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Estrogens and male reproduction: a new concept

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Abstract

The mammalian testis serves two main functions: production of spermatozoa and synthesis of steroids; among them estrogens are the end products obtained from the irreversible transformation of androgens by a microsomal enzymatic complex named aromatase. The aromatase is encoded by a single gene (*cyp19*) in humans which contains 18 exons, 9 of them being translated. In rats, the aromatase activity is mainly located in Sertoli cells of immature rats and then in Leydig cells of adult rats. We have demonstrated that germ cells represent an important source of estrogens: the amount of P450arom transcript is 3-fold higher in pachytene spermatocytes compared to gonocytes or round spermatids; conversely, aromatase activity is more intense in haploid cells. Male germ cells of mice, bank voles, bears, and monkeys express aromatase. In humans, we have shown the presence of a biologically active aromatase and of estrogen receptors (α and ß) in ejaculated spermatozoa and in immature germ cells in addition to Leydig cells. Moreover, we have demonstrated that the amount of P450arom transcripts is 30% lower in immotile than in motile spermatozoa. Alterations of spermatogenesis in terms of number and motility of spermatozoa have been described in men genetically deficient in aromatase. These last observations, together with our data showing a significant decrease of aromatase in immotile spermatozoa, suggest that aromatase could be involved in the acquisition of sperm motility. Thus, taking into account the widespread localization of aromatase and estrogen receptors in testicular cells, it is obvious that, besides gonadotrophins and androgens, estrogens produced locally should be considered to be physiologically relevant hormones involved in the regulation of spermatogenesis and spermiogenesis.

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Introduction

It is well known that normal testicular development and the maintenance of spermatogenesis are controlled by gonadotrophins and testosterone whose effects are modulated by a complex network of locally produced factors, with estrogens being obviously involved. Estrogens have long been considered to be specific female hormones; however, the presence of estrogens in the male gonad has been well documented since the publication of Zondek (1) more than 70 years ago showing the presence of estrogen

in stallion urine (for a review, see Ref. 2). Indeed the androgen/estrogen balance is essential for normal sexual development and reproduction in mammals. In the mammalian testis, the maintenance of this balance is under a fine tuning via endocrine and paracrine factors, but is also related to aromatase activity. Aromatase, an enzymatic complex, ensures androgen conversion to estrogens and is localized in the endoplasmic reticulum of various tissues including placenta, gonads, brain, bones, and adipose tissue.

Besides the well-known negative effect exerted by these female hormones on the secretion of gonadotrophins, estrogens play a major role *in situ* and appear to be important not only in women but also in men, especially taking into account the data obtained from men genetically deficient in aromatase (for a review, see Ref. 3). Moreover, in several epidemiological studies decreased sperm counts and increased male reproductive tract disorders (cryptorchidism, hypospadia, testicular cancer) have been attributed to a deleterious effect of endocrine disruptors with either estrogenic or antiandrogenic actions (4,5). Therefore, the capacity of the testis to synthesize estrogens has been extensively studied. Over the last two decades the role of estrogens in male reproduction has been seriously revisited especially in primates taking into account the existence of specific estrogen receptors (ERα and ERß) which are distributed all along the genital tract (for reviews, see Refs. 2,6-9).

Presence of aromatase in testicular cells of mammals and humans

The aromatase gene

Aromatase is composed of two proteins: a ubiquitous NADPH-cytochrome P450 reductase and a cytochrome P450 aromatase (P450arom), which contains the heme and the steroid-binding pocket. In humans, P450arom is the product of a single gene located in region q21.1 of chromosome 15 and called *cyp19*, which belongs to the cytochrome P450 gene family. The *cyp19* gene is more than 123 kb in length with a coding region of 9 exons (II-X) and 9 nontranslated exons I (Figure 1). Expression of the *cyp19* gene is regulated by tissue-specific promot-

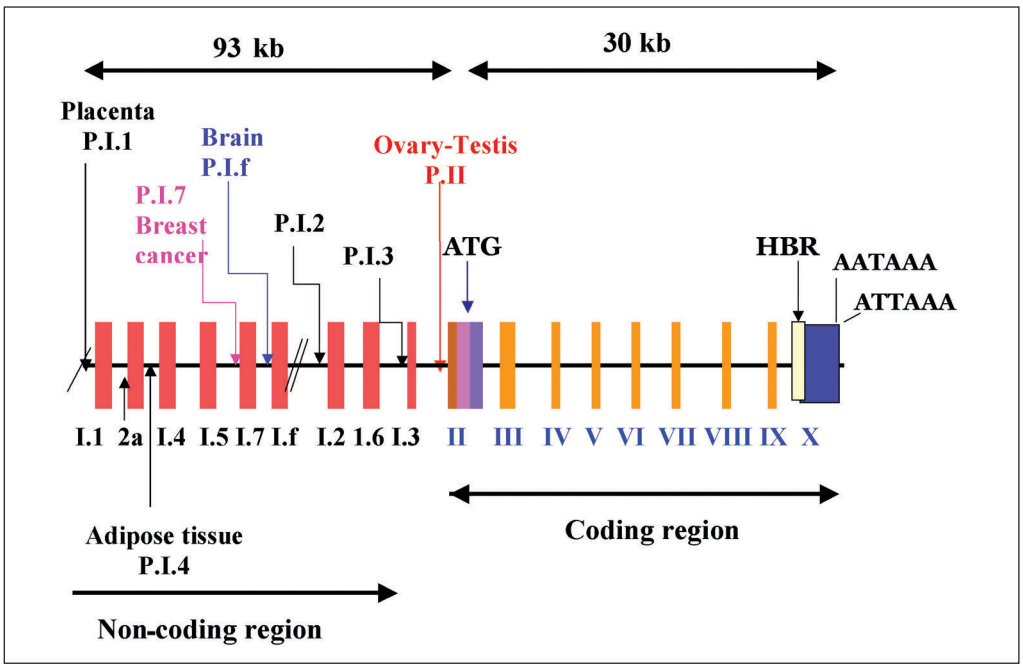

Figure 1. Schematic presentation of the human aromatase gene. P = promoter.

ers producing alternate 5'-untranslated exons I that are then spliced onto a common 3'-splice acceptor site in exon II, upstream of the translation starting site (for reviews, see Refs. 10-12). Therefore, there is generation of *cyp19* variants with different 5'untranslated regions giving rise to different mRNAs; however, the coding sequences are identical and give rise in humans to a single protein composed of 503 amino acids with a molecular mass of 55 kDa. It is of note that P450arom is encoded by a single *cyp19* gene in most species except for pigs in which three distinct genes encode three aromatase isoenzymes (13) and for fish in which two *cyp19* genes (specifically expressed in the brain and gonads) have been identified (14).

Presence of a biologically active aromatase in male germ cells

As abundantly documented in the literature, it is difficult to find a tissue completely devoid of aromatase gene expression (10). Despite the large number of experiments carried out both *in vitro* and *in vivo* in different strains of various species, an agreement on the precise localization of aromatase activity at different ages has not always been evident. Indeed, studies investigating the testicular site of androgen aromatization in rat testicular tissues and/or in isolated cells have shown that Leydig cells express aromatase. In the rat there is an age-related change in the cellular distribution of aromatase activity, which is mainly found in Sertoli cells in immature animals and in Leydig cells in adults (7,9). Nevertheless, for the first time in 1993, Nitta et al. (15) reported the presence of aromatase in male germ cells of the mouse. During the same period, with the tools to check the expression of the aromatase gene being available in our laboratory, we decided to carefully re-examine the source of estrogens in adult rat testicular cells. As a matter of fact, we demonstrated an additional source of estrogens in purified pachytene spermatocytes (PS), round spermatids (RS) and spermatozoa of adult rats (16,17), in agreement with the data of Janulis et al. (18) showing a stronger immunostaining for aromatase in elongated spermatids. The amount of P450arom mRNA decreases according to the stage of germ cell maturation, being higher in younger than in mature rat germ cells. Conversely, aromatase activity is higher in spermatozoa than in PS or RS. Even if adult Sertoli cells may express aromatase (16), it is well known that germ cells exert a negative control on P450arom gene expression (19), leading to the conclusion that roughly half the testicular aromatase activity is located in germ cells (20,21). We have recently extended our studies, showing that preleptotene spermatocytes and spermatogonia contain aromatase, whereas peritubular cells are devoid of P450arom (Figure 2). Thus, we demonstrated that aromatase is constitutively expressed in all testicular cells except myoid cells in the rat (9; Silandre D, Delalande C, Carreau S, unpublished results). It is noteworthy that the epididymal cells of the rat also contain aromatase (22). Similarly, aromatase has been demonstrated in the germ cells of the bank vole, and its expression is photoperiod-dependent, i.e.,

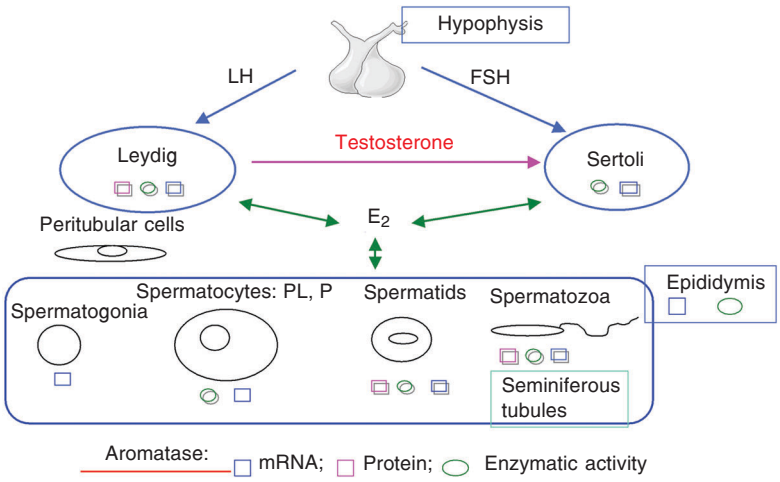

Figure 2. Localization of aromatase in adult rat testicular cells. PL = preleptotene; P = pachytene; E_2 = estradiol.

much more intense under long-light cycle conditions which coincide with the breeding season (23). Identical data have been reported for the bear (24).

In the stallion, many estrogens are synthesized by Leydig cells (for a review, see Ref. 25). In contrast to the data of Hess and Roser (26), Hejmej et al. (27) have recently immunohistolocalized aromatase not only in Leydig cells, but also to epididymal cells and to the germ cells present in the lumen of the epididymis. In the boar testis the main source of estrogens is in the Leydig cells (28).

Aromatase in primate testes

In humans, Leydig cells have long been considered to be the only source of estrogens (29); however, *in vitro* both Leydig cells and Sertoli cells produce estrogens (for a review, see Ref. 30). It has also been claimed that spermatozoa can convert pregnenolone into estrogens (31). After obtaining data on the expression of aromatase in male germ cells of the rat, we decided to check for the presence of aromatase in ejaculated spermatozoa from healthy donors. In a second approach, we hypothesized that aromatase could be used as a marker of sperm quality (especially motility) and thus we measured the amount of P450arom transcripts in motile and immotile spermatozoa. In addition, since no information was available on the source of estrogens in testicular germ cells, we examined by RT-PCR the putative expression of *cyp19* in immature germ cells (spermatocytes and spermatids) obtained from semen samples with large number of round cells (32). Briefly, the quality of the germ cell preparations was carefully analyzed in order to eliminate any sample contamination with leukocytes via the expression of CD45 transcript and with Sertoli cells via the presence of the Sertoli cell factor mRNA. All samples containing detectable levels of these transcripts were eliminated; conversely the presence of *c-kit* mRNA in round cells was used as a positive control for the presence of germ cells. We detected the presence of P450arom transcripts in both immature germ cells and ejaculated spermatozoa. The sequence analysis of the PCR products obtained was identical and showed more than 98% identity to the published sequence of human P450arom (33). Using Western blots and a specific monoclonal antibody against a highly conserved region of aromatase we demonstrated the presence of aromatase in both immature germ cells and ejaculated sperm cells, with the staining intensity being more abundant in spermatozoa containing cytoplasmic droplets.

Moreover, we demonstrated that the amount of P450arom transcripts was 30% lower in immotile than in motile spermatozoa from several samples individually prepared by density gradient purification; in addition, the aromatase activity was 50% greater in the motile fraction compared to immotile spermatozoa. Our observations showing that aromatase was expressed both as a transcript and as a biologically active protein in spermatozoa from normal donors agree with other data (34). These latter observations are correlated with the immunolocalization of aromatase in cytoplasm surrounding elongated spermatids in man (35) and aromatase has also been revealed in cytoplasmic droplets of ejaculated human spermatozoa (36). Recently, Carpino et al. (37) have immunolocalized aromatase in the epithelial cells of human efferent ducts and to the proximal caput epididymis, suggesting an additional source of estrogens in the male genital tract. Similar observations have been made on rhesus monkeys, whose testis and, to a lesser extent, epididymis contained two P450arom transcripts, one of them being truncated (38).

Regulation of aromatase gene expression

In order to obtain insights about the role of estrogens in male reproduction and espe-

cially within seminiferous tubules, it is necessary to study the regulation of the *cyp19* gene (for a review, see Ref. 39). Thus, using RACE-PCR we demonstrated that promoter II directs the expression of the aromatase gene whatever the testicular cell type studied in the rat (40). In the testis, follicle-stimulating hormone and luteinizing hormone both act to increase the concentrations of intracellular cyclic AMP (cAMP), thereby inducing expression of P450arom which in turn requires the transcription factors cAMP response element-binding protein, cAMP response element modulator and steroidogenic factor-1 (SF-1). SF-1 belongs to the nuclear orphan receptor superfamily and regulates the transcription of steroidogenic genes, among them P450arom via its interaction with numerous co-activators such as cAMP response element-binding protein, DAX-1, SOX-9, and WT1. In addition, we have shown that liver receptor homologue-1, an SF-1 homologue, which is present both in Leydig cells and germ cells, but not in Sertoli cells, increases the P450arom gene expression in a mouse Leydig cell line (41). Numerous functional motifs have been identified in P.II (11); in this context it is worth noting that truncated transcripts of the P450arom gene giving rise to putative inactive proteins have been described in PS and RS of adult rats (42). Moreover, it is now clear that not only P.II drives the aromatase gene in rat testis but two additional promoters, P.I.f (brain promoter) and a new one that we called P.I.Tr (testis rat; Silandre D, Delalande C, Carreau S, unpublished results), are involved. We have also demonstrated that the nutritional status of fetuses (43) and aging (Hamden K, Silandre D, Delalande C, El Feki A, Carreau S, unpublished results) can modulate aromatase gene expression in male rats. In mice it has been shown that aromatase expression is controlled by three different promoters, among them a testis-specific promoter (44). In human gonads, only the P.II promoter has been reported to control aromatase gene expression thus far (45).

Estrogen receptors in the male genital tract

In order to exert a biological role, testicular estrogens should interact with ERs, which in turn modulate the transcription of specific genes. Until 1996, the only data on ERs concerned ERα; however, with the discovery of a novel ER denoted ERß, the localization of ERs has been reexamined, and it has been shown that the α and ß forms are not always present in the same cells (or are present in different amounts) within the male genital tract. The distribution of the mRNAs coding for the two types of ERs in the male rat gonad has been studied (46) and Saunders et al. (47) have shown the presence of ERß in pachytene spermatocytes and spermatids. ERß has also been immunolocalized in Leydig cells and in the seminiferous tubules of the bank vole (23).

Mutembei et al. (48) have shown that ERß is ubiquitously distributed in the testicular cells of the boar including Leydig cells and is absent only in elongated spermatids. Conversely ERα is very weakly expressed in Leydig cells and is much more abundant in young germ cells (spermatogonia and PS). Therefore, species differences are obvious and the pig seems to have a unique distribution of ERs consitent with the large amounts of estrogen produced in boar testes (for a review, see Ref. 25).

In immature germ cells of men we have identified both in terms of transcripts and proteins the two main isoforms of ERs, not only the full-length one but also some variants (32). Besides the wild type of ERα, an additional transcript related to the exon1-deleted variant of ERα with a 46-kDa molecular mass has been reported in ejaculated sperm. Concerning ERß, two isoforms (in terms of transcript and protein) corresponding to the expected sizes (full-length and

shorter one) were detected in germ cells, whereas in spermatozoa only the PCR product was found (49). Aquila et al. (50) have described the presence of ERα and ERß in human ejaculated spermatozoa both as transcripts and as proteins corresponding to the well-characterized ER forms. These discrepancies with our studies could be due to the different methodologies used. Recently, Aschim et al. (51) reported the presence of several splice variants of ERß in human testicular cells but the proteins have not been yet identified and thus their specific functions remain to be elucidated. Solakidi et al. (52) have also demonstrated by confocal analysis the presence of the two main ERs in ejaculated sperm. In primates, both ERs have been demonstrated but it is clear that Leydig cells mainly express ERα whereas in seminiferous tubules ERß and ERα are present especially in PS and RS (for a review, see Ref. 53).

Thus, a general statement can be made: ERα is mainly localized in Leydig cells whereas ERß is found in the seminiferous tubules and mainly in germ cells.

Conclusions: estrogens and male fertility

In conclusion, the net biological effect of estrogen in the testis was long thought to be mainly negative. In view of the widespread distribution of ERs in male gonads and an additional expression of aromatase in germ cells, the role of estrogens in male reproduction is more complex than previously realized. Regarding the role of estrogens in male gamete maturation, we have shown not only the existence of estrogen sources in various germ cells but also the presence of ERα and ERß and, in addition, the presence of a truncated form of ERα (probably localized on the membrane) in ejaculated human sperm. Indeed, the motility and number of spermatozoa are reduced in diminished aromatase-deficient men (for a review, see Ref. 3). In general, an impairment of fertility is observed in men with a defect of the aromatase gene. These observations of decreased sperm motility in men with aromatase deficiency, together with our data showing a significant decrease of aromatase in immotile human spermatozoa, could suggest that aromatase is involved in the acquisition of sperm motility. In mouse as well as in man it has been shown that estrogens are positively involved in sperm capacitation and acrosome reaction (54). The existence of ERs on the sperm membrane (55) and in the mitochondria (52) is likely to be relevant for a role of estrogens in male gamete motility.

Consequently, the production of estrogens by germ cells and the existence of ERs represent good markers which should help to understand the physiological role of estrogens in human spermatogenesis. As also suggested by Ostermeier et al. (56), the genetic fingerprint of fertile men could include the analysis of mRNA profiles and thus aromatase and ERs may be helpful. Nevertheless, it is clear that further studies are necessary to elucidate the real impact of estrogen on human male reproduction, although it is obvious that several steps are involved (54). Even if numerous estrogen-targeted genes remain to be defined, there is now a body of evidence in favor of a positive role for estrogens in male reproduction via genomic and rapid membrane effects (for a review, see Ref. 57).

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