA synthesis reaction mix

Reagent	Volume(µL)
NZY RT master mix	10
NZY RT enzyme mix	2
DEPC-H_2O	Up to 20

5.Real-time PCR

Real-time PCR (qPCR) was used to analyze the expression of Dhh, c-kit/SCF and Fas System in SeT and in SCs in different experimental conditions. Characteristics of gene-specific primers, as well as housekeeping genes are indicated in Table III.2. GAPDH and ß-actin housekeeping genes were used to normalize gene expression. Primer concentration and annealing temperature were optimized before each assay and specificity of the amplicons was determined by melting curve analysis. Efficiency of the reactions was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:10, and 1:100).

Amplification reactions were prepared in a final volume of 20 µl as described in Table III.3. SYBRgreen master mix was obtained from Fermentas Life Sciences (Ontario, Canada). Reaction mix was distributed to PCR-plate wells, and then the necessary volume of DNA was added. Reactions were carried out on a thermocycler (iCycler iQTM system, Biorad). After

initial denaturation at 95ºC for 5 min, the following cycling conditions were used (40 cycles): 95ºC for 10s, 55ºC, 58ºC or 60ºC for 30s and 72ºC during 10 s. The specificity of the amplified fragments was checked by melting curves analysis. For this reactions were heated from 55ºC to 95ºC with 10 s holds at each temperature. Samples were run in triplicate for each assay.

Normalized expressions values of Dhh c-kit, SCF, FasR and FasL, were calculated according to a published mathematical model proposed by Vandesompele and collaborators [149].

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Table III.2 Primers characterization: sequences, annealing temperature (AT) and size of amplified fragment.

Gene	Sequence	AT (ºC)	Size (bp)
Dhh	sense: 5'-TAA TGG TAG TCT ATC AGT AGT AG -3´ antisense: 5'- GAG CGT TCT TGT CCT TAC -3´	55	180
c-kit	sense: 5'- CCG TCT CCA CCA TCC ATC C -3' antisense:5'- TTC GCT CTG CTT ATT CTC AAT CC -3'	60	143
SCF	sense: 5'- ATG GCT TGG GAA ATG TCT G -3' antisense: 5'- GCT GAT GCT ACG GAG TTA C -3'	58	193
FasR	sense: 5'- GCA ACA CCA AAT GCA AGA AA -3' antisense: 5'-GGA TTC CAG ATT CAG GGT CA -3'	60	118
FasL	Sense: 5'- GGT GGC TCT GGT TGG AAT GG -3' Antisense: 5'- ATG ATA CTC TAA GGC TGT GGT TGG -3'	60	103
GAPDH	Sense: 5'- GTT CAA CGG CAC AGT CAA -3' Antisen:se 5'- CTC AGC ACC AGC ATC ACC -3'	60	177
ß-actin	Sense: 5'- ATG GTG GGT ATG ATG CAG -3' Antisense: 5'- CAA TGC CGT GTT CAA TGG -3'	60	79

Table III.3 Composition of the qPCR reaction mix.

Reagent	Quantity (μL)
SYBR green Mix	10
Gene-specific sense primer 5nM[a] or 7,5nM[b]	0,8 or 1,2
Gene-specific antisense primer 5nM[a] or 7,5 nM[b]	0,8 or 1,2
Sterile water	Up to 19 μL

[a]FasR, FasL, c-kit Dhh; [b]SCF

6. Protein Extraction

Sertoli cells and SeT were collected from culture flasks as described above and homogenized in an appropriate volume (2,5 mL for each culture flask or 100 µL for each 3 mg of tissue) of Radio-Immunoprecipitation Assay buffer (RIPA) (1x PBS, 1%NP-40, 0,5% sodium deoxycholate, 0,1% SDS, 1 mM PMSF) supplemented with 1% protease inhibitor cocktail, aprotinin (10 µg/mL) and 100 mM sodium orthovanadate. The lysates were left to 15 minutes on ice and the suspension was centrifuged at 14000 *g* for 20 minutes at 4ºC. The supernatant was collected to new tubes and total protein concentration determined using the Bradford assay [150].

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7.Western Blot

50 µg of total protein extracts from SeT and SCs were heat-denatured and fractionated on a 12% SDS-PAGE at 120 V/gel during 90 minutes. After electrophoresis, proteins were electrotransferred to a PVDF membrane at 750 mA during 75 minutes. The membranes were blocked in a Tris-buffered saline solution (TBS) with 0,05% Tween 20 containing 5% skimmed dried milk for 90 minutes. The membranes were then incubated overnight at 4ºC with rabbit anti-SCF (1:500, Santa Cruz Biotechnology, SC-9132), or rabbit anti-FasL (1:500 Santa Cruz Biotechnology, sc-6237), or rabbit anti-c-kit (1:500, Santa Cruz Biotechnology, Sc-168), or rabbit anti-FasR (1:500, Santa Cruz Biotechnology, Sc-1023) primary antibodies. Mouse anti-actin primary antibody was used to normalize differences in protein loading (1:5000, Sigma-Aldrich, A 5441). Immune-reactive proteins were detected separately with goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2007) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology, Sc 2008). Detection was performed with ECF reagent (GE, Healthcare, Weßling, Germany) and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were obtained using the Quantity One Software (Bio-Rad), according to standard methods.

8. Statistics

The statistical significance of mRNA and protein expression variation among the experimental groups was assessed by two-way ANOVA, followed by Bonferroni post-test. All experimental data are shown as mean ± SEM (n=5 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $p<0.05$ was considered significant.

III. Results

1. Morphology of Seminiferous Tubules in Culture During Experimental Conditions

The maintenance of the integrity of cultured SeT using our experimental approach was demonstrated by Gonçalves J. [146]. A representative image of SeT morphology (Fig. IV.1) shows maintenance of SeT epithelium after 72h of culture, with presence of germ cells in distinct development stages. Spermatogonias, primary spermatocytes, elonged spermatides and spermatozoa were identified in H&E stained sections (Fig. IV.1).

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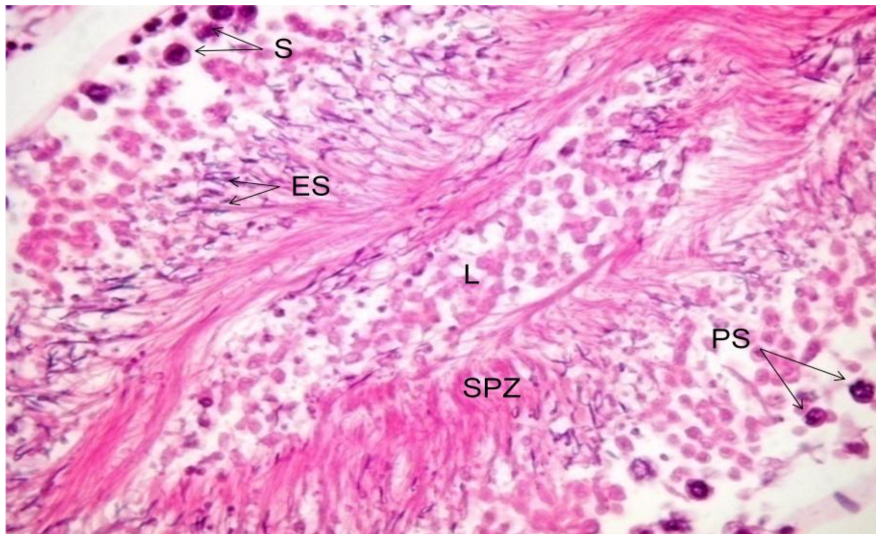

Figure IV.1. Representative histological section showing morphology of rat seminiferous tubules after 72h of *ex vivo* culture (H&E staining, 100× amplification). S – Spermatogonia, PS -Primary spermatocyte, ES - Elongated spermatid, SPZ- spermatozoa, L – Lumen. Adapted from Gonçalves J. [146].

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2. Effect of E_2 on the Expression of Dhh, SCF/c-kit and Fas System in Seminiferous Tubules Cultured *Ex Vivo*

Seminiferous tubules from 90 days-old rats were cultured in presence (100 nM) or absence (control) of E_2 during 24h, and the expression of Dhh, SCF, c-kit, FasL and FasR was analyzed by qPCR and Western Blot.

Expression of survival genes in SeT displayed a distinct pattern in response to E_2 treatment. Dhh mRNA levels did not change upon stimulation with E_2, being its values not significantly different between experimental groups (Fig. IV.2). On the other hand, both mRNA (Fig. IV.2) and protein levels of SCF (Fig. IV.3) were increased in E_2-treated group comparatively to the control. However, only SCF protein expression was significantly different (1,3-fold variation relatively to control group, $p<0,05$). Considering the expression of SCF receptor, the c-kit, E_2 treatment induced a significant decrease in its mRNA ((Fig. IV.2) and protein levels (Fig. IV.3). In both cases, fold-reduction relatively to control was approximately 0,4 (Fig. IV.2 and Fig. IV.3) with p values $<0,001$.

The expression of apoptotic genes, namely the Fas system, FasL and FasR, was increased in SeT cultured in the presence of 100 nM E_2. Although no significant differences were observed in mRNA expression of both FasL and FasR in response to E_2-stimulation (Fig. IV.2), a significant increase in protein expression was found (Fig. IV.3). Protein expression of FasL and FasR in E_2-treated group increased approximately 1,5-fold relatively to the control with p values, respectively, $<0,05$ and $<0,01$.

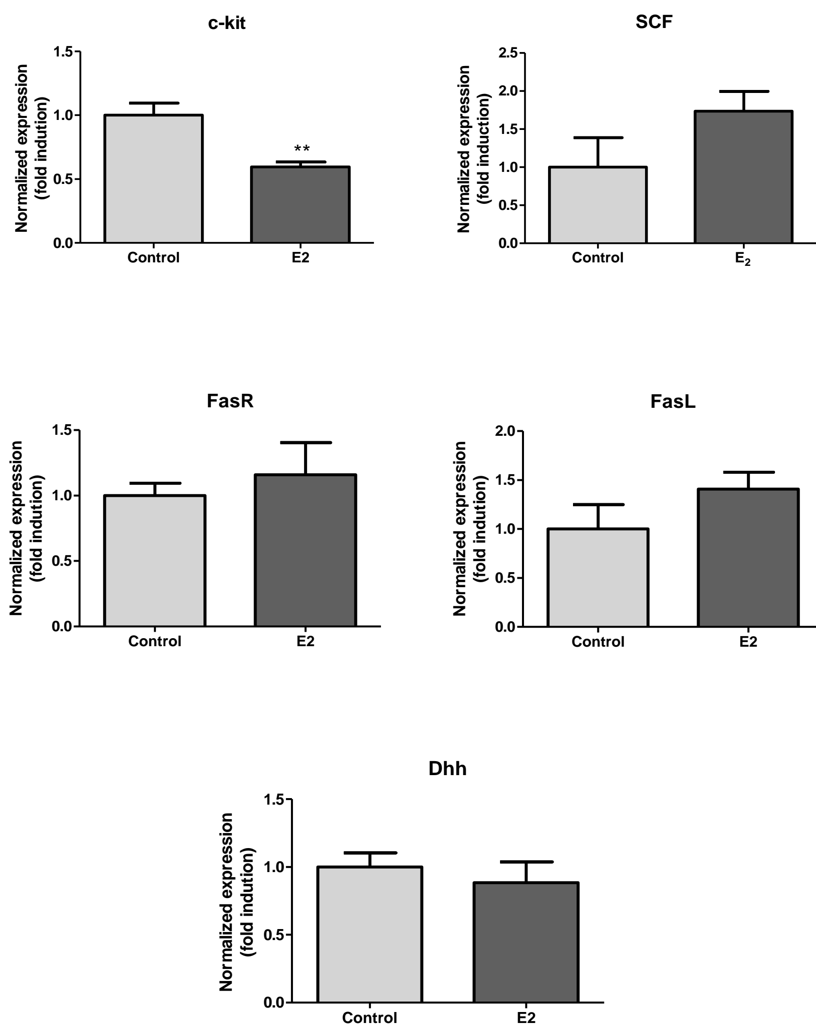

Figure IV.2. Effect of 100 nM E_2 on c-kit, SCF, FasR, FasL and Dhh mRNA expression in rat seminiferous tubules cultured *ex vivo* (24 h) determined by qPCR after normalization with ß-actin and GAPDH housekeeping genes. Results are expressed as fold-variation relatively to 0 nM E_2 (control). Error bars indicate mean ± SEM (n≥4). ** p<0,001.

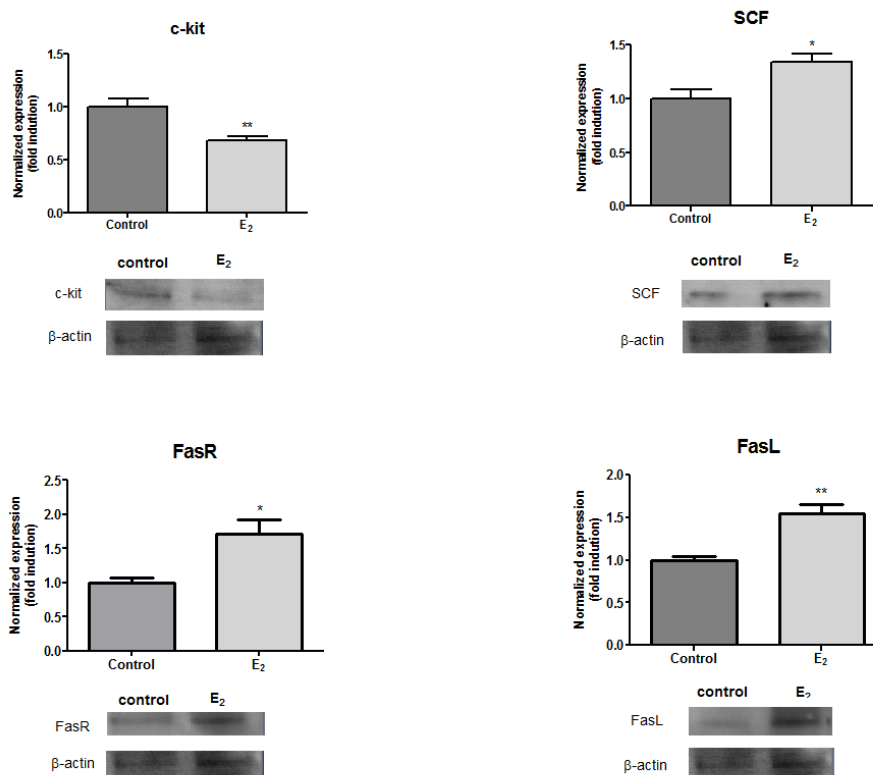

Figure IV.3. Effect of 100 nM E_2 on c-kit, SCF, FasR and FasL protein expression in rat seminiferous tubules cultured *ex vivo* (24 h) determined by Western Blot after normalization with β-actin housekeeping protein. Images of representative immunoblots for β-actin and target genes are provided. Results are expressed as fold-variation relatively to 0 nM E_2 (control). Error bars indicate mean ± SEM (n≥4). * p<0,05 ** p<0,01.

3. Effect of E_2 on the Expression of SCF and FasL in Sertoli cells Cultured *In Vitro*

Sertoli cells primary cultures were obtained from 20 days-old rats as described in Material and Methods section following slightly modifications of the protocols described by Oliveira et al [147] and Laurentino et al [148]. A previous report of our research group [148] demonstrated that under the experimental conditions, the purity of isolated SCs is greater than 95%, as assessed by means of immunohistochemistry analysis using SC specific markers (Fig. IV.4).

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Confluent SCs cultures were treated for 24 h with serum-free culture medium containing E_2 (0,1 nM or 100 nM) or GPER, ERα and ERβ specific agonists, respectively, G1 (100 nM), PPT (100 nM) and DPN (100 nM). This approach allowed determining the effect of estrogens controlling FasL and SCF expression in SCs, as well as establishing the ER involved in such regulation.

17β-estradiol treatment (100 nM) significantly increased SCF mRNA expression (Fig. IV.5) in more than 2-fold relatively to untreated control group ($p<0,005$). Stimulatory effects on SCF transcripts levels were also observed with G1 and PPT (Fig. IV.5) agonists, with G1 being the strongest regulator of SCF expression. 100 nM of G1 induced a 5-fold increase in SCF expression relatively to the control ($p <0,001$).

Considering FasL, although both 100 nM of E_2 and G1 increased its mRNA expression (Fig. IV.5), only G1 produced statistically significant effects relatively to control. Again, G1 induced a strong effect increasing FasL transcript levels (3,5-fold increase in comparison with control, $p<0,005$).

When using a physiological doses of E_2 (0,1 nM) no changes were observed on the expression of SCF and FasL.

Expression analysis of SCF and FasL proteins in cultured SCs in response to E_2 is underway.

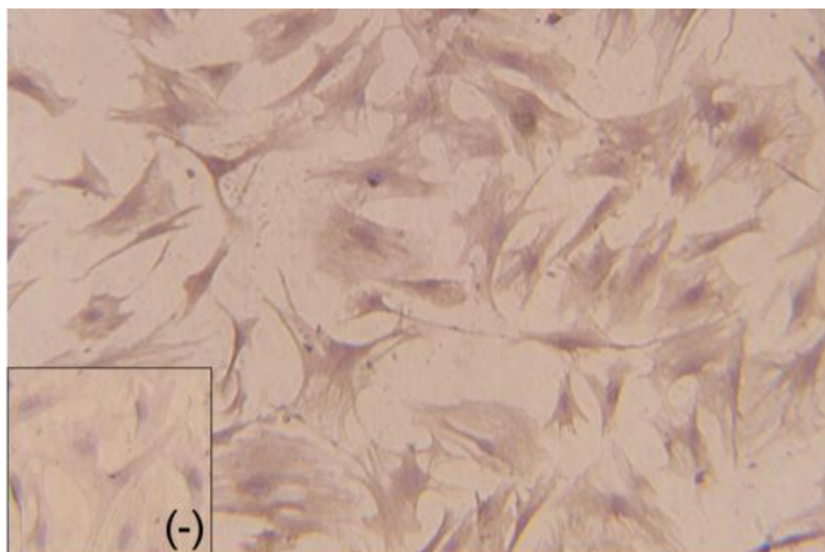

Figure IV.4. Immunohistochemistry analysis of Sertoli cells (SCs) in culture using a primary antibody against vimentin, a SC specific marker. (-), negative control obtained by omission of the primary antibody. Adapted from Laurentino [148].

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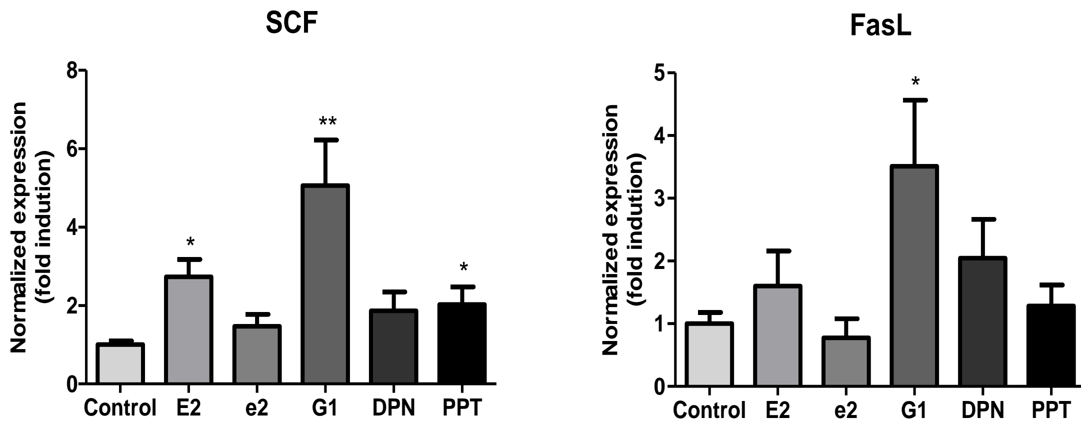

Figure IV.5 Effect of E_2 (0,1 nM (e2) and 100 nM (E_2)), and selective ER agonists (G1, DPN and PPT) on SCF and FasL mRNA expression in rat cultured Sertoli cells determined by qPCR after normalization with β-actin and GAPDH housekeeping genes. All agonists were used at 100 nM. Results are expressed as fold-variation relatively to 0 nM E_2 (control). Error bars indicate mean ± SEM (n≥4). * $p<0,05$, ** $p<0,001$.

IV. Discussion

Estradiol has been traditionally view as the “female” hormone while testosterone is widely recognized as the “male” hormone. However, due to considerable experimental an clinical evidences this idea has been changed in the last years [151], with the emergence of estrogens as important regulatory elements in male reproductive function. The explosion on the interest of estrogens roles in the male happened with the demonstration that male fertility is impaired in mice lacking ERs [122] and aromatase [124], and in human subjects with mutations on genes encoding these proteins [94, 118]. Exposure to estrogens in the environment is another important reason for the large interest in the role of estrogens in male reproduction, and many reports describe their negative effects on male reproductive development with impairment of spermatogenesis and decreased sperm counts [152]. Moreover, increased E_2 levels have been detected in the seminal plasma of infertile men [114, 124], which still sharpens the curiosity to disclose the functions and the underlying molecular mechanisms of this steroid hormone in spermatogenesis.

Spermatogenesis is the complex cellular process involving mitosis, differentiation, and meiosis that give rise to the male haploid germ cells [10, 11]. SCs, confer the physical and biochemical support to the developing germ cells at different stages of development, playing a central role in spermatogenic process. SCs secrete a number of locally produced autocrine and paracrine factors [27], nourishing germ cell and highly coordinating their development [31].

However, SCs have a limited capacity for the number of germ cells they can support and for achievement of a successful spermatogenesis it is important to preserve the appropriate ratio of germ cell:SCs [35]. It has been shown that regular apoptosis of spermatogenic cells is required to maintain the correct testicular homeostasis. However increased germ cells death can result in defective spermatogenesis leading to infertility [153]. Therefore, germ cell apoptosis has been shown to play an important role in controlling sperm output in many species [36]. Germ and SCs can communicate using two vias, a direct via ligand/receptor-mediated interactions or through paracrine factors. It is accepted that SCs play a crucial role controlling germ cells fate, by secretion of a number of locally produced autocrine and paracrine factors that can control germ cells death or survival. Among others, Dhh [38] and SCF [39] have been pointed as important germ cell survival regulators. SCF is a membrane-bound factor, which has its receptor, the c-kit, on the surface of adjacent germ cells [39]. Thus, enhanced or diminished expression of c-kit could have a profound impact on germ cell survival. Also, the interplay between FasL in SCs and FasR in germ cells has been identified as a mechanism employed to determine germ cell death [40]. In the same way as it could happens for SCF/c-kit, abnormal expression of FasR may disrupt SC:germ cells communication towards increased rates of apoptosis.

Although not always producing concordant results (Table I.2), many studies have suggested estrogens as important regulators of testicular apoptosis. Moreover, reports exist demonstrating the capacity of E_2 in regulating the expression of Dhh, SCF, c-kit, FasL and FasR in several tissues [38, 40, 50, 52, 65]. However, the effect of estrogens controlling the testicular expression of these factors has remained poorly known.

In the present study we investigated the effect of E_2 stimulation on the expression of Dhh, SCF, c-kit, FasL and FasR in *ex vivo* tissue cultures of rat SeT and primary SCs cell cultures. Using 100 nM of E_2 no effect was observed on the mRNA expression of Dhh (Fig. IV.2) in SeT. Considering the expression of c-kit, E_2 treatment induced a significantly decrease in its mRNA (Fig. IV.2) and protein levels (Fig. IV.3). c-kit is a growth factor receptor transducing growth regulatory signals [46] that lead to maintenance of the self-renewal and differentiation of spermatogonias ratio [47]. It has been shown that mice with a mutant form of c-kit are incapable of PI3K recruiting and are sterile caused by reduced proliferation and increased apoptosis in the spermatogonia [154]. Through PI3K pathway, c-kit/SCF facilitate the upregulation and nuclear accumulation of cyclin D3 as well as spermatogonia proliferation suggesting that cyclin might be one of the targets of c-Kit/SCF pathway within the testis [56, 155]. Inactivation of c-kit caused an impairment of spermatogonia self-renewal [156], and thus, the decreased expression of c-kit may indicate the apoptotic fate of germ cells.

Like other cell types, the male spermatogenic cells are sensitive to external signals and to their internal milieu by activating intracellular signaling pathways that drive cells to death. The Fas system is known to be involved in apoptotic process of spermatogenic cells [157], thus we studied the effect of E_2 stimulation on the expression of FasL and FasR in SeT, and on the expression of FasL in SCs. FasL and FasR expression was increased in cultured SeT in the presence of 100 nM E_2 (Fig. IV.3). Estrogenic stimulation of SCs with G1 also enhanced the expression of FasL (Fig. IV.5). Previous *in vivo* studies also demonstrated that administration of DES induces apoptotic death in spermatogenic cells, which may be triggered by increased expression of Fas system [135]. The increased expression of FasL in SCs (Fig. IV.5), concomitant with an increased response of germ cells to death signals, since FasR expression is augmented in germ cells (Fig. IV.4) strongly indicate that germ cells may be determined to undergo apoptosis.

On the other hand, 100 nM of E_2 increased SCF expression in SeT (Fig. IV.4) and SCs (Fig. IV.5). SCF is a cytokine produced in SCs which has been considered a survival factor essential for development of germ cells [52, 53]. SCF increased expression was also described in other tissues when some lesion happens or some apoptotic stimulation are present [158], which would have the effects stimulating cell proliferation [159]. In addition, it is widely accepted that SCs have the ability to adapt its physiology to the needs of germ cells and maintenance of spermatogenesis [160]. Therefore, the observed increase of SCF expression in SeT could reflect the response of SCs in presence of increased apoptosis of germ cells to counteract

reduction of germ cells number and maintain spermatogenesis. The 0,1 nM physiological dose produced no effects on the expression of death and survival factors. In fact, other *in vitro* studies showing the effect of E_2 as apoptosis inducer have used doses of 10^{-8} (10 nM) and 10^{-7} M (100 nM) with about 80% reduction in cell survival at 10 h after initiation of E_2 treatment [136]. In opposition, reports of E_2 as a survival agent inhibiting germ cell apoptosis have used lower doses of 10^{-10} M (0,1 nM) and 10^{-9} M (1nM) [130]. Herein, we also demonstrated that pharmacological doses of $E_{2,}$ as could be found in the testis of infertile men [114, 124], seem to induce germ cell apoptosis. In contrast, physiological concentrations E_2 could have a beneficial effect in spermatogenesis.

Classical estrogens actions involve ligand-binding to intracellular ERα and ERβ, dissociation of chaperone complexes, receptor phosphorylation, receptor dimerization (ERα/ERα, ERα/ERβ and ERβ/ERβ dimmers), nuclear translocation, DNA binding and, finally, modulation of transcriptional activity [79]. More recently, the GPER, a membrane-bound G-protein receptor was identified mediating estrogen-signal transduction events [99]. GPER has been localized in both germ and SCs of human testis [103] and in mouse spermatogonia cell line where after activation with E_2 it has been linked to the control of cell proliferation [161].

To start elucidating the mechanisms underlying the estrogenic control on survival and death factors expression we stimulated SCs with GPER, ERα and ERß specific agonists, respectively, G1 (100 nM), PPT (100 nM) and DPN (100 nM). The estrogen analogue for GPER, G1 was the main regulator of FasL and SCFexpression in SCs (Fig. IV.5). The effect of G1 was considerably higher comparatively with 0 nM E2 and ERα and ERß agonists. Royer et al [134] also demonstrated that estrogens effects inducing spermatocyte apoptosis were mediated by GPER, which activate the rapid EGFR/ERK/c-jun signaling cascade, triggering the apoptotic mitochondrial pathway involving increased Bax expression and reduction of cyclin A1 and B1 levels [162].

In sum, our results showed that pharmacological doses of E_2 unbalanced SCF/c-kit and Fas system communication between SCs and germ cells. The observed decrease in c-kit expression coupled with increased expression of Fas system may suggest a diminished survival and increased apoptosis of germ cells in the epithelium of SeT, which could be relevant for long-term maintenance of spermatogenesis. Noteworthy, increased serum and intratesticular E_2 levels have been detected in infertile men [114, 124]. Furthermore, increased rates of apoptosis and altered expression of several apoptosis-related genes has been found in the testis with defective phenotypes of spermatogenesis [36, 133, 163-165].

Based on our results we propose a model (Fig. V.1) that may explain male infertility syndromes related with hyperestrogenism. Increased levels of E_2 in intratesticular fluids could be related with augmented rates of germ cell apoptosis in consequence of increased expression of Fas system (FasL and FasR) and diminished expression of c-kit. If SCs response

increasing expression of SCF does not counteract Fas death stimulation, a depletion of germ cells will occur with consequent impairment of spermatogenesis and infertility.

Finally, this work contributed to a better understanding of the mechanisms involved in male infertility syndromes, particularly those that are related with abnormal concentrations of E_2. Moreover, it produced scientific information, which could be relevant for medical management and treatment of male infertility targeting estrogen signaling mechanisms.

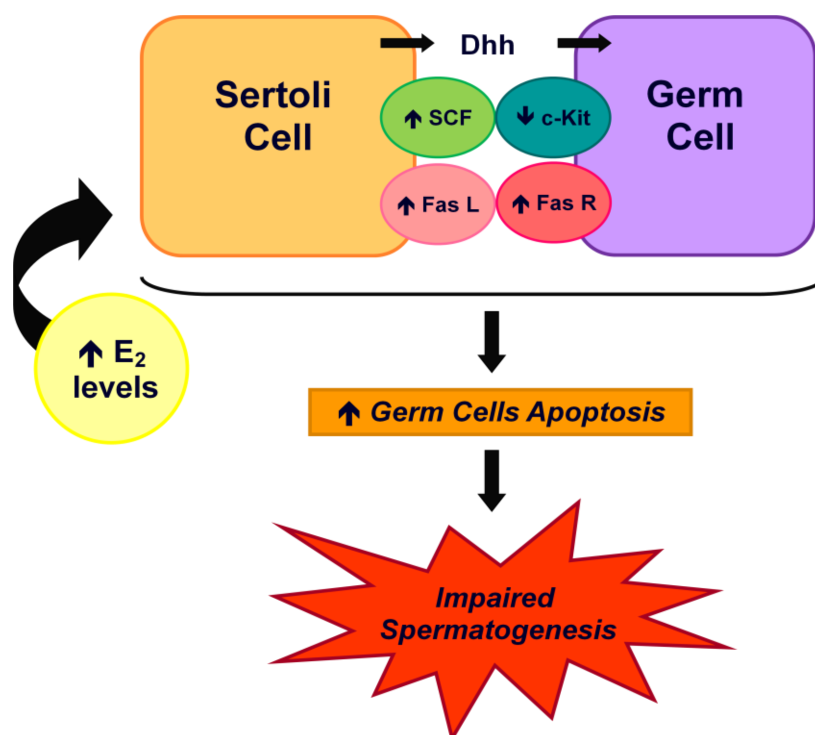

Figure V.1 Role of E_2 perturbing Sertoli cells (SCs):germ communication with induction of germ cell apoptosis. Increased intratesticular E_2 levels, which have been reported in infertile men [114, 124]), may thus be responsible for an increased expression of Fas system (FasL and FasR) and diminished expression of c-kit, with augmented rates of germ cell apoptosis. SCs respond increasing SCF expression to maintain germ cell survival. If SCF/c-kit actions do not counteract Fas response germ cells will be depleted with impairment of spermatogenesis and consequently male infertility.

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V. Conclusion

In the present study it was showed that E_2 stimulation has the ability to disturb SCs and germ cells communication by modulating the expression of survival (SCF/c-kit) and death (Fas system) regulators. This was achieved by a double approach using *ex vivo* SeT cultures and SCs primary cultures, producing concordant results. The observed decrease in c-kit expression coupled with increased expression of Fas system may suggest a diminished survival and increased apoptosis of germ cells in the epithelium of SeT, which could be relevant for long-term maintenance of spermatogenesis. Although apoptotic conditions were present, since apoptotic related genes was increased and c-kit expression decreased, still remains pending the confirmation of germ cells death in response to high concentrations of E_2. Further studies using assays for measurement of apoptosis will disclose this question determining whether or not germ cells number is decreasing. In this case, the whole SeT cultures and co-cultures of germ cells and SCs could be the most suitable systems.

As it is known that high E_2concentrations are present in the testes of infertile men, our future studies could augment the knowledge on male infertility causes, and contribute to establish new treatments of infertility that could attempt to decrease estrogens concentration, for example inhibiting aromatase expression.

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VI. References

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VII. List of Communications

Alves MR, Correia S, Laurentino S, Cavaco JE, Oliveira PF and Socorro S. Genes under estrogenic control in the testis: impact on apoptosis and male fertility. *XXXVIII Jornadas Portuguesas de Genética* 4-5 Junho 2013, Porto, Portugal.

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