Teasing out the role of aromatase in the healthy and diseased testis

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Scientific discoveries over the past decade have shifted the stereotypical view of androgens as male hormones and estrogens as female hormones. It is now recognized that a delicate balance of both androgens and estrogens, a process controlled by aromatase, is fundamental for normal testicular development and fertility. While the site-specific actions of these two classes of steroids within the testis are becoming better documented, the role and regulation of estrogen biosynthesis by aromatase within the testis remains unclear. The majority of data comes from a wide range of animal species, particularly genetically modified mouse models; aromatase knockout (ArKO) and overexpressing (AROM⁺), with limited information on humans, however the existence of congenital aromatase mutations has provided some insight into its effects on individual parameters of the testis. This review dissects out the localization and activity of aromatase in the healthy and diseased testis, addresses the cellular insult to the testis that occurs in its absence and over abundance and proposes potential molecular mechanisms of aromatase regulation in the testis.

Introduction

It has become increasingly apparent that a precise balance of both androgens and estrogens is fundamental for normal sexual development and function in animals and humans (reviewed in ref. 1).² This balance is governed by cytochrome P450 aromatase, which when complexed with NADPH-cytochrome P450 reductase, catalyzes the irreversible conversion of C₁₉ androgenic steroid substrates, testosterone and androstenedione, into the C₁₈ estrogens, estradiol and estrone, within the endoplasmic reticulum (reviewed in ref. 3). Human aromatase is encoded by the 123 kb *CYP19A1* (cytochrome P450, family 19, subfamily A, polypeptide 1) gene located on the long arm of chromosome 15 (15q21.1).⁴ This gene contains a coding region across 9 exons, starting with exon II, and is expressed across a variety of tissue types within the human body, including the testis (reviewed in ref. 3). The expression of aromatase in different locations within

the body is under the control of at least 8 different tissue-specific promoters, which are situated upstream of a series of 11 alternative 5' untranslated first exon Is (reviewed in ref. 3). However, regardless of the site of tissue expression, each transcript generates a highly conserved 55 kDa aromatase protein as the alternative 5' untranslated exon Is are differentially spliced into a common 3' splice acceptor site located 39 bp upstream of the translational start site (reviewed in ref. 5).

There is no question that steroidogenesis plays a fundamental role in the regulation of male fertility. The involvement of testosterone in male fertility has long been established however the importance of estrogens has only come to light over the last decade (reviewed in ref. 6). More recently, exposure to environmental estrogens has been interlinked to poor semen quality and many male reproductive tract disorders including cryptorchidism, hypospadias and testicular cancer.⁷⁻⁹ From this, many questions have arisen regarding the specific contribution of aromatase to testicular development and disease. Aromatase mRNA has been identified in all somatic cells, germ cells and spermatids within the testis and moreover spermatozoa in the epididymis across species (reviewed in ref. 10), however we know from earlier work that transcript levels are not a direct indicator of enzymatic activity.¹¹ To understand the contribution of aromatase to testicular development and disease, we need to pinpoint its cellular localization within the testis, its expression at these sites, the cellular effects that occur in its absence and over abundance and elucidate the molecular mechanisms that underpin each of these events. This review synthesizes the data available, highlights important gaps in our understanding and attempts to develop new concepts for the role of aromatase in testicular function.

Intratesticular Sites of Aromatization in the Healthy Testis

Animals. Data from animal models has provided the basis of our understanding on the site(s) of aromatization within the testis, although quality data are now emerging for the human. It is generally accepted that Leydig cells are the main site of aromatization in sexually mature animals, however it is becoming apparent that other testicular cell types may be alternative sites of estrogen biosynthesis. Aromatase has been immunolocalized to, and found to be enzymatically active in, pachytene spermatocytes and round, elongating and elongated spermatids within the seminiferous epithelium as well as spermatozoa within the lumen of the rat,^{11,12}

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mouse¹³ and rooster.¹⁴ The importance of aromatase and/or estrogen action in this location for successful spermatogenesis has been demonstrated in the rat and monkey where administration of an aromatase inhibitor resulted in significant decreases in the round and elongated spermatid numbers.^{15,16} Furthermore, active aromatase has been immunolocalized in spermatozoa within the epididymis of mice¹⁷ and roosters¹⁴ and the ejaculate of pigs.¹⁸ In the rat, it has been demonstrated that there is a developmental-related switch in the cellular location of the main aromatization site, switching from Sertoli cells in sexually immature rats to Leydig cells in mature rats.¹¹ In contrast, other studies have claimed there is no switch in species such as the stallion,¹⁹ bison,²⁰ Shiba goat²¹ and pig.²² Interestingly, many seasonal breeding species do exhibit a switch in aromatase localization, however this switch occurs between their breeding and non-breeding seasons including the black bear,²³ sika deer,²⁴ ground squirrel,²⁵ bank vole^{26,27} and raccoon dog.²⁸ However, there is still variability in the testicular cell types that express aromatase between these species. For example, during breeding periods in the ground squirrel, intense aromatase immunodetection is evident in all testicular cell types but in the sika deer aromatase is only evident in Leydig cells. Nonetheless, during the non-breeding period in both species, no aromatase immunodetection is evident in any testicular cell type.

These species differences could be explained by the variation in environmental cues that induce reproductive cyclicity; for example, photoperiod, temperature, weather, nutrition and/or age. The extent of testicular fluctuations between breeding seasons affects testicular size, seminiferous tubule diameter, sperm progression and somatic cell differentiation and function, however whether these cyclic changes are orchestrated by changes in steroidogenesis are yet to be elucidated. In addition to developmental and seasonal related changes in cell specific aromatase localization, new data in rats demonstrates that there are stage related changes in aromatase gene expression, with maximum expression reported between stages XIII and III,²⁹ however currently there is no quantitative evidence for stage related changes in aromatase protein expression, though qualitative observations in the stallion have provided promise that a link may exist.¹⁹ Although an understanding of the developmental stage and cell specificity of the enzyme would be telling, there is a degree of uncertainty as to the exact cellular source of aromatase within the testis across animal species, which is probably attributed to differences in antibody specificity used across these studies. Employing a validated, highly specific, monoclonal antibody generated against a peptide within aromatase that is conserved across species such as the one generated by Turner et al. in combination with stringent isotype controls, would help eliminate incongruities in future studies and provide confidence in understanding the exact site of aromatization in the testis across species throughout developmental time-periods, different seasons and spermatogenic stages.

Humans. Data on aromatase localization during certain developmental periods is restricted due to the lack of accessibility of clinical samples. Nonetheless valuable information can be

gleaned from case studies and it appears that aromatase is differentially expressed in a developmental manner in males.

Fetal. Aromatase has been immunolocalized to Leydig, Sertoli and germ cells between 13 and 22 weeks of gestation and is depleted by 35 weeks.³¹ The increase in aromatase immunoreactivity during this time coincides with the period of interstitial cell (Leydig cell precursors) hyperplasia, a normal part of testis development essential to meet the steroidogenic needs of the human fetal testis once they have ceased proliferation.³²

Infant. During the neonatal period in testicular specimens obtained from boys aged between 1 and 21 d, aromatase has been immunolocalized in both Leydig and germ cells with inconsistent staining in Sertoli cells across patient samples.³³ The same study also showed that aromatase immunolocalization between 1 mo and 5 y of age in germ and Sertoli cells is consistent with the neonatal results, however between 1 and 7 mo of age, the intensity of Leydig cell immunoreactivity decreases and between 1 and 5 y the Leydig cell aromatase immunoreactivity is absent.³³ In contrast, another study has claimed that at 3 and 8 mo of age no aromatase immunoreactivity is present in any testicular cell type.³⁴

Pubertal. In the testes of boys at 15 years of age, studies show that aromatase is biologically active however curiously in the same samples no aromatase immunoreactivity could be detected,³⁴⁻³⁶ making it impossible to pinpoint the exact site of aromatase activity at this time. Reasons why the biochemical results may not concur with the immunohistochemical results may be that the enzyme protein level is very low and thus undetectable by the antibody, the immune probe used is of poor specificity or it is plausible that the aromatase enzyme may not be expressed before puberty.³⁴ Understanding the role of aromatase during normal pre-pubertal testicular development is fundamental in elucidating what is occurring in pre-pubertal boys presenting dysfunctional aromatase activity, as seen in Peutz-Jegher's syndrome.³⁷

Adult. It is thought that the main site of aromatization and primary source of estrogen biosynthesis in the adult testis is the Leydig cells,³⁸ and not in Sertoli or germ cells.^{34,36,39-41} In accord, this correlates with data on aromatase activity in Leydig cells and the absence of activity in Sertoli and germ cells.³⁴ While conflicting data does exist regarding the presence of aromatase in germ cells of the human testis (reviewed in ref. 42),^{30,36,39,43} it is clear that active aromatase is present in ejaculated spermatozoa.^{43,44} However, there is a degree of uncertainty as to the precise location of aromatase in spermatozoa, with studies claiming it is solely restricted to the flagellum,⁴⁴ that it's present in both the flagellum and head⁴⁵ and that it occupies numerous sites stretching the entire length of the spermatozoon (reviewed in ref. 1). The role of aromatase in spermatozoa is thought to be in regulating sperm motility, as it has been noted that immobile human spermatozoa contain markedly reduced levels of aromatase activity compared with their mobile counterparts,⁴³ whether this can be attributed to changes in mitochondrial function is worth consideration. This concurs with findings from the aromatase deficient mouse (ArKO) (reviewed in ref. 6), and human,^{46,47} highlighting a potential link between local estrogen production and sperm motility. New data report an association between a

CYP19A1 gene polymorphism, which impairs aromatase activity, and reduced sperm motility.⁴⁸ It is well recognized that estrogens are essential for luminal fluid secretion and re-absorption to generate an optimum environment for spermiation and sperm motility acquisition.⁴⁹ The fact that aromatase has been immunolocalized to human efferent ductules, the site of luminal fluid re-absorption, and the proximal ductus epididymis, which aids in sperm motility acquisition, further supports this established link and suggests that spermatozoa themselves may be capable of regulating local estrogen concentrations via aromatase.⁵⁰ Moreover, clinical studies suggest that excess estrogens can also have detrimental effects on sperm quality parameters, including sperm concentration, motility and morphology, as evidenced by the exposure to bisphenol A (BPA), an estrogen-like chemical found in plastics⁷ and other endocrine disruptors (reviewed in ref. 51). It is important to note, endocrine disruptors may impact sperm quality via non-estrogenic actions.

Aged. It is well known that variations in the balance of sex steroids are a typical feature of aging (reviewed in ref. 52). In healthy men, there is a progressive decline in free testosterone levels^{52,53} and an increase in estradiol levels with age.⁵⁴ This results in a testosterone/estradiol imbalance, which has been linked to an increase in aromatase immunoreactivity.² For that reason it has been postulated that there may be age-related changes in the cellular distribution and activity of testicular aromatase. While this has been evidenced in the rat,^{11,29} no studies have yet demonstrated this in men.^{36,39} Curiously, the opposite has been found, where the highest levels of testicular aromatase activity³⁶ and immunointensity³⁴ have been reported in young men. In addition, the role aromatase plays in longevity has been investigated, and it has been found that the *CYP19* gene in conjunction with the *ESR1* gene is involved in life-span determination as genetic polymorphisms involving the *CYP19**T allele favor a longer than normal life expectancy, inferring that aromatase may play a role in the longevity-fertility trade-off mechanism.⁵⁵

Intratesticular Site of Aromatization in the Diseased Testis

It is too early to speculate whether changes in aromatase expression and activity are a cause or effect of infertility or a mere consequence of hormone and gene perturbations. Although data are limited, here we attempt to afford an appreciation of aromatization in the subfertile and infertile testis using case reports implicating aromatase in various testicular pathologies. In comparison to healthy settings, the site of aromatization in testicular pathologies appears to be shared between different testicular cell types. For example, in an adult patient with Klinefelter's syndrome (KS), aromatase immunostaining is evident in Leydig cells and Sertoli cells.⁵⁶ The increase in the number of aromatization sites in this patient suggests a higher increase in the androgen to estrogen conversion, as supported by the fact that this patient presents low bioavailable testosterone and relatively high serum estradiol levels. While no data on biological activity was available from this study, others have reported that aromatase is four times higher in testes of men with KS resulting in similar intertesticular steroid

patterns.⁵⁷ In other diseased settings, the site of aromatization is restricted to and increased in the cell type(s) that are compromised in the disease, as evidenced in Sertoli³⁶ and Leydig⁵⁸ cell tumors, and intratubular germ cell neoplasia.⁴¹ The localization of aromatase in these settings suggests there are additional sources of estrogen production in the testis, which may function to feed tumor growth. Indeed, the role of estrogen as a potent proliferative agent has been demonstrated in tumors of the breast⁵⁹ and testis.⁶⁰ Although there is a marked increase in aromatase activity in these two tumor types resulting in the increased production of estrogens, upon closer inspection it has been elucidated that in breast tumorigenesis there is a switch in aromatase promoters, from promoter I.4 to promoters I.3 and II,³ whereas in the testis the amount of aromatase expression is not a result of using different tissue-specific promoters, but rather from activation of (or failure to inhibit) upstream regulatory elements of the same promoter (II).³⁵

Testicular Function in Models of Aromatase Deficiency and Excess

The generation of the aromatase knockout (ArKO) and aromatase overexpressing (AROM⁺) mouse models, as well as the discovery of naturally occurring congenital mutations in humans that result in a global deficiency or excess of aromatase, has provided researchers with powerful opportunities to study the role of aromatase on the morphological development of the testis and its consequent reproductive capacity. Dissecting out the testicular cell specific role of aromatase will only be possible with the availability of conditional mouse models.

Mouse models. There are a number of mouse models of disrupted estrogen action that have been comprehensively discussed (reviewed in ref. 61). Most notably, the differences between the ArKO and estrogen receptor α knockout (ER α KO) testicular phenotypes and the mechanisms underpinning them have been reviewed excellently in reference 6 and 61, and thus will not be described in detail here. However, in brief, the ArKO model arguably shows the first line of evidence that disruption of estrogen action directly impairs the spermatogenic process, while the ER α KO shows spermatogenetic disruption is due to the indirect consequence of back pressure of luminal fluids, as estrogen regulates fluid reabsorption in the head of the epididymis.

ArKO. The ArKO mouse was generated via collaboration with our laboratory through targeted disruption of the *Cyp19a1* gene.⁶² As a result, these mice have undetectable estrogen levels and elevated testosterone levels.⁶² Up until around 4.5 mo of age, the histological architecture of male ArKO mice is generally indistinguishable from their wild type littermates⁶² and thus fertility appears unaffected,⁶ however it has been documented that their sexual behavior is impaired as evidenced by reduced time to first mount and frequency of mounting.^{6,63} From this point, their fertility progressively deteriorates with age, with their ability to reproduce being severely compromised at 1 y of age.⁶⁴ As no change in Sertoli cell number has been reported^{6,64} (although Sertoli cell function has yet to be tested), it appears this is a result of significant reductions in round and elongated spermatid

numbers and the fact that spermatozoa in these mice display decreased motility, as evidenced by the proven inability to fertilize oocytes in vitro.⁶⁵

Despite this, seminiferous heterogeneity still exists across different ArKO mice at the 1 y time point, as mice with qualitatively normal spermatogenesis have also been observed, however the interstitial morphology of all 1 y old mice is consistent, featuring Leydig cell hypertrophy and hyperplasia.⁶²

AROM⁺. The AROM⁺ mouse bears the human ubiquitin C promoter/human P450 aromatase fusion gene.⁶⁶ AROM⁺ male mice are hypoandrogenic and hyperestrogenic, and are infertile, with significantly reduced testis weights that are cryptorchid.⁶⁶ At 4 mo of age, spermatogenesis is arrested at meiosis and similar to what is seen in the ArKO, albeit at 1 y of age, the interstitium is largely comprised of hypertrophic and hyperplastic Leydig cells. The AROM⁺ male phenotype was followed up at 9 and 15 mo of age, and in some instances in both age groups Leydig cell adenomas were present, however no malignant or metastatic tumors were observed.⁶⁷ Interestingly, spermatogenic disruption worsened with age. At 9 mo there was a notable reduction in the numbers of all germ cell types and by 15 mo the seminiferous tubules were atrophic and nearly all germ cell populations were depleted.⁶⁷ Giant multinucleated cells were present in the interstitium of all age groups and immunohistochemical analysis revealed that these cells were in fact macrophages, and increased in number with increasing age.⁶⁷ Given the fact that intratesticular estradiol concentrations also increased with age in these mice, it is likely that the surge in aromatization is indirectly exacerbating the testicular phenotype as the effect of heightened estradiol levels on the testis have been well documented. For example, administration of estrogens or xenoestrogens on rats not only results in a similar testicular phenotype as the AROM⁺ mice, but also results in reduced Sertoli cell numbers.⁶⁸ However it is prudent to note that the impaired testicular phenotype in the AROM⁺ mouse may not be solely a result of elevated aromatase action; it may in part be an effect of cryptorchidism.

These mouse models have been of tremendous value in advancing our rudimentary understanding of aromatase in spermatogenesis. These models have the potential to create new information about the role of aromatase in the developing and adult testis and of the genes and proteins that regulate aromatase signaling. Aromatase inhibitors are now being used to understand the role of aromatase in testicular biology. It has been shown in rats that neonatal exposure to exemestane results in a moderate decrease in Sertoli cell number but with no differences in sperm output and fertility as assessed by older morphometric approaches to quantitation.⁶⁹ In fish, (the three-spot wrasse), treatment with long-term exemestane, resulted in decreased spermatogonial proliferation suggesting aromatase plays a role in supporting early germ cell development.⁷⁰ Whether these cellular effects are a result of a direct effect on the developing germ cells or via the Sertoli cell remains unknown.

Clinical Cases

Aromatase deficiency. Aromatase deficiency in males as a result of inactivating mutations in the *CYP19A1* gene is an extremely rare condition, with only 9 cases reported to date.^{46,47,71-77} As expected, these patients have undetectable estrogen levels, with normal to high levels of testosterone and gonadotropins. While the extra-gonadal clinical features for this condition are reportedly very similar across all cases (reviewed in ref. 78), there are no consistent findings with respect to the testicular phenotype (Table 1). Testicular size spans small,⁴⁶ normal^{47,71} and large⁷⁶ and in some cases cryptorchidism is evident.^{73,74} The lack of patient consent for human ejaculate samples and testicular biopsies hampers the ability to draw solid conclusions about the effect of aromatase deficiency on spermatogenesis, but from the data that is available it appears a lack of aromatase is associated with Sertoli cell only syndrome,⁷⁴ hypospermatogenesis,⁷⁵ oligospermia,^{46,47} reduced sperm motility⁴⁷ and complete sperm immobility,⁴⁶ many of which are in concert with findings from the ArKO model (reviewed in ref. 6). Further qualitative analyses on the histological architecture of the testis noted seminiferous tubules of normal appearance⁷⁵ and those that were hypotrophic.⁷⁴ The direct effect of aromatase deficiency on fertility on all except one of the aromatase deficient men⁴⁶ is yet to be reported. Nevertheless, based on the fact that this man could not father children, what has been noted on the sperm parameters of the other men with aromatase deficiency and the reported observations in the ArKO mouse, it is foreseeable that their reproductive ability would be severely impaired.

Aromatase excess. Overproduction of aromatase in men has been associated with two reportedly rare syndromes: aromatase excess and Peutz-Jeghers (PJS). Both of these syndromes result in an amplified aromatization rate leading to an elevated production of estrogens, however they differ by the location in which this occurs. Aromatase excess syndrome is typically due to sporadic or familial polymorphisms in the *CYP19A1* gene that results in an increase in aromatization in the adipose tissue and probably elsewhere.^{79,80} This clinically induces pre-pubertal gynecomastia with no measurable effect on the testis.^{79,80} In comparison, the genetic makeup of PJS frequently comprises inactivating mutations of the serine/threonine kinase 11 (*STK11*) tumor suppressor gene located on chromosome 19p13.3 which encodes for the protein kinase, LKB1⁸¹ and consequently predisposes individuals to benign and malignant tumors of many organ systems, including the testis.⁸² This mutation is presumably playing the pathogenic role in tumor development in patients with PJS however the molecular mechanisms by which this occurs is yet to be founded.⁸³ In terms of the testis, the sex cords are the site of tumorigenesis and a total of 35 boys with PJS have presented with testicular Sertoli cell tumors.^{36,37,39,59,81,84-100} In boys with PJS, there is an increase of aromatase expression and activity in these tumors, resulting in excess estradiol production that not only acts to feed local tumor growth,^{36,39} but also accelerates testicular and extra-gonadal morphogenesis. For instance, the obvious clinical manifestations are enlarged testes, pre-pubertal gynecomastia and feminizing precocious puberty, and it has become increasingly

Table 1. Summarizes the reproductive phenotypes and hormonal profiles of available data from reported men with aromatase deficiency.

Case report	Age	Mutation	Estradiol (pg/ml)	T (ng/dl)	Free T (%)	FSH (mIU/ml)	LH (mIU/ml)	Aromatase Activity In vitro	Reproductive Phenotype
Morishima et al. 1995	24	Point mutation at bp 1123 (C→T) in exon IX Results in cysteine instead of arginine at position 375	<0.7 (10-50)	2015 (30-263)	4.7 (2.0-4.8)	28.3 (5.0-9.9)	26.1 (2.0-9.9)	0.2	Macroorchidism
Carani et al., 1997	31	Point mutation at bp 1094 in exon IX Results in glutamine instead of an arginine at position 365	<10.0 (10-40)	390 (360-900)	N/A	13.6 (1.7-8.9)	8.9 (1.4-8.9)	0.4	Microorchidism Severe oligospermia 100% immobile spermatozoa
Hermann et al., 2002	27	Frameshift mutation and premature stop codon 8bp downstream from the splicing acceptor site before exon VI Results in C→A substitution in intron V	<20.0 (10-50)	899 (270-870)	N/A	11.0 (1.0-7.0)	6.0 (2.0-10.0)	0.0	Normal testicular size Oligospermia 17.4 million/ ml (>20) Reduced sperm motility (10%)
Maffei et al., 2004	29	Point mutation at the last nucleotide of exon V (G→A) Results in spliceosome unable to recognise a splicing site	Undetectable <1.5 (30-90)	380 (380-900)	N/A	23.0 (1.2-7.8)	3.7 (1.5-9.2)	1.0	Bilateral cryptorchidism Azoospermia Low testicular volume (10-11ml)
Bouillon et al., 2004	17	Frameshift mutation due to C-base deletion in exon V Results in premature stop codon after 21 codons	Undetectable <5.0	1305	3.2	8.0	8.0	N/A	Normal
Maffei et al., 2007	25	Two point mutations: the first at bp 380 (T→G) in exon IV and the second at bp 1124 (G→A) in exon IX Results in arginine instead of methionine - 127 and histidine instead of arginine - 375 respectively	Undetectable <5.0 (<40)	581 (300-900)	N/A	10.1 (1.2-7.8)	6.75 (1.5-9.2)	0.0	Severe penile phimosi Hypospermatogenesis
Lanfranco et al., 2008	26	Frameshift mutation due to 23bp deletion in exon IV and point mutation in the first nucleotide of intron IX Results in premature stop codon at position 361 and possibly alternative splicing respectively	Undetectable (20-40)	839 (2.99-10.0)	5	12.5 (1.2-10.0)	3.1 (1.0-7.0)	N/A	Right cryptorchidism

Italics show the normal ranges of hormone profiles if provided. N/A denotes data are not available.

recognized that both of these result from the increase in testicular aromatization.⁸¹ The tumors are typically benign and considered the equivalent of the ovarian sex cord tumor with annular tubules (SCTAT), commonly seen in females with PJS.³⁷ Interestingly, AROM⁺ mice do not develop sex cord tumors, rather Leydig cell adenomas, which are exacerbated as a result of increasing testicular estradiol concentration.⁶⁷ Microscopically, SCTATs result in clusters of enlarged annular seminiferous tubules nestled among seminiferous tubules of normal size and appearance.¹⁰⁰ The enlarged seminiferous tubules are composed of hypertrophied Sertoli cells enclosed by a thickened basement membrane, with no evidence of germ cells.¹⁰⁰ Moreover, the tumors in boys with PJS can in some instances show signs of calcification distributed throughout the tumor, which is a hallmark for the diagnosis of a large cell calcifying Sertoli cell tumor (LCCSCT).¹⁰⁰ Together, SCTAT and LCCSCT classically comprise the two tumor types observed in boys with PJS. In both these tumor types the cascade of intracellular events that leads to the aberrant expression of aromatase driven by proximal promoter II (PII) remains largely unknown. Nonetheless, here we attempt to synthesize possible molecular mechanisms of action for the expression of aromatase in both the healthy and diseased testis.

Molecular Mechanisms Controlling Aromatase Gene Expression in the Testis

In the healthy and diseased testis, it is PII that controls aromatase mRNA transcription.^{35,39,101-103} Many potential extracellular regulators of PII driven aromatase gene expression in the testis have been identified: follicle-stimulating hormone (FSH),¹⁰⁴

luteinizing hormone (LH),¹⁰⁵ insulin-like growth factor-1 (IGF-1),¹⁰⁶ thyroid hormone (TH),¹⁰⁷ transforming growth factor- β 1 (TGF β 1)^{83,108} and epidermal growth factor (EGF),^{109,110} (see Fig. 1). While these extracellular regulators have been identified, the independent or convergent intracellular pathways they operate through to drive PII aromatase transcription remain largely unknown. Nonetheless, there are two response elements within PII that together with their transcription factors have been identified to drive aromatase transcription in the testis and provided some insight into the molecular mechanism(s) behind these events. The *first* is steroidogenic factor response element (SFRE).¹¹¹ SFRE (consensus sequence CAA GGT C; position -90) requires the binding of a monomeric orphan member of the nuclear receptor family of transcription factors to induce aromatase expression.^{103,112} Steroidogenic factor-1 (SF-1; NR5A1) is one member of this family that binds to this response element in vitro and stimulates the expression of aromatase reporter genes in Leydig cell lines.^{103,113,114} While these findings infer SF-1 can regulate aromatase expression in the testis, the localization of SF-1 within the testis is restricted to particular cell types. Immunolocalization studies have revealed that SF-1 is present in Sertoli and Leydig cells prior to puberty but only Leydig cells post-puberty in the rat.¹¹⁵ This data are in concert with the immunolocalization of aromatase during these time periods, where it is clear that aromatase switches from Sertoli to Leydig cells at puberty.^{11,29} The equivalent localization of SF-1 and aromatase supports the in vitro data and highlights a role for SF-1 regulated aromatase expression in the testis. Another related orphan member of the nuclear receptor family, liver receptor homolog-1 (LRH-1; NR5A2), has also been shown to bind to SFRE and

Figure 1. Model of the molecular mechanisms regulating PII-driven aromatase gene expression in the testis. This model is based on data available from a variety of testicular cell types and is a compilation of many papers and across many species.

regulate testicular PII aromatase transcription *in vivo*.¹¹⁶ While LRH-1 is localized to Leydig cells throughout all stages of development, unlike SF-1, LRH-1 protein is expressed in gonocytes and spermatogonia prior to puberty and pachytene spermatocytes and round spermatids post-puberty¹¹⁶ and is not localized to the Sertoli cells at any stage of development.^{111,116} These data suggest that both SF-1 and LRH-1 regulate PII transcription independently in Sertoli and germ cells and cooperatively in Leydig cells, the primary site of steroidogenesis. To extend on this, another orphan member of the nuclear receptor family, Dax-1, exhibits an inhibitory effect on PII aromatase gene expression in normal settings however in the absence of Dax-1 in rats, Leydig cell tumors develop and a concomitant increase in aromatase gene expression results.¹¹⁷ The precise extracellular regulator(s) that lead to the interaction of orphan transcription factors to the SPRE remain unknown however two have been postulated: (1) IGF-1: can regulate aromatase gene by stimulating SF-1 binding to the SFRE on PII in rat Leydig cell tumors¹⁰⁶ and (2) TH/TH-receptor complex is able to compete with SF-1 in binding to the SFRE response element on PII in rats.¹⁰⁷

The *second* is cyclic AMP responsive element (CRE)^{112,113} which contains 3 CRE-like sequences (position -335: TGA ACT CA, position -231: TGA AAT CA and position -169: TGC ACG

TCA).¹¹² These bind both CRE-binding protein (CREB) and cyclic AMP (cAMP) responsive element modulator (CREM). It is generally considered in the healthy testis that FSH and LH are the main extracellular regulators that act by increasing the intracellular concentration of cAMP leading to the activation of protein kinase A (PKA) and triggering the phosphorylation of transcription factors such as CREB and CREM which bind to the CRE-like motifs and induce PII aromatase transcription.¹⁰¹ While data linking the cAMP/PKA signal transduction pathway to specific testicular cell types is inadequate, it is clear that CREB and CREM exhibit cyclical expression in both germ and Sertoli cells throughout spermatogenesis which correlates with the fluctuations in cAMP signaling induced by FSH and LH.¹¹⁸ In line with this, studies have shown that it is the cAMP/PKA pathway that controls aromatase expression in spermatozoa.^{44,119,120} In vitro studies have shown that in Leydig tumor cell lines, the aberrant aromatase expression does not occur through this cAMP-dependent pathway, rather other transcription factors specifically expressed by the tumor bind the CRE-like motifs and drive PII aromatase transcription in a cAMP-independent manner.¹¹²

In addition to FSH and LH, two other extracellular regulators have been shown to drive PII aromatase gene expression through

the CRE: (1) as a result of overexpression of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) stimulates the cAMP/PKA dependent pathway in mouse Leydig cell tumors¹²¹ and (2) in a similar manner, the xenoestrogen BPA increases rat Leydig cell aromatase expression by upregulating COX-2.¹²² Furthermore, a recent study has shown elegantly that aromatase expression is regulated by $1\alpha,25(\text{OH})_2$ vitamin D₃ (1,25D) in day 30 rat Sertoli cells cultures with the data suggesting the existence of a genomic and non-genomic activation of the vitamin D receptor involving at least the PKA pathway.¹²³

Another extracellular regulator of PII aromatase gene expression that doesn't act through SFRE or CRE is TGF β 1. There is an isolated TGF β response element on PII within the testis (consensus sequence: GAG TTG GGA G, position -422).¹⁰¹ TGF β 1 displays an inhibitory effect on aromatase gene expression in pachytene spermatocyte and round spermatid cultures¹⁰⁸ however in comparison, in a Sertoli cell tumor sample from a patient with aberrant aromatase expression due to PJS, the TGF β 1 protein displays high expression levels.⁸³ This apparent switch in TGF β function has been reflected in a model of breast cancer progression, where it is evident TGF β can switch from a tumor suppressor to a pro-metastatic factor.¹²⁴

At present, data showing the molecular mechanisms controlling PII aromatase gene transcription in the testis are limited, although a valuable exercise is to use information from other tissues and organs to provide potential clues from well characterized aromatase regulated tissues such as the breast and prostate,¹²⁵ and indeed many factors that have been implicated in progression of disease in these tissues are known to be present in the testis. Nonetheless, majority of data suggests that is the conjoint action of SF-1/LRH-1 and phosphorylated CREB binding to their corresponding response elements on PII that contribute to constitutive active aromatase expression. Additionally, these transcription factors also appear to act independently by regulation of aromatase expression in different testicular cell types, in both the healthy and diseased testis, potentially offering an explanation for differences in patterns and levels of aromatase expression seen in different testicular cell types.

Summary

The precise testicular aromatase genotype-phenotype relationship is still much of a mystery to medical researchers and clinicians. While the aromatase protein is highly conserved across

species, it seems that the main site of aromatization depends on the species, the stage of development, the breeding season and whether the tissue is healthy or diseased. Nonetheless, what is clear is that normal testicular development and function requires aromatase, and that the location, time, quantity and duration of aromatase action is vital for spermatogenesis and full fertilizing capacity. The discovery of alternative sites of aromatase activity and therefore estrogen biosynthesis in cell types such as spermatocytes, spermatids and spermatozoa, suggests that estrogens are not only endocrine regulators of spermatogenesis but may also exert its effects in a paracrine/autocrine manner. This is particularly evident throughout post-meiotic germ cell maturation in the testis and spermatozoa maturation in the epididymis, as well as in diseased settings, where it is clear that local aromatase translocation and aberrant activity occurs in testicular cell types that are compromised in the disease, consequently exacerbating the testicular insult. The existence of congenital dysfunctional aromatase cases has provided some insight into the role aromatase plays on the development and function of the testis. In terms of aromatase deficiency, although the testicular phenotype presented by each patient is not identical, there are commonalities that indicate aromatase is required for normal testicular size and sperm quantity and motility. However, when aromatase exceeds normal levels, as in the case of some patients with PJS, aromatase turns into a potent proliferative agent and aids in the development and progression of Sertoli cell tumors. Whether or not too much aromatase has an influence on fertility in PJS patients remains unknown, however given the recorded effects of exogenous estrogens on sperm parameters,^{7,51} it is likely their fertilizing capacity would be compromised. Suffice to say, deciphering the specific roles of aromatase and estrogens and their underlying cellular and molecular mechanisms in the healthy testis, would be extremely valuable in advancing our understanding of how these mechanisms become compromised in the diseased testis, to ultimately reveal potential therapies for aromatase driven testicular disease.

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