Review

Oestrogens and spermatogenesis

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The role of oestrogens in male reproductive tract physiology has for a long time been a subject of debate. The testis produces significant amounts of oestrogenic hormones, via aromatase, and oestrogen receptors (ERs) α (ESR1) and ER β (ESR2) are selectively expressed in cells of the testis as well as the epididymal epithelium, depending upon species. This review summarizes the current knowledge concerning the presence and activity of aromatase and ERs in testis and sperm and the potential roles that oestrogens may have in mammalian spermatogenesis. Data show that physiology of the male gonad is in part under the control of a balance of androgens and oestrogens, with aromatase serving as a modulator.

Keywords: testis; oestrogen; spermatogenesis; oestrogen receptor; germ cell

1. INTRODUCTION

The mammalian testis is a complex organ that serves two important functions: synthesis of steroids and production of spermatozoa that are controlled by gonadotrophins and numerous locally synthesized factors (Saez 1994) and among them oestrogens (see review, Carreau *et al.* 1999). Aromatase, which is the last step in the steroidogenesis pathway leading to the formation of oestrogens from androgens, is localized in the cellular endoplasmic reticulum of numerous tissues. The role of oestrogens in the physiology of male reproductive tract of mammals has for a long time been a subject of debate, even though more and more evidence suggests that oestrogens are involved via their specific receptors (see reviews of O'Donnell *et al.* 2001; Hess 2003; Carreau *et al.* 2008). Indeed, 70 years ago, Zondek (1934) discovered an oestrogenic hormone in stallion urine; but at the beginning, oestrogen production by testicular tissues was more a curiosity and it was only 30 years later that several publications provided evidence that testes are able to synthesize and secrete oestrogens. Jayle *et al.* (1962) were the first to demonstrate that the human testis produce oestrogens and moreover hCG stimulates that production, whereas castration exerts an opposite effect. Hendry *et al.* (1983) confirmed the testicular source of oestrogens and reported higher levels in the spermatic vein of men. Consequently, the presence of large quantities of oestrogens in the rete testis fluid and the spermatic vein of numerous mammals has been reported (reviewed by Hess 2003). Therefore, besides a well-known negative feedback of oestrogens in the hypothalamus–hypophysis complex, it has become more obvious that testicular oestrogens could play a role locally in the male gonad and the reproductive tract, especially

after the discoveries of patients genetically deficient in aromatase (see review of Rochira *et al.* 2005) and the oestrogen receptor (ER) α knockout mouse (review by Hess *et al.* 2002). Moreover, decreased sperm counts and increased male reproductive tract disorders (cryptorchidism, hypospadias and testicular cancer) in men have been described and related to a deleterious effect of endocrine disruptors (Toppari *et al.* 1996; Skakkebaek 2004). In this review, we will summarize the data concerning aromatase sources in the male genital tract, the presence of ERs in testis and the roles of oestrogens in mammalian spermatogenesis. There will also be a special emphasis on recent knowledge obtained with the presence of GPR30 in germ cells (Sirianni *et al.* 2008).

2. OESTROGEN SOURCES IN MAMMALIAN TESTICULAR CELLS

(a) Cyp19 gene

The aromatase enzyme complex comprises two proteins: a specific cytochrome P450 (P450arom) encoded by the *CYP19* gene and a ubiquitous NADPH cytochrome P450 reductase. In humans, *Cyp19* gene is located in the 21.2 region of the long arm of the chromosome 15 and is approximately 123 kb length (Sebastian & Bulun 2001). This gene contains nine exons (exons II–X) encoding for a unique protein with a molecular weight of 55 kDa (Nakajin *et al.* 1986) and upstream of exon II, a regulatory region is present (Simpson *et al.* 1994). The 5' region contains 10 promoters, which are used according to the needs and the characteristics of the tissue. However, regardless of the promoter used, each untranslated first exon is spliced into a common splice junction localized 39 bp upstream of the translational start site (AUG; Simpson *et al.* 1997). In mouse, the *Cyp19* gene is localized on chromosome 9, and Golovine *et al.* (2003) have shown that three promoters specifically control aromatase gene expression.

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One contribution of 17 to a Theme Issue 'The biology and regulation of spermatogenesis'.

Figure 1. Age-related changes on the use of P450 aromatase transcripts in rat testis.

In rat, the *CYP19* gene is situated on chromosome 8 and up to now three promoters have been described: the promoter PI.f used in brain (Yamada-Mouri *et al.* 1996) but also in testis (Silandre *et al.* 2007), the promoter PII (Young & McPhaul 1998), which is the main one directing aromatase gene expression in gonads (Lanzino *et al.* 2001; Pezzi *et al.* 2004), and the promoter PI.tr only used in testis (Silandre *et al.* 2007). Indeed, in figure 1, we have provided a schematic presentation of these promoters used according to age in the rat gonad, which suggests a very precise control of the expression of aromatase and the amount of oestrogens produced.

(b) Testicular oestrogens in mammals

As abundantly documented in the literature, it is difficult to find a tissue completely devoid of aromatase gene expression (Simpson *et al.* 1994); indeed, it is now well established that the mammalian testis is able to produce oestrogens (table 1; Hess 2003; Hess & Carnes 2004; Carreau *et al.* 2006). Early in the study of testicular aromatase, it was thought that its expression was in Sertoli cells during development in the rodent species, but then only in Leydig cells in the adult testis (see reviews of Hess & Carnes 2004; Carreau *et al.* 2007b). In some species such as pig (see review by Conley & Hinshelwood 2001) and human (Inkster *et al.* 1995), a testicular P450arom mRNA was thought to be present only in Leydig cells.

Despite the large amount of data published in different species, the precise localization of aromatase has been a subject of debate. Some of the variations observed between immunohistochemicalization and enzyme activity in cell culture could result from an absence of endocrine and/or paracrine regulation (Carreau 1994), such as after *in vivo* germ cell depletion or lack of cell–cell contacts in culture dishes (Papadopoulos *et al.* 1987; Carreau *et al.* 1998).

The availability of antibodies allowed for immunohistochemicalization of aromatase in Leydig cells of young and adult rats (Kurosumi *et al.* 1985). After being purified, aromatase was cloned and primers made available for checking transcripts together with measurements of aromatase activity and immunohistological studies. Thus, with these tools Nitta *et al.* (1993) were the first to demonstrate that mouse germ cells express aromatase. In male rat gonad, several distinct sites of aromatization have been reported and oestrogen synthesis evolves with age: in mature rat, Leydig cells are considered as the major source of oestrogens, whereas in the immature animal, this synthesis is in Sertoli cells (Rommerts *et al.* 1982; Papadopoulos *et al.* 1986). This has been confirmed *in vitro* using specific primers to detect aromatase transcripts (Levallet & Carreau 1997; Genissel & Carreau 2001).

Additionally, we have shown in adult rats that germ cells represent a new source of oestrogens (Janulis *et al.* 1996, 1998; Levallet *et al.* 1998a). The expression of

Table 1. Aromatase expression (+), absence (–) or undetermined (?) in testicular cells of mammals (only the main references).

species	Leydig cell	Sertoli cell	germ cell	references
mouse	+	+	+	Nitta <i>et al.</i> (1993)
rat	+	+	+	Janulis <i>et al.</i> (1996), and Levallet <i>et al.</i> (1998a,b)
bank vole	+	+	+	Bilinska <i>et al.</i> (2001)
boar	+	?	+/-	Conley & Hinshelwood (2001) and Raeside & Renaud (1983)
bear	+	?	+	Tsubota <i>et al.</i> (1997)
bison	+	+	+	Kopera <i>et al.</i> (in press)
deer	+	?	+	Schon & Blottner (2008)
horse	+	+/-	+	Hejmej <i>et al.</i> (2007) and Sipahutar <i>et al.</i> (2003)
monkey	+	?	+?	Pereyra-Martinez <i>et al.</i> (2001)
human	+	+	+	Lambard <i>et al.</i> (2003, 2004) and Payne <i>et al.</i> (1976)

the aromatase gene is higher in meiotic prophase cells (pachytene spermatocytes) than in round spermatids or testicular spermatozoa; conversely, aromatase activity (measured by the tritiated water release method) is greater in haploid germ cells than in the younger spermatocyte germ cells, a finding also confirmed by the immunolocalization of the protein that was intensively expressed in elongated spermatids (Levallet *et al.* 1998b) and newly released sperm (Janulis *et al.* 1998). The presence of aromatase mRNA has been reported in gonocytes, spermatogonia as well as in preleptotene spermatocytes (Silandre *et al.* 2007). However, in purified myoid cells, we were unable to detect any aromatase transcript by RT-PCR and therefore, the aromatase gene is present in all stages of germ cell development (figure 2) so far studied (Carreau *et al.* 2008).

In male bank vole, aromatase has also been described not only in somatic cells but also in germ cells and the protein is quantitatively more abundant during the breeding season corresponding to the full development of spermatogenesis (Bilinska *et al.* 2001; Kotula-Balak *et al.* 2003). Similar observations in terms of seasonal changes in the expression of steroidogenic enzymes have been made in the black bear by Tsubota *et al.* (1997). In bison (Kopera *et al.* in press) and the roe deer (Schon & Blottner 2008), somatic and germ cells expressed aromatase. In boars, all data available are in agreement that Leydig cells represent the only source of oestrogens (Raeside & Renaud 1983; Mutembei *et al.* 2005), which was confirmed in the minipig (Weng *et al.* 2005).

Figure 2. Aromatase immunohistochemical staining in (a) the adult mouse testis and (b) caput epididymis. In testis, staining is intense in elongate spermatids (ES) and spermatozoa in the seminiferous tubule lumen (Sp). All other germ cells and Sertoli cells show weaker staining. Leydig cells (LC) are also positive in the interstitial space. In the epididymal lumen, aromatase is strongly positive in the cytoplasmic droplet of the sperm tail (arrows). The epididymal epithelium (Epid) may also show some weak staining in some cells. Scale bars, (a) 25 μ m; (b) 10 μ m.

The stallion was the first mammal in which oestrogens were discovered to be produced by the testis (Zondek 1934). Later data have confirmed that the testicular source of oestrogens in horse resides quite exclusively in the Leydig cells (Eisenhauer *et al.* 1994), but recent data are in favour of a putative source of oestrogens in the seminiferous tubules (Sipahutar *et al.* 2003), recently supported by Hejmej *et al.* (2007) even though Hess & Roser (2004) were not able to demonstrate a positive staining in germ cells.

In monkey (Pereyra-Martinez *et al.* 2001) and the human testis (Carreau *et al.* 2010), aromatase was found not only in the Leydig cells (Payne *et al.* 1976) but also in Sertoli cells (Foucault *et al.* 1992), as well as in immature germ cells (Lambard *et al.* 2003) and ejaculated spermatozoa (Aquila *et al.* 2002; Lambard *et al.* 2004; Carreau *et al.* 2010). As can be seen on figure 3 there is a dual immunohistolocalization of aromatase in ejaculated spermatozoa with strong staining in the midpiece and an annular presence of aromatase at the limit between the acrosomal membrane and the nucleus. Thus, the localization of aromatase in the midpiece and in the acrosomal membrane with ERs could be related to a potential role for oestrogen in mitochondria and energy production for motility, as well as for participation in capacitation and/or the acrosomal reaction of sperm (Solakidi *et al.* 2005; Aquila *et al.* 2004).

3. OESTROGEN RECEPTORS IN TESTIS

It has been known for many years that oestrogen binds to proteins in male reproductive tissues, especially the epididymis, and now there is clear evidence that ER α (ESR1) and ER β (ESR2) are localized in specific cells of the testis, efferent ductules and epididymis, with species specificity (see reviews by Hess 2000, 2003, 2004; Hess *et al.* 2001, 2002; Hess & Carnes 2004; Sierens *et al.* 2005). ER β has a rather ubiquitous expression throughout the male reproductive system,

but ER α has greater specificity, with only two regions of the male reproductive system consistently being demonstrated to express ER α in every species, namely Leydig cells of testis and epithelium of efferent ductules, the region connecting rete testis to the head of the epididymis (Hess *et al.* 2001; Hess 2002). Immunohistochemical staining for ER α is more intense in efferent ductule epithelium than in any other tissue and its mRNA expression in this tissue is 3.5-fold higher than even in uterus (Hess *et al.* 1997b), which traditionally has been the standard for ER expression.

In testis, the reports of ER α and ER β expression are highly variable (table 2), with major differences between species, as well as between individuals within a species. Results also differ between immunohistochemical localization of the receptors and mRNA analysis of testicular tissues and cells (Saunders *et al.* 2001; Hess & Carnes 2004; Carreau *et al.* 2007a). This confusion in the literature over ER localization in cells of the testis, especially for ER α , has made it more difficult to draw conclusions and to compare data between species. The differences appear to be due primarily to tissue preservation techniques and antibodies used for immunohistochemistry (Hess *et al.* 2002; Lucas *et al.* 2008). However, localization of ER β , which is typically more abundant than ER α , has been more consistent.

In mouse testis, ER α was found only in Leydig cells and some peritubular myoid cells (figure 4), whereas ER β was found in some Leydig cells, Sertoli cells and some germ cells, particularly spermatocytes (Zhou *et al.* 2002; Kotula-Balak *et al.* 2005). In general, ER α has been noted in the interstitial space, whereas ER β is most often located within the seminiferous epithelium. However, using frozen sections and new antibodies against the amino- or carboxy-terminal regions of ER α , very solid data revealed that ER α is also expressed in Sertoli cells and some germ cells of the rat (Lucas *et al.* 2008). In the same study, 17 β -oestradiol treatment of cultured immature Sertoli cells resulted in a translocation of both ER α and ER β from the nucleus to the plasmalemma (Lucas *et al.* 2008). Based on these new data, the entire field of ER localization in the male reproductive system may require reinvestigation to more accurately determine oestrogen responsive cell types in the different tissues and among different species.

In human testis, most early reports suggested no labelling for ER α in any of the testicular cells (Pelletier & El-Alfy 2000; Saunders *et al.* 2001; Mäkinen *et al.* 2001; Sierens *et al.* 2005). However, an occasional paper suggested that ER α could be found in germ cells, particularly spermatocytes and spermatids (Pentikäinen *et al.* 2000; Lambard *et al.* 2004). Even as early as 1981, it was suggested that human spermatozoa showed specific binding sites for 17 β -oestradiol near the plasma membrane (Cheng *et al.* 1981a,b). Then in 1998, it was shown that the spermatozoon contained both mRNA and protein for ER α (Durkee *et al.* 1998) and more recent studies have confirmed this finding of mRNA and protein for ER α as well as ER β in human ejaculated sperm (Durkee *et al.* 1998; Aquila *et al.* 2004; Lambard *et al.* 2004; Solakidi *et al.*

2005). Sperm appear to express the expected size (66 kDa) of ER α , but also a shorter isoform of 46 kDa (Lambard *et al.* 2004; Solakidi *et al.* 2005). This smaller form was only found in spermatozoa and is located on the membrane (Lambard *et al.* 2004), which is consistent with this protein being similar to GPR30 (G protein-coupled receptor 30), an integral membrane protein capable of mediating the rapid effects of oestrogen through a non-genomic pathway (Filardo *et al.* 2002, 2007; Revankar *et al.* 2005).

4. OESTROGENS AND THE REGULATION OF SPERMATOGENESIS

It has been known for many years that oestrogen has an essential role in regulating the hypothalamus–pituitary–testis axis and thus indirectly regulates the luteinizing hormone (LH) and testosterone (T) balance through a feedback loop (O'Donnell *et al.* 2001). It is also now well recognized that testicular and germ cell oestrogen has a direct role in the regulation of downstream physiology, as the highest concentration of ER α is found in epithelial cells lining the efferent ductules (Hess *et al.* 2002; Hess 2003), whose primary function is to reabsorb luminal fluid and increase the concentration of sperm before they enter the epididymis (Hess 2002). However, evidence for oestrogen having a direct action on the seminiferous epithelium has been lacking. Opinions favouring a role for oestrogen in spermatogenesis have typically come from indirect evidence (Hess *et al.* 2002; Hess 2003; Hess & Carnes 2004; Carreau *et al.* 2007a,b, 2008): (i) prenatal exposure to oestrogens, (ii) expression of aromatase in the testis, (iii) expression of ER α and ER β in the testis, (iv) various knockout animal models, and (v) anti-oestrogen treatment (table 3). Others have carefully examined the important role of oestrogen in the development of testis and male reproductive tract (Sharpe *et al.* 2000; O'Donnell *et al.* 2001; Sharpe 2001, 2006; Albrecht *et al.* 2009); therefore, this review will examine oestrogen's potential function only in the adult testis and the regulation of spermatogenesis.

(a) *Oestrogen receptor knockout*

The original ER α knockout mouse, the neo-ERKO (Lubahn *et al.* 1993; Eddy *et al.* 1996), generated by inserting a *neo* gene into exon 2, produced the first evidence that a classical ER was essential for male fertility. However, spermatogenesis was reportedly normal up to about 10 weeks and subsequent degeneration of the seminiferous epithelium and eventual tubular atrophy appeared to be related to the accumulation of fluid in the rete testis (Eddy *et al.* 1996). A more detailed study of the neo-ERKO mouse revealed that fluid accumulation was due to the failure of efferent ductules to reabsorb luminal fluids, which then backed up into the rete testis and seminiferous tubules (figure 5), eventually inducing degeneration of the seminiferous and testicular atrophy (Hess *et al.* 1997a, 2000; Zhou *et al.* 2001). In these early studies of the neo-ERKO mouse, the only evidence suggesting a direct effect on the seminiferous epithelium was data showing a decrease in fluid secretion, presumably by the Sertoli cell (Hess *et al.* 1997a).

Table 2. ESR1 (ER α) and ESR2 (ER β) present (+) or absent (–) in the testis, examined by immunohistochemistry, *in situ* hybridization and RNA analysis. Peritub, peritubular myoid; spgonia, spermatogonia; spcyte, spermatocyte; sptid, spermatid; /, not determined.

species	cell types															
	whole testis		Leydig		peritub		Sertoli		spgonia		spcyte		sptid		sperm	
	ER α	ER β	ER α	ER β	ER α	ER β	ER α	ER β	ER α	ER β	ER α	ER β	ER α	ER β	ER α	ER β
rat ^a	+	+	+	+/-	+/-	+/-	+/-	+	-/+	+/-	-	+/-	-/+	+/-	+	-
mouse	+	+	+	+	+/-	+/-	-	+	-	+	-	+	-	+	/	/
and vole ^b																
dog ^c	/	/	+	-/+	+	-/+	-	-	-	+	-	+	-	+	-	-
cat ^d	/	/	+/-	+	-	+	-	-	-	+	-	+	-	+	-	-
goat ^e	+	/	-	/	-	/	-	/	-	/	-	/	-	/	-	-
boar ^f	+	+	-/+	+	/	/	-	+	+	+	+	+	-/+	+	/	/
bird ^g	+	/	/													
fish ^h	+	+	+	+	/	/	/	/	/	/	+	+	+	+	+	+
monkey ⁱ	+/-	/	+/-	+	-	+	-	+	-	+	-	-/+	-	+	-	-
man ^j	+/-	+	-/+	+/-	-	+	+/-	+/-	-	+	+/-	+	+/-	+/-	+/-	+/-

^aEcheverria *et al.* (1994), Fisher *et al.* (1997), Kuiper *et al.* (1997, 1998a,b), Mowa & Iwanaga (2001), Oliveira *et al.* (2003), Pelletier (2000), Saberwal *et al.* (2002), Saunders *et al.* (1997, 1998), Shughrue *et al.* (1998), van Pelt *et al.* (1999) and Zhai *et al.* (1996). One report has shown convincing evidence of ER α localized in the seminiferous epithelium (Lucas *et al.* 2008).

^bBilinska *et al.* (2001), Iguchi *et al.* (1991), Jefferson *et al.* (2000), Rosenfeld *et al.* (1998), Shibayama *et al.* (2001), Takao *et al.* (2003), Terakawa *et al.* (1985) and Zhou *et al.* (2002).

^cNie *et al.* (2002).

^dNie *et al.* (2002).

^eGoyal *et al.* (1997) and Mansour *et al.* (2001).

^fMutembei *et al.* (2005) and Rago *et al.* (2004).

^gGonzalez-Moran *et al.* (2008).

^hBouma & Nagler (2001), He *et al.* (2003), Huang *et al.* (in press), Nagler *et al.* (2007), Vinas & Piferrer (2008), Wu *et al.* (2001), Zhang *et al.* (2008) and Zhu *et al.* (2008).

ⁱFisher *et al.* (1997), Heikinheimo *et al.* (1995), McKinnell *et al.* (2001), Pelletier *et al.* (1999), Saunders *et al.* (2001) and West & Brenner (1990).

^jAquila *et al.* (2004), Berensztein *et al.* (2006), Brandenberger *et al.* (1997), Carreau *et al.* (2006), Denger *et al.* (2001), Enmark *et al.* (1997), Gaskell *et al.* (2003), Lambard *et al.* (2004), Makinen *et al.* (2001), Mosselman *et al.* (1996), Pelletier (2000), Pentikainen *et al.* (2000), Rago *et al.* (2006), Sar & Welsch (2000), Saunders *et al.* (2001, 2002), Sierens *et al.* (2005), Solakidi *et al.* (2005), Takeyama *et al.* (2001) and Taylor & Al-Azzawi (2000).

Figure 3. Localization of aromatase in ejaculated human spermatozoa using confocal microscopy. CD 46 (red): specific marker of inner acrosomal membrane; chromatin is localized with DAPI (blue) and the aromatase is revealed by a polyclonal antibody (green). Adapted from the publisher.

Subsequent studies showed that oestrogen's major effect was on the regulation of ion transporters and aquaporin water channels in efferent ductules (Zhou *et al.* 2001;

Oliveira *et al.* 2005; Ruz *et al.* 2006), but effects on these molecules in testis was not found.

The neo-ERKO mouse resulted in the presence of a truncated ER α protein (Lubahn *et al.* 1993; Couse *et al.* 1995); therefore, the floxed-ERKO mouse, generated by homologous recombination and Cre recombinase-mediated excision of exon 3, resulted in complete deletion of ER α transcripts downstream of exon 2 and complete ER α and ER β null mutants (Dupont *et al.* 2000). However, the male phenotype of this complete ER α knockout was essentially identical to that of the neo-ERKO, although a more recent floxed-ER α KO mouse has shown a 30 per cent decrease in testis weight at day 84 (Chen *et al.* 2009) compared with the neo-ERKO, which showed increased testis weight at day 81 (Hess *et al.* 1997a). The ER β KO, neo- or floxed-, mice have shown no significant effects on spermatogenesis (Couse *et al.* 1997; Dupont *et al.* 2000), although one study has shown Leydig cell hyperplasia and an increase in the number of spermatogonia (Gould *et al.* 2007). The double ER α β KO mice appear to be essentially the same as the ER α KO mice (Couse *et al.* 1997; Dupont *et al.* 2000), suggesting that ER β , although having widespread expression in the testis and male reproductive tract, has a limited role in testicular function. Thus, the question of whether oestrogen plays a

Table 3. Experimental animal models in the study of testicular oestrogen. A, infertility or decreased fertility or delayed infertility; B, increased or decreased LH and/or testosterone; C, change in testis weight or testicular atrophy; D, seminiferous tubular disruption; E, Leydig cell effects; F, efferent ductule luminal dilation; G, decreased efferent ductule epithelial height; H, decreased efferent ductule function +, present; –, not observed; I, decreased water and ion transport molecules; J, effects on sperm counts/motility; K, effects on prostate/seminal vesicles; /, not determined; esulfotransKO, oestrogen sulphotransferase knockout; ArKO, aromatase knockout.

experimental model	A	B	C	D	E	F	G	H	I	J	K
ER α KO: neo-ERKO and floxed-ERKO ^a	+	+	+	+	+	+	+	+	+	+	–
ER β KO ^b	–	–	–	–	–	–	–	–	–	–	+
ER $\alpha\beta$ KO ^c	+	+	+	+	+	+	+	+	+	+	+
ArKO: degeneration of testis with age ^d	+	–/+	–/+	+	+	–	–	–	–	+	+
NERKI (mutation of ER α DNA-binding domain) ^e	+	–	+	–	–	–	–	–	–/+	–	/
ENERKI (mutation of ER α ligand-binding domain) ^f	+/-	+	+/-	+/-	+	–	–	–	–	+/-	+
ENERKI+PPT (synthetic ER α agonist) ^g	–	–	–	–	–	–	–	–	/	/	/
arom overexpression ^h	+	+	+	+	+	/	/	/	/	/	+
ICI 182,780 ⁱ	+	+/-	+/-	+/-	+	+	+	+	+	+	+
tamoxifen ^j	–/+	+	+	+/-	+	/	/	/	/	+	+/-
raloxifene ^k	–	–	–	–	–	/	/	/	/	–	+
esulfotransKO ^l	–	/	+	+	+	/	/	/	/	+	+
aromatase inhibitor ^m	+	+	+	+	/	/	/	/	/	+	–
isoflavones (soy) ⁿ	–	–	–	–	–	/	/	/	/	–	+

^aAkingbemi *et al.* (2003), Dupont *et al.* (2000), Eddy *et al.* (1996), Hess *et al.* (1997a,b, 2000), Lee *et al.* (2000, 2001), Lubahn *et al.* (1989, 1993), Mahato *et al.* (2000, 2001), Nakai *et al.* (2001), Ogawa *et al.* (2000), Prins *et al.* (2001) and Zhou *et al.* (2001).

^bDupont *et al.* (2000), Gustafsson & Warner (2000), Kregel *et al.* (1998), Risbridger *et al.* (2001) and Weihua *et al.* (2001).

^cCouse *et al.* (1999) and Dupont *et al.* (2000).

^dFisher *et al.* (1998) and Robertson *et al.* (2001, 2002).

^eMcDevitt *et al.* (2007, 2008) and Weiss *et al.* (2008).

^fSinkevicius *et al.* (2008, #15519; 2009, #17036), McDevitt *et al.* (2007, #15322) and Li & Rahman (2008).

^gSinkevicius *et al.* (2009).

^hFowler *et al.* (2000), Gill *et al.* (2001), Hiramatsu *et al.* (1997), Luthra *et al.* (2003) and Simpson (2003).

ⁱMouse (Hess *et al.* 1997a,b; Lee *et al.* 2000; Cho *et al.* 2003), rat (Oliveira *et al.* 2001, 2002), prostate (Huynh *et al.* 2001; Turner *et al.* 2001; Ho 2004), human sperm (Aquila *et al.* 2004).

^jAdamopoulos *et al.* (1997), Belmonte *et al.* (1998), Brigante *et al.* (1985), Buvat *et al.* (1983), Chou *et al.* (1992), Corrada *et al.* (2004), Danner *et al.* (1983), Dony *et al.* (1985), Du Mond *et al.* (2001), Gill-Sharma *et al.* (1993, 2001, 2003), Gopalkrishnan *et al.* (1998), Kotoulas *et al.* (1994), Li (1991), Minucci *et al.* (1997), Nam *et al.* (2003), Noci *et al.* (1985), Padmalatha Rai & Vijayalaxmi (2001), Parte *et al.* (2000), Robinson *et al.* (1990), Rozenboim *et al.* (1986, 1989), Saberwal *et al.* (2002), Schill & Landthaler (1981) and Sethi-Saberwal *et al.* (2003).

^kHoyt *et al.* (1998) and Neubauer *et al.* (1993, 1995).

^lQian *et al.* (2001).

^mHayes *et al.* (2000, 2001), Leder *et al.* (2004), Luthra *et al.* (2003), Mauras *et al.* (2000), Omura *et al.* (2001), Panno *et al.* (1995), Shetty *et al.* (1998), Smith *et al.* (2002), Trunet *et al.* (1993), Turner *et al.* (2000) and Ulisse *et al.* (1994).

ⁿFaqi *et al.* (2004), Morrissey & Watson (2003), Mitchell *et al.* (2001) and Robertson *et al.* (2002).

Figure 4. (a) ER α and (b) ER β in the adult mouse testis. Immunohistochemical localization of ER α using perfusion fixation with neutral buffered formalin and the NCL-ER-6F11 antibody (Novocastra, Newcastle upon Tyne, UK) gave strong staining in Leydig (Lc), other interstitial cells and peritubular myoid cells (Pc) of the testis. Staining for ER β was more intense in some spermatocytes (Spc) than other germ cells, but all germ cells, except the elongated spermatids (Es) were positive, including the round spermatids (Rs). Scale bar, 50 μ m.

direct role in spermatogenesis was unanswered by the ER null mice.

(b) Aromatase knockout

The phenotype observed in the neo-ERKO mice generated considerable interest in the aromatase knockout (ArKO) mouse, in which the natural oestrogen ligand was removed. However, the ArKO mouse did not result in a neo-ERKO phenotype (Robertson *et al.* 1999, 2002; O'Donnell *et al.* 2001) and spermatogenesis appeared normal until the males began to age (table 3). Thus, it appears that oestrogen is important for the long-term maintenance of spermatogenesis, particularly the production of round spermatids and formation of the acrosomal granule, suggesting a role for oestrogen in the differentiation of spermatocytes (Robertson *et al.* 2002). Testis weight was not reduced in ArKO males until about 1 year, but degeneration of round spermatids began to appear as early as 12–14 weeks (Robertson *et al.* 1999, 2002). It was hypothesized that the standard soy-based diets may contain enough oestrogenic compounds to prevent a complete

Figure 5. Wild-type (WT) and neo-ER α KO adult mice testes. (a) WT seminiferous tubules at 90 days of age show normal spermatogenesis with full complement of spermatogenesis. (b) In the ER α KO at 90 days, some tubules are dilated (double arrow) and all seminiferous epithelia are decreased in height. (c) At 180 days of age, total atrophy of all seminiferous tubules can be found in the ER α KO testis, with some tubules showing denuded epithelia. The rete testis (RT) is severely dilated from birth and enlarges into adulthood. (d) Higher magnification of seminiferous tubules from 180 days ER α KO testis showing Sertoli cell (SC)-only tubules. Scale bars, 50 μ m.

oestrogen knockout in the ArKO mouse; therefore, the Simpson group carefully evaluated these males when fed soy+ and soy– diets (Robertson *et al.* 2002). Hence, the study suggested that dietary phytoestrogens may be ‘agonistic’ in the absence of endogenous oestrogen (ArKO mice), but ‘antagonistic’ when present with endogenous oestrogens (wild type (WT)). We have recently demonstrated (Hamden *et al.* 2008) that phytoestrogens, probably via their antioxidant role against the reactive oxygen species, are helpful in protecting the male reproductive functions, especially during ageing.

Removing soy phytoestrogens from the diet of ArKO mice has provided the most significant data to date to demonstrate that oestrogen does indeed have a role in the maintenance of spermatogenesis, at least after 12–14 weeks of age. Total withdrawal of oestrogens (endogenous and dietary soy) did not produce spermatogenic disruptions prior to 14 weeks, but at 1 year the disruption of spermatogenesis was more severe than in the ArKO + soy males, as there was a significant decline in spermatocytes, round and elongated spermatid numbers (Robertson *et al.* 2002). At 14 weeks, soy-deficient diets produced

decreased seminiferous tubular diameters in the ArKO mice, but increased in tubular lengths in both the ArKO and WT mice, which were explained by the separate effects of oestrogen on germ cells versus Sertoli cells. There was also an increase in the number of Sertoli cells in the ArKO + soy males.

It was surprising that the ArKO mouse did not induce a neo-ERKO phenotype (Robertson *et al.* 1999, 2002; O'Donnell *et al.* 2001). The major testicular effects observed in the neo-ERKO were caused by the back-pressure of fluid owing to the failure of efferent ductules to reabsorb luminal fluids downstream (Hess *et al.* 1997a, 2002; Zhou *et al.* 2001; Hess 2003). In ArKO males, testicular degeneration with ageing is independent of the efferent ductule abnormalities. It was hypothesized that ER α expression in the male reproductive tract, where its expression is 3.5-fold greater than uterus (Hess *et al.* 1997b; Robertson *et al.* 2002), could be independent of its endogenous ligand and thus would remain in efferent ductule tissues in the ArKO male (Hess & Carnes 2004). Toda *et al.* (2008) tested this hypothesis, demonstrating that ER α and other smaller bands in Western blot analysis were indeed present in the ArKO tissues. Table 4 lists several potential explanations for the differences between ArKO and neo-ERKO male testicular phenotypes, including the possibility that oestrogen function is a combination of activities through its receptors, both ligand dependent and independent, as well as rapid effects through a membrane receptor via growth factor receptors (Levin 2009a,b).

(c) Direct effects of oestrogen on seminiferous epithelium

Several studies have now presented data supporting the hypothesis that oestrogen does have a direct role in the regulation of spermatogenesis. An indirect role through the hypothalamus–pituitary–testicular feedback loop has been well established (O'Donnell *et al.* 2001; Balasiner *et al.* 2006; Ebling *et al.* 2006) and direct effects on Leydig cell function are well known, as ER α is typically expressed in these cells with most fixation methods and antibodies (Hess *et al.* 2002; Lucas *et al.* 2008). The anti-oestrogen ICI 182,780 inhibits T production in WT Leydig cells but not in neo-ERKO cells (Akingbemi *et al.* 2003). In mice expressing human aromatase, Leydig cell hypertrophy was observed without elevated LH (Strauss *et al.* 2009). Others have shown oestrogen-dependent regulation of Leydig cell *Cyp17a1* (Tong *et al.* 2004) and *Star* (Houk *et al.* 2004). Although the immunolocalization of ER α has not been consistent in the seminiferous epithelium, the ArKO-soy data clearly suggested a direct effect of oestrogen on the seminiferous epithelium in the absence of efferent ductule dysfunction (Robertson *et al.* 2002; Toda *et al.* 2008).

ERKO mice lack ER α throughout the development and therefore do not provide an answer to the question of whether the receptor is essential for adult testicular function. Thus, the observed changes in the ERKO male reproductive system could be due to developmental defects and not adult dysfunction. This hypothesis

Table 4. Possible explanations why the ArKO male mouse phenotype does not equal that of the ER α knockout mouse.

1. ER α is expressed in efferent ductule epithelium in the absence (ArKO) of oestrogen (Oliveira *et al.* 2004; Toda *et al.* 2008).
2. Aromatase is normal, and thus oestrogen levels, in the absence of ER α in the male.
3. ER ligands other than oestrogens are active in the tissues (Picciarelli-Lima *et al.* 2006).
4. Because ER α is present in ArKO ductules, ER α is activated in a ligand-independent pathway (Power *et al.* 1991; Salomonsson *et al.* 1994; Weis *et al.* 1996; Wang *et al.* 2001; O'Malley 2005; McDevitt *et al.* 2007; Sinkevicius *et al.* 2008, 2009).
5. Maternal oestrogens during development are sufficient to permit normal development of rete testis and efferent ductules in the ArKO.
6. Seminiferous tubular fluid secretion may be reduced in ArKO males (Robertson *et al.* 2002; Toda *et al.* 2008).
7. Dietary phytoestrogens may be sufficient to activate ER α -mediated pathways in ArKO males (Robertson *et al.* 2002).
8. Dietary phytoestrogens show stimulatory effects on epididymal sperm counts in the absence of ER α , but inhibitory effects on sperm movement (Ruz *et al.* 2006).
9. Oestrogen function in the male reproductive tract is a combination of receptor-mediated transcriptional activity, both ligand dependent and independent, as well as rapid effects through a membrane receptor (Revankar *et al.* 2005; Carreau *et al.* 2006; Ruz *et al.* 2006; Filardo *et al.* 2007; McDevitt *et al.* 2007, 2008; Sinkevicius *et al.* 2008, 2009; Weiss *et al.* 2008; Noel *et al.* 2009).

was tested by treating adult animals with the pure anti-oestrogen ICI 182,780 or Faslodex (AstraZeneca, Macclesfield, Cheshire, UK), which blocks both ER α and ER β and also downregulates the receptor protein (Wakeling & Bowler 1991, 1992; Wakeling 1995; Sun *et al.* 2002; Berry *et al.* 2008). Anti-oestrogen treatment of the adult rodent simulated many of the ER α KO mouse phenotypes (table 5), but there were differences between rats and mice in their testicular responses. In rat, the anti-oestrogen exposure increased testicular weight by 45 days, with dilation of seminiferous tubules, which was due to fluid accumulation in efferent ductules and rete testis (Oliveira *et al.* 2001, 2002). Testicular swelling was followed by total atrophy of the seminiferous tubules, with ageing.

The mouse efferent ductules and rete testis also responded to ICI 182,780, similar to those of the rat and resembled the neo-ERKO model, except the anti-oestrogen that did not induce the transient increase in testicular weight and total atrophy (Lee *et al.* 2000; Cho *et al.* 2003). Although effects on the efferent ductules were noted as early as 4 days after treatment (Cho *et al.* 2003), effects on the testis were delayed, similar to those seen in the ER α KO (Eddy *et al.* 1996; Hess *et al.* 1997a; Weiss *et al.* 2008), but seminiferous tubular atrophy was heterogeneous and focal, as seen in ArKO mice (Fisher *et al.* 1998; Robertson *et al.* 2001, 2002; Toda *et al.* 2008), and not due to fluid back-pressure. Others have also found apparent direct effects of ICI 182,780 on seminiferous epithelium (Gancarczyk *et al.* 2004; Anahara *et al.* 2006), including effects within 6 days of treatment (Anahara *et al.* 2006).

The ERKO and ArKO animal models suggested that direct effects of oestrogen may be occurring in the seminiferous epithelium, but definitive evidence for this hypothesis was not found until new animal and tissue models were designed. Lucas *et al.* (2008) used improved immunohistochemistry and *in vitro* culture of isolated Sertoli cells to show specific staining for ER α in immature and adult rat Sertoli cells, which demonstrated that both ER α and ER β migrate to the cell membrane within 10 min following treatment with 17 β -oestradiol (figure 6). These data are contrary to nearly every report in the literature over

Table 5. Comparisons of ER α knockout mice and ICI 182,780 anti-oestrogen-treated male mice and rats.

characteristic	neo- or floxed-ERKO ^a	ICI-182,780 ^{b,c}
rete testis dilation	yes	yes
seminiferous tubule dilation	yes	no
Sertoli cell ER α migration to membrane	not determined	yes
decrease cortactin in Sertoli cells	not determined	yes
transient increase in testis weight	yes	yes ^d
seminiferous epithelial degeneration	yes	yes, with age
testicular atrophy	yes	no ^e
increased testosterone	yes	yes
decreased Leydig cell function	yes	yes
efferent ductule dilation	yes	yes
abnormal efferent duct epithelium	yes	yes
normal expression of ER β in efferent ductules	yes	yes
decrease in AQP9	yes	yes
decrease in NHE3 (SLC9A3)	yes	yes
decrease in carbonic anhydrase II (CA2)	yes	yes
increase in CFTR ^f	yes	yes
decreased cauda sperm number	yes	yes
decreased sperm motility determined	yes	not
decreased fertility	yes	yes, with age

^aER α knockout (Lubahn *et al.* 1993; Eddy *et al.* 1996; Hess *et al.* 1997a,b, 2000; Dupont *et al.* 2000; Lee *et al.* 2000, 2001; Mueller & Korach 2001; Zhou *et al.* 2001; Akingbemi *et al.* 2003; Ruz *et al.* 2006; Toda *et al.* 2008; Weiss *et al.* 2008; Chen *et al.* 2009).

^bFaslodex formulation (AstraZeneca, Macclesfield, Cheshire, UK); 5 mg per mouse per week for five weeks.

^cAkingbemi *et al.* (2003), Anahara *et al.* (2006), Cho *et al.* (2003), Lee *et al.* (2000, 2001), Lucas *et al.* (2008) and Oliveira *et al.* (2001, 2002, 2003, 2005).

^dLess increase than in neo-ERKO.

^eFocal seminiferous tubular atrophy but not total testicular atrophy.

^fCystic fibrosis transmembrane conductance regulator.

Figure 6. (a) ER α (ESR1) colocalized with a Sertoli cell nuclear marker (green) under basal conditions (control) or (b) after incubation with E2 (0.1 nM) for 10 min. Some of the ER α migrates from the nucleus into the Sertoli cell membrane after oestrogen treatment. Scale bar, 30 μ m. Adapted from fig. 6 in Lucas *et al.* (2008).

the past 10 years and thus require that future studies incorporate improved methods for preserving ER for tissue localization.

Another novel animal model that has changed our understanding of oestrogen action in the testis came from the combination of ERKO mice and mice having mutations either in the DNA-binding domain (Weiss *et al.* 2008) or in the ligand-binding domain of ER α (Sinkevicius *et al.* 2008, 2009). The absence of ligand binding (ENERKI) demonstrated that both oestrogen-independent as well as oestrogen-dependent signalling pathways are working in the male reproductive tract (Sinkevicius *et al.* 2009).

In the ENERKI, ligand-independent ER α is capable of activating the efferent ductules and preventing fluid accumulation, possibly working through the PI3K-AKT pathway (Lee-Kwon *et al.* 2001). However, oestrogen-dependent ER α signalling was shown to be important for the progression of normal germ cell development (Sinkevicius *et al.* 2009), in support of the earlier ArKO findings (Robertson *et al.* 2001, 2002). The absence of DNA binding (NERKI) resulted in a rescue of the neo-ERKO phenotype, producing normal spermatogenesis, but persistent effects on aquaporin-1 in efferent ductules, although without inducing fluid accumulation (Weiss *et al.* 2008). Thus, it now appears that indeed oestrogen and its major receptor, ER α , have important roles in the regulation of spermatogenesis, particularly with ageing and that this activity occurs through both rapid non-classical membrane-associated/growth factor receptors (Levin 2009a; Santen *et al.* 2009), as well as classical transcriptionally mediated pathways. Future studies are required to better understand the separation of these pathways and their potential interactions with other steroid receptors that coexisting in the same cell types.

(d) Rapid effects of oestrogen and the role of GPR30

A large body of evidence has demonstrated that oestrogens can function not only through the classical genomic effect but also can trigger rapid responses

that involve transduction pathways different from those activated by ERs (Levin 2005, 2009a). Recently, several studies showed that the GPR30 is able to mediate oestrogen action in both rodents and humans target tissues (Filardo *et al.* 2002; Maggiolini *et al.* 2004; Revankar *et al.* 2005; Thomas *et al.* 2005; Levin 2009b).

The recent demonstration that GPR30 is expressed in a spermatogonia mouse cell line highlights a role for this receptor after activation with oestradiol in the control of mouse spermatogonia cell proliferation (Sirianni *et al.* 2008). Our most recent study (Chimento *et al.* in press) demonstrated that the rat pachytene spermatocytes express ER α , ER β and GPR30. For the first time, we showed that oestrogens, through both GPR30 and ER α , are able to activate the rapid EGFR/ERK/c-jun signalling cascade, which in turn triggers an apoptotic mitochondrial pathway involving an increase in Bax expression correlated to a reduction of cyclin A1 and B1 levels and thus, induces spermatocyte apoptosis. It is well known that pachytene cells before further differentiation can proliferate or die through apoptosis (Perrard & Durand 2009). Moreover, taking into account the presence of a biologically active aromatase in spermatocytes (Carreau *et al.* 2010), it will be very interesting to study the molecular mechanisms under oestradiol control that are involved in this important stage of spermatogenesis.

In mice, it has been claimed that GPR30 is not involved in oestrogenic responses of the reproductive organs (Otto *et al.* 2009). Indeed, these authors generated *Gpr30*-deficient mice and showed that mutant male and female are fertile. However, it is noteworthy that data are missing about the spermatogenetic process and a careful examination of oestrogenic response was carried out only in uterus and mammary gland. Although GPR30 is not an ER and considerable controversy remains regarding its role in oestrogen-mediated activity, others have suggested that it may collaborate with the membrane ER α to mediate signalling through the growth factor receptor pathway (Levin 2009b).

5. TESTICULAR TUMOURS AND OESTROGENS

The overexpression of aromatase, which leads to an oestrogen-androgen imbalance, leads to Leydig cell hyperplasia, dysmorphic seminiferous tubules and disrupted spermatogenesis (Li *et al.* 2001). It is clear that according to the mouse model, age is important since if the overexpression of aromatase is developed during gestation, 100 per cent of the males are infertile but conversely if the promoter is activated at puberty only 50 per cent of the males are fertile.

In human seminomas, excess oestrogens induce an impairment of spermatogenesis (Nakazumi *et al.* 1996), as also observed in mice overexpressing aromatase (Fowler *et al.* 2000; Li *et al.* 2001). In addition, we have reported (Roger *et al.* 2005) the expression of a functional aromatase complex in seminoma cells and confirmed that the JKT-1 cell (a pure human testicular seminoma cell line), similar to tumour seminoma cells and human normal testicular basal germ cells, expresses ER β , including ER β 1 and ER β 2, but not

ER α . Seminoma cells are able to respond to oestrogens through a possible autocrine or paracrine loop and thus, these female hormones contribute to human testicular germ cell cancer proliferation by rapid activation of ERK1/2 and PKA through a membrane ER, as demonstrated recently (Bouskine *et al.* 2008). This non-genomic effect confirms a new basis for understanding the oestrogenic control of spermatogenesis and evaluating the role of exposure to endocrine disruptors (xenoestrogens) during malignant transformation of testicular germ stem cells. In addition, a very recent work (Rago *et al.* 2009) has identified ER β 1 and ER β 2 in seminomas and embryonal carcinoma. Together with the greater presence of ER α in Leydig tumours cells (Carpino *et al.* 2007), and the existence of membrane ER, it is obvious that oestrogens are functioning in testicular cancers but further studies are necessary to elucidate the mechanisms of action involved.

6. CONCLUSION

It is clear from this review that the testis is equipped with the ability to produce significant amounts of oestrogenic hormones, via aromatase, and expresses ER α and ER β in Leydig cells as well as the seminiferous epithelium. Oestrogenic activity appears to involve not only the classical genomic pathway, but also the newly discovered rapid membrane receptor pathway and possibly non-classical nuclear ER-tethering pathways (Sun *et al.* 2002; Harrington *et al.* 2003; Rodriguez *et al.* 2004). This activity of oestrogen on spermatogenesis appears to involve germ cell proliferation, differentiation and the final maturation of spermatids, as well as germ cell survival and apoptosis. The final steps of spermatid maturation, spermiogenesis, appear to be particularly sensitive and dependent upon oestrogen, especially after several waves of spermatogenesis have occurred. Based upon these new findings, it will be important to focus new studies on oestrogen-regulated genes associated with spermatogenesis and germ cell differentiation. There still remains a lack of knowledge concerning nuclear receptor cofactors in the testis and their combined activities when present together in cells containing both androgen and ERs. Such studies will help us to better understand the consequences of exposure to environmental endocrine disruptors, as well as provide a potential target for the development of a non-androgen male contraceptive.

Together with the existence of a functional aromatase, the intracrine role of oestrogens should be therefore considered in the immature germ cells as well as in the final steps of spermatozoa maturation (Carreau *et al.* 2010). Even if numerous oestrogen-targeted genes are still missing, there are now evidences in favour of a positive role of oestrogens in the male reproduction via genomic and rapid membrane effects. Age-related studies are also necessary in order to fully understand the control of the aromatase expression and the role of oestrogens in male reproduction, as aromatase and oestrogens are probably involved in promoting sperm development and acquisition of fertilizing capacity.

Finally, new studies are suggesting that testicular cancers in men may have an oestrogen component that will need to be addressed from both an aromatase perspective, as well as receptor expression and activity or the ultimate balance between androgens and oestrogens in the male.

We are greatly indebted to our collaborators Drs Silandre and Delalande (Carreau). All this work has been supported by funding from the French Ministry of Education and Research, a Polonium grant and the Région Basse-Normandie (Carreau); CICCR, a programme of CONRAD, Eastern Virginia Medical School and AstraZeneca (Hess).

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