17 β -estradiol signaling and regulation of Sertoli cell function

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In this review, we will present an overview of estrogen actions in the testis from immature and adult animals, with special emphasis on signaling mechanisms involved in the 17 β -estradiol regulation of Sertoli cell function in immature rats. 17 β -estradiol activates Sertoli cell proliferation in immature rats by a mechanism that involves the translocation of the estrogen receptors ESR1 and ESR2 to the plasma membrane, phosphorylation of epidermal growth factor receptor and activation of mitogen-activated protein kinase 3/1. Activation of the G protein-coupled estrogen receptor (GPER) also induces phosphorylation of mitogen-activated protein kinase 3/1 via epidermal growth factor receptor transactivation, which in turn increases expression of the antiapoptotic protein BCL2 and decreases the expression of proapoptotic protein BAX, indicating an antiapoptotic role of E2-GPER in immature rat Sertoli cells. In conclusion, ESRs and GPER can mediate rapid 17 β -estradiol signaling in Sertoli cells, and modulate transcriptional events important for Sertoli cell function and maintenance of normal testis development and homeostasis. Our findings are important to clarify the role of estrogen in a critical period of testicular development and to direct further studies, which may contribute to better understand the causes of male infertility.

Spermatogenesis is a complex process controlled by a network of endocrine and other regulatory factors. Somatic cells (Sertoli cells) play a key role in the control of germ cell development. Androgens are recognized as a major factor to support male germ cell development, and Sertoli cells are important targets for androgen action (reviewed in ref. 1 and 2). Estrogens also play key roles in the development and maintenance of the reproductive function and fertility, and also have an important role in pathological processes observed in tissues from the reproductive system (reviewed in ref. 3–7). Thus, there is a delicate balance between androgens and estrogens to maintain normal reproductive function.

In this review, we will present an overview of the estrogen actions in the testis, with special emphasis on signaling mechanisms involved in the 17 β -estradiol (E2) regulation of Sertoli cell function.

Estrogen Receptors Signaling and Mechanism of Action

The three main endogenous estrogens are 17 β -estradiol (E2), estrone (E1) and estriol (E3), of which E2 is the predominant and most active. E2 is produced from testosterone by aromatase enzyme cytochrome P45019 A1, encoded by the *CYP19* gene (reviewed in ref. 8).

Cellular signaling of estrogens is mediated through two estrogen receptors ESR1 and ESR2 (also known as ER α and ER β , respectively), both belonging to the nuclear receptor family of transcription factors. ESR1 and ESR2 are encoded by two different genes located on chromosomes 6 and 14, respectively, and contain five distinct functional domains. Both receptors are expressed in a wide range of tissues and cell types throughout the body. The expression of these receptors varies in different tissues during development, aging or disease state. Several splice variants have been described for these receptors, but whether all the variants are expressed as functional proteins is not clear (reviewed in ref. 9–11).

Nuclear (genomic) modulation of target gene transcription by ESRs is a complex and highly dynamic process. After E2 or synthetic ligands bind to the ligand binding domain, ESR dissociates from its chaperone proteins, undergoes a conformational change and dimerizes. These activated ESR-dimer complexes bind to target sites on DNA, either directly, to specific estrogen response elements (EREs), or indirectly, through other transcription factors, such as activating protein-1 (AP-1), specificity protein 1 (Sp-1), nuclear factor κ B (NF κ B) and signal transducer and activator of transcription (STAT) 5. After binding to DNA, the dimer interacts with coregulatory proteins, such as chromatin modulators, co-activators and corepressors, important for the modulation of gene expression. The nature of the ligand is a key determinant of coregulatory protein recruitment and of the distinct biological response to ESR ligands.^{9–13} Furthermore, the nature and concentration of the ligand determines whether homodimers, heterodimers or a combination of the two is formed, and distinct patterns of gene regulation are observed.^{14,15}

In addition to the well established transcriptional effects of E2 mediated by the classical nuclear ESRs, estrogens also mediate rapid effects, occurring within seconds or minutes. These rapid effects may be mediated by: (1) ESR1 and ESR2 localized at or near the plasma membrane after exposure to ligand

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(reviewed in ref. 16–19); (2) truncated variants of ESR1 called ER α -46,²⁰ and ER α -36,^{21,22} and/or (3) G protein-coupled estrogen receptor (GPER or GPR30) (reviewed in ref. 23–26). These rapid responses include activation of different downstream signaling pathways, for example, the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase (PI3K) pathways, eNOS activation, cyclic AMP production and intracellular calcium mobilization, which in turn can modulate nuclear transcriptional events in a variety of normal or transformed cell lines (reviewed in ref. 16–26). How ER localizes to the cell membrane is not clear. Studies suggest that receptor post-translational lipid modifications, such as palmitoylation, could play a role facilitating membrane localization of ER (reviewed in ref. 18, 19 and 27).

Estrogen Synthesis and Function in the Male

In males E2 is present in low concentrations in blood, but its concentration in semen and rete testis can reach values even higher than in female serum.^{28,29} The role of estrogen in male reproduction had remained unclear until the first studies with mice with targeted disruption of ESRs (*Esr1*^{-/-}).^{30,31} *Esr1*^{-/-} male is infertile, its testes appeared normal until puberty but began to degenerate afterwards and became atrophic at adult age, and they also presented abnormalities in spermatogenesis and sperm concentration. *Esr2*^{-/-} mouse, in which exon 3 of *Esr2* was deleted by Cre/LoxP-mediated excision, is also sterile.³²

The main source of estrogens in the testis is the local conversion of androgens to estrogens by the cytochrome P450 aromatase, encoded by the *CYP19* gene (reviewed in ref. 6 and 33). Aromatase is localized in the cellular endoplasmic reticulum and catalyzes the last step in the steroidogenic pathway. Depending on the species, aromatase can generally be detected in Leydig and Sertoli cells, in spermatogonia, spermatocytes, elongate spermatids and spermatozoa (reviewed in ref. 6, 33 and 34). More recently, the presence of aromatase mRNA in gonocytes and preleptotene spermatocytes has also been reported.³⁴ In immature rats, Sertoli cells are the major source of estrogen but Leydig cells are the main source in adult animals.^{35,36} Expression of aromatase is stimulated by follicle-stimulating hormone (FSH) and the maximum stimulatory effect of FSH in aromatase gene expression occurs in 20-d old rat Sertoli cells and seems to parallel Sertoli cell differentiation. Leydig cells are the main source of estrogen in humans, although Sertoli cells are also able to synthesize E2 in vitro. Expression of the aromatase gene has not been detected in purified myoid cells from mice (reviewed in ref. 37).

A mutation in either exon V or IX of the *CYP19* gene causes a deficiency in aromatase function. Eight men have been described to harbor one of these mutations.^{38,39} These men have undetectable estrogen and normal to high levels of testosterone and gonadotropins. The aromatase knockout mouse presents a similar phenotype, and infertility in *Cyp19*^{-/-} animals is mainly caused by a defect of germ cell function.^{40,41}

Estrogen Receptors in the Testis

In the testis, the reports of ESR1 and ESR2 expression are highly variable, with major differences between species, as well as between individuals within a species. Results also differ with respect to mRNA expression in testicular tissues and cells, and immunohistochemical localization of the receptors (reviewed in ref. 5, 6 and 34). In testis from immature (15 and 28 d-old) and adult (120 d-old) rats, the presence of *Esr1* transcript and of two splicing variants of *Esr2* are observed: the *Esr2_v1* isoform in testis from 15 d-old rats⁴² with high similarity with the human *ESR2*,⁴³ and the *Esr2_v2* in testis from 15, 28 and 120 d-old rats,⁴² which contains an in-frame insert of 54 nucleotides, only described in tissues from rodents (reviewed in ref. 9). *Esr2_v2* encodes a protein that differs from the isoform 1 of ESR2 only by an insertion of 18 aminoacids in the ligand binding domain. A more recent study confirmed the presence of these isoforms of *Esr2*, and that the expression of *Esr1* transcripts does not change in the whole testis from 10 and 30 d-old rats, whereas it increases between 30 and 70 d-old rats.⁴⁴ In adult rats, levels of *Esr1* transcript are higher in seminiferous tubules than in Leydig cells; in germ cells, *Esr1* expression is higher in round spermatids than in pachytene spermatocytes and also in stages VII to XIV, suggesting a role of estrogens in those haploid steps of spermatogenesis.⁴⁴ Furthermore, the presence of another *Esr1* transcript, with a deletion of exon 4, has been observed in the testis from adult rats.⁴⁴ In the adult rat testis, *Esr2* transcript is expressed at the same level in seminiferous tubules and Leydig cells, whereas this transcript is higher in round spermatids than in pachytene spermatocytes.⁴⁴

In protein gel blot analysis only one protein band is distinguished with an antibody that recognizes the C-terminal region of the ESR1 in the whole adult rat testis, in purified germ cells⁴⁵ and in immature rat Sertoli cells.⁴² Furthermore, one protein band is distinguished with an antibody that recognizes the N-terminal region of the receptor that is common to both ESR2 isoforms.⁴² In the testis ESR1 is immunolocalized in Sertoli cells from immature and adult rats. Although germ cells from immature rats are ESR1-negative, some spermatids are positive for this receptor in the adult animal.⁴² Our laboratory also has detected ESR2 specific immunostaining in multiple cell types in rat testis, including Sertoli-Leydig cells, and in some, but not all, germ cells.⁴²

In human testis, some studies were unable to detect ESR1 (reviewed in ref. 7), while others report the expression of ESR1 and ESR2 (reviewed in ref. 6, 36 and 46). An isoform of the human ESR1 has been identified that lacks the first exon, which encodes the N-terminal 173 amino acids (A/B region) present in ESR1 (66 kDa). This isoform, called ER α -46, is an effective ligand-inducible transcription factor, but an inhibitor of ESR1 in a cell context where the transactivating function of AF-1 predominates over AF-2.⁴⁷ ER α -46 protein has been detected in human immature germ cells and spermatozoa.³⁶

There are conflicting data regarding the presence of mRNA for GPER in human and rodent testis (reviewed in ref. 48). *Gper* transcript is observed in the testes from 5, 15 and 120 d-old

rats.⁴⁹ *Gper* transcript is also observed in the mouse spermatogonial GC-1 cell line,⁵⁰ in rat pachytene spermatocytes and round spermatids^{45,51} and in primary culture of Sertoli cells.⁴⁹

17 β -estradiol Signaling and Regulation of Sertoli Cells

Sertoli cells are known to divide in the fetal rat testis, proliferating maximally on day 20 post conception, with a progressive decrease in division until postnatal day 21, when division ceases.⁵² The Sertoli cell population remains relatively constant in adult mammals, and each Sertoli cell supports approximately 30–50 germ cells at different stages of development in the seminiferous epithelium.⁵³ However, recent studies have shown that Sertoli cells are capable of dividing even in adult animals, including rats,⁵⁴ mice⁵⁵ and humans,^{55,56} under experimental and even physiological conditions.

Recent studies have shown that in the microenvironment of the seminiferous epithelium, wherein each Sertoli cell supports germ cells at different stages of development, locally produced autocrine and paracrine factors are involved in spermatogenesis (reviewed in ref. 57–59). As mentioned before, Sertoli cells are the major source of estrogens in immature rats, although Leydig and germ cells synthesize these hormones in adult animals (reviewed in ref. 6 and 36). Thus, a better knowledge about the role of 17 β -estradiol and its receptors in the regulation of the homeostasis and functions of Sertoli cell will be important for the control of male fertility.

Our laboratory has studied the presence of E2 receptors and the effects of E2 in Sertoli cells from 15 d-old rats. The presence of ESR1 and ESR2 in the nuclei of cultured Sertoli cells has been observed.⁴² E2 at physiological concentration activates a translocation of ESRs to the plasma membrane mediated by SRC (SRC family of tyrosine kinases), which results in the phosphorylation of EGFR (epidermal growth factor receptor) and mitogen-activated protein kinase 3/1 (MAPK3/1, also known as ERK1/2). E2-ESRs also induce PI3K-AKT activation (Royer C, et al. unpublished data). Moreover, activation of ESR1 and/or ESR2 by E2 is involved in the proliferation of immature Sertoli cells,⁴² and the mitogenic effect of E2-ESRs involves upregulation of CCND1 (Cyclin D1) in these cells.⁴⁹ Study using estrogen non-responsive ESR1 knock-in (ENERK1) mice, which have a point mutation in the ligand-binding domain of ESR1 that significantly reduces interaction with endogenous estrogens, without affecting ligand-independent ESR1 pathways mediated by growth factors, confirmed that estrogen-dependent ESR1 signaling is required for germ cell viability, most likely through support of Sertoli cell function.⁶⁰

The GPER (mRNA and protein) is present in rat Sertoli cells as reported by our laboratory.⁴⁹ GPER immunostaining was

Figure 1. Identification of GPER in Sertoli cells. Specific immunostaining for GPER using rabbit antibody generated against a synthetic peptide derived from the C-terminal of deduced amino acid sequence of human GPER polypeptide (red). Negative control was performed using normal rabbit serum at the same dilution of antibody or in the absence of primary antibody.

mostly found in the intracellular region but not in the nucleus of Sertoli cells (Fig. 1). To identify the specific cellular localization of this receptor in the Sertoli cells, we performed co-localization studies in confocal microscopy, using an anti-GPER antibody (a kind gift from Edward J. Filardo, Rhode Island Hospital) and markers for plasma membrane (pan-cadherin), endoplasmic reticulum (ER-tracker Blue-White DPX), Golgi apparatus (BODIPY-TR-Ceramide) and mitochondria (JC-1). GPER was preferentially immunodetected in the endoplasmic reticulum and Golgi apparatus, whereas almost no colocalization was observed using plasma membrane or mitochondria markers (Fig. 2). These results are consistent with report in various cell types (reviewed in ref. 24). In general, seven-transmembrane receptors are well expressed within the endoplasmic reticulum and submembranous vesicles, and these localizations have been associated with their trafficking to the plasma membrane during receptor biosynthesis (reviewed in ref. 61) and their endocytic trafficking after agonist stimulation (reviewed in ref. 62).

In rat Sertoli cells, activation of GPER induces transactivation of EGFR via activation of SRC by G-protein β γ subunits that promotes a metalloprotease-dependent release of EGFR ligands, with a consequent activation of MAPK3/1 phosphorylation, which in turn triggers an increase in the expression of antiapoptotic protein BCL2 and a decrease in the expression of proapoptotic protein BAX, indicating an antiapoptotic effect of E2-GPER in rat Sertoli cells.⁴⁹ Furthermore, E2-GPER through EGFR-MAPK3/1-phospho-CREB (cyclic AMP response element-binding protein) may regulate the proapoptotic protein BAX in cultured Sertoli cells from 15 d-old rats (Royer C, et al. unpublished data). It is important to emphasize, that low doses of bisphenol A stimulate human seminoma (JKT-1) cell proliferation by allowing a rapid membrane-initiated activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) signaling pathways associated

Figure 2. For figure legend, see page 5.

Figure 2. Colocalization of GPER with the plasma membrane, endoplasmic reticulum, Golgi apparatus and mitochondria markers in Sertoli cells by confocal laser scanning microscopy. Specific immunostaining for GPER was observed in the Sertoli cells (red in A, B, D, and green in C). (A) Nuclei were stained with DAPI (blue) and the plasma membrane was marked with anti-pan-cadherin antibody conjugated with FITC (green). No colocalization of the red and green labels is shown in merged images (at right). (B) Nuclei were stained with Yo-Pro-1 (green) and the endoplasmic reticulum was marked with ER-tracker Blue-white DPX (blue). Colocalization of the red and blue labels is shown in merged images (magenta, at right). (C) Nuclei were stained with DAPI (blue) and the Golgi apparatus was marked with BODIPY-TR-Ceramide (red). Colocalization of the green and red labels is shown in merged images (yellow, at right). (D) Nuclei were stained with DAPI (blue) and the mitochondria were marked with JC-1 (green). No colocalization of the red and green labels is shown in merged images (at right). Scale bar as indicated. The data shown are representative of three to five independent experiments.

Figure 3. Putative mechanism of estrogen action in rat Sertoli cells. Estrogen interaction with classical nuclear ESRs induce translocation of these receptors to the plasma membrane, activation of SRC, which phosphorylates and activates EGFR, leading to activation of MAPK3/1 and gene transcription involved with Sertoli cell proliferation (pathway indicated by black arrows).⁴² Estrogen interaction with GPER leads to activation of SRC by $\beta\gamma$ subunits of a G protein, activation of a metalloproteinase, which releases HB-EGF from the plasma membrane, leading to the activation of EGFR, MAPK3/1, and gene transcription involved with inhibition of Sertoli cell apoptosis (pathway indicated by red arrows).⁴⁹ See text for more details.

with phosphorylation of the transcription factor CREB and the cell cycle regulator retinoblastoma protein (Rb).⁶³ In human testes, a positive GPER immunostaining is detected in Leydig and in Sertoli cells while all germ cells are immunonegative. Furthermore, neoplastic cells of the Sertoli cell tumor, Leydig cell tumor, seminoma and embryonal carcinoma are all immunopositive.⁶⁴ Analysis of GPER expression in several types of post-pubertal testicular germ cell tumors confirmed high level of GPER expression in all intratubular germ cell tumors, seminomas and embryonal carcinomas, but low level in teratomas.⁶⁵

In conclusion, ESRs and GPER can mediate the rapid E2 actions in Sertoli cells, which in turn can modulate nuclear

transcriptional events (Fig. 3) important for Sertoli cell function and maintenance of normal testis development and homeostasis. Our findings are important to clarify the role of estrogen in a critical period of testicular development, and to direct further studies, which may contribute to better understand the causes of male infertility.

Concluding Remarks and Future Perspectives

Signaling initiated by extra-nuclear pathways may help modulating nuclear transcriptional responses by priming signaling molecules, coactivators or corepressors for more robust nuclear

response. Additional studies evaluating the biological role of the membrane-initiated estrogen receptors signaling, either alone or in conjunction with ESRs nuclear transcriptional function will be necessary for our overall understanding of estrogen receptor biological function in Sertoli cells and also in testicular germ cell tumors.

Disclosure of Potential Conflicts of Interest

The authors have nothing to declare.

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