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Aromatase Expression in the Ovary: Hormonal and Molecular Regulation

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Abstract

Summary—Estrogens are synthesized by the aromatase enzyme encoded by the *Cyp19a1* gene, which contains an unusually large regulatory region. In most mammals, aromatase expression is under the control of two distinct promoters a gonad- and a brain-specific promoter. In humans, this gene contains 10 tissue-specific promoters that are alternatively used in various cell types and tumors. Each promoter is regulated by a distinct set of regulatory sequences and transcription factors that bind to these specific sequences. The cAMP/PKA/CREB pathway is considered to be the primary signaling cascade through which the gonad *Cyp19* promoter is regulated. Very interestingly, in rat luteal cells, the proximal promoter is not controlled in a cAMP dependent manner. Strikingly, these cells express aromatase at high levels similar to those found in preovulatory follicles, suggesting that alternative and powerful mechanisms control aromatase expression in luteal cells and that the rat corpus luteum represents an important paradigm for understanding alternative controls of the aromatase gene. Here, the molecular and cellular mechanisms controlling the expression of the aromatase gene in granulosa and luteal cells are discussed.

Keywords

aromatase; ovary; granulosa cells; luteal cells; gene expression

1. Introduction

Estrogens are crucial for female and male fertility, as proved by the severe reproductive defects observed when their synthesis [1] or actions [2] are blocked. In the ovary, locally produced estradiol acts in concert with the gonadotropins secreted from the anterior pituitary to provide for successful folliculogenesis and steroid production. As a secreted hormone, estradiol modulates the structure and function of female reproductive tissues, such as the uterus and oviduct. Estradiol is also one of the principal determinants of pituitary neuron functioning and is critical in enabling these cells to exhibit fluctuating patterns of biosynthetic and secretory activity and to generate the preovulatory surge of luteinizing hormone (LH). Estradiol also contributes to cyclical variations in sexual female behavior. Therefore, the coordinated and cell-specific expression of the aromatase (*Cyp19a1*) gene in the ovary plays a key role in the normal progress of the menstrual/estrous cycle.

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In humans, estradiol also participates in pathological processes such as breast, endometrial, and ovarian cancers. Much attention has been given to the regulation of the aromatase gene and its implication in the development and progression of human diseases. Several excellent reviews in this area have been recently published [3-5]. The present review focuses on the molecular, cellular, and normal physiological mechanisms regulating the expression of the aromatase gene in the ovary with a particular emphasis on the rat.

2. Aromatase Expression during Ovarian Development

In rodents, aromatase is restricted to the gonads and the brain. In the ovaries of sexually mature animals, aromatase expression is limited to the follicle and the corpus luteum. In these structures, the expression of this gene is controlled in a cell-specific, temporal, and spatial manner that limits estradiol production only to mural granulosa cells of healthy large antral follicles and to luteal cells. However, aromatase expression has also been reported in fetal and immature ovaries.

2.1 Fetal ovaries

In many species, ovaries acquire the enzymatic capacity to produce estrogen during embryonic life as determined by the capacity of fetal ovaries to convert radiolabeled androgen to estrone and estradiol [6-8] or by the amplification of aromatase cDNA by PCR [9]. Aromatase expression in fetal ovaries is extremely low; although it can be increased by treatment with an analog of cAMP but not by follicle stimulating hormone (FSH) [8,10]. Whether this minute expression of aromatase in fetal ovaries plays any role in the regulation of ovarian development is not clear since estrogens do not appear to be critical for the normal development of the ovary. Neither deletion of the aromatase gene (ArKO) [11] nor mutations of the aromatase protein [12] affect ovarian formation. Although in these cases maternal estrogens could compensate for the lack of local estradiol synthesis, estrogen receptor α and β (ER α and β or Esr1 and Esr2) knockout mice show no defects on fetal ovarian development [13], suggesting that, at least in mice, estrogens do not participate in this process.

2.2 Neonatal ovaries

Aromatase activity and mRNA can be found at days 5 and 8 after birth in the granulosa cell layer of growing follicles [10,14,15]. Aromatase expression progressively increases in both preantral and small antral follicles in infantile ovaries [Fig. 1a and reference 14]. In 21-day-old rats, aromatase is no longer expressed by growing follicles, but is confined to healthy large antral follicles [14]. Aromatase expression in neonatal ovaries requires normal levels of gonadotropins [16]. This evidence suggests that the appearance of aromatase activity and mRNA in the neonatal rat correlates closely with the development of primary and secondary follicles. Although aromatase activity is present in preantral follicles of prepubertal ovaries, estrogen production at this stage of development must be limited since these follicles do not possess all the components of the 'two cell, two gonadotropins' model.

The structure of the ovary in immature ArKO animals has not been assessed; however, at 10 weeks of age, the ovaries of these mutant animals contain follicles of all types (primordial, primary secondary, and antral); although many antral follicles appeared histologically unhealthy [17,18], these results suggest that estrogen is not a prerequisite for the initiation of follicle growth. However, the ovaries of ArKO mice maintained on a phytoestrogen-free diet degenerate with age and develop testicular tissue, indicating that estrogen is necessary to maintain the structure of the ovary in adult animals [19].

In immature ER α and ER β knockout mice, LH levels are elevated [13]. The high levels of LH result in a premature maturation of the ovary characterized by the presence of adult-like follicles

in prepubertal females [13]. Indirectly, this result suggests that, in prepubertal animals, ovarian estradiol contributes to maintain normal levels of gonadotropins via its well-known regulatory feed-back in the pituitary gland.

2.3 Mature ovaries

In contrast to neonatal ovaries, at first proestrus and in older animals, aromatase is no longer expressed by growing follicles, and its expression is restricted to large antral healthy follicles and preovulatory follicles [Fig. 1b and ref. 14]. Laser microdissection of preantral and antral follicles has recently confirmed these results [20]. Within the granulosa cell layer of antral follicles, aromatase expression is highest in the mural granulosa cells at the outer edge of the follicle when compared to granulosa cells closest to the antral cavity [14,21]. Moreover, aromatase is not expressed in cumulus granulosa cells [14,21]. The marked compartmentalization of aromatase expression within the granulosa layer is particularly striking in mature follicles from rats at proestrus (Fig. 1). The molecular basis and physiological implication of the differential expression of aromatase in mural and cumulus granulosa cells are not clear.

As with the LH receptor [22], aromatase in cumulus granulosa cells is probably silenced by oocyte-derived compounds. Factors produced by the oocytes such as BMP-15 [23] and GDF-9 [24,25] inhibit the FSH-induced increase in aromatase expression. In addition, in GDF-9 deficient mice, which manifest an arrest of preantral follicular growth, there is a premature expression of aromatase in preantral follicles [26].

Recently, it was suggested that the tumor-suppressor gene BRCA1 may contribute to the specific distribution of aromatase in antral follicles. In contrast to aromatase, BRCA1 expression is restricted to the cumulus cells of antral follicles [27]. Ectopic expression of BRCA1 decreases aromatase in a granulosa tumor cell line [28]. Although BRCA1 seems to bind the aromatase promoter [29], the region where BRCA1 binds and the mechanism by which BRCA1 represses aromatase remains to be elucidated. Nevertheless, it is possible that BRCA1 does not regulate aromatase, and the differential expression of BRCA1 and aromatase in mural and cumulus cells is only a consequence of the different phenotypes of these two cells. Dias et al. [30] recently demonstrated that oocyte-stimulated signaling opposes the action of FSH, whereas FSH stimulates the expression of mural marker transcripts and suppresses the expression of cumulus markers. Thus, oocytes and FSH establish opposing gradients of influence that define the cumulus and mural granulosa cell phenotypes. Strikingly, in rat preantral granulosa cells, estrogen amplification of the stimulatory effect of FSH (see below) occurs only in the presence of oocytes [31], suggesting that preantral granulosa cells respond differently to oocyte-secreted molecules.

2.3.a Factors affecting aromatase expression in granulosa cells

It is well accepted that FSH is the major inducer of aromatase activity in granulosa cells. However, the stimulatory effect of FSH is subject to modulations by numerous compounds. A list of some of these compounds and their actions on FSH-induced aromatase expression can be found in Table I. The role of estradiol, androgens, and insulin growth factor-I will be discussed in detail.

It has long been recognized that estradiol augments the actions of FSH on granulosa cells, and both hormones are necessary to establish a fully differentiated and healthy preovulatory follicle. Estradiol acts in an autocrine manner to enhance FSH actions. This feed-forward effect of estradiol is believed to play a key role in follicle dominance. At least in the rat, one of the effects of estradiol is to enhance aromatase stimulation by FSH [32,33]. Estradiol actions in rodent granulosa cells are mediated by the activation of ER β [34]. Accordingly, in immature

ER β knockout mice, the stimulation of aromatase expression by FSH is impaired [35]. Since estradiol alone does not affect aromatase expression [32] and no ER response elements are present in the aromatase promoter, it is not known if ER β is able to directly affect the activity of the aromatase promoter.

Androgens also enhance the effect of FSH on aromatase expression. In fact, it was demonstrated early on that testosterone is more effective than estradiol in enhancing FSH induction of aromatase expression in rat granulosa cells, whereas dihydrotestosterone, a nonaromatizable androgen, is as effective as estradiol [32,36]. This evidence indicates that theca cell androgens act not only as a substrate of estrogen synthesis but also modulate FSH action via activation of androgen receptors [32,36]. *In vitro* studies suggest that androgens enhance FSH-stimulated steroidogenesis by increasing cAMP levels [37,38]. In the rat, androgen receptor expression is highest in preantral/early antral follicles and gradually decreases as follicles mature at the time that aromatase expression increases [39]. These findings suggest that androgens enhance FSH action at an early stage of follicular development, but during the final stages of follicular development they mainly serve as a substrate for estrogen synthesis [36]. Aromatase expression and estradiol levels are normal in the ovaries of androgen receptor knockout mice [40], suggesting that androgens are not crucial for aromatase expression *in vivo*.

In murine granulosa cells, FSH actions are greatly potentiated by insulin-like growth factor-1 (IGF-1). IGF-1 acts via its receptor on the granulosa cells to enhance the FSH-induced stimulation of aromatase activity [41-43]. IGF-I also amplifies the synergism observed between FSH and testosterone in aromatase expression [38]. In mice, aromatase mRNA is found only in IGF-I and FSH receptor positive follicles [44]. Further evidence of a functional link between the IGF system and stimulation of aromatase by FSH is provided by the fact that IGF binding protein (IGFBP-4) is a potent inhibitor of FSH-induced estradiol production by murine [45] and human [46] granulosa cells.

IGF-I likely acts primarily by augmenting the capacity of granulosa cells to respond to FSH since FSH receptor expression is reduced in IGF-1 knockout mice [44]. In human granulosa cells, however, IGF-I alone increases estradiol production to levels comparable to those induced by FSH, although together these hormones also have a synergistic effect [47-49].

2.3.b Intracellular signaling pathway and aromatase expression

Early studies clearly established that cAMP is the main intracellular messenger mediating FSH stimulation of aromatase expression [10,50-52]. The increase in intracellular cAMP levels induced by FSH leads to the activation of the cAMP-dependent protein kinase A (PKA). Fitzpatrick and Richards identified two regions in the aromatase promoter that mediate the stimulatory effect of cAMP/PKA on the aromatase promoter [53,54]. One of these elements is a hexameric sequence recognized by nuclear orphan receptors [53,55]. The second and more distal element was identified as a cAMP-response element-like sequence (CLS) [54]. Since the characterization of these two binding sites, other sites have been shown to participate in the control of aromatase expression in granulosa cells (see below).

FSH activates several other intracellular signaling pathways, including those for the extracellular regulated kinases (ERKs), p38 mitogen-activated protein kinases (MAPKs), and phosphatidylinositol-3 kinase (PI3K) [reviewed in ref. 56]. Of these pathways, PI3K, which activates protein kinase B (PKB or viral proto-oncogene 1; Akt), also participates in the induction of aromatase expression by FSH [57,58]. For instance, aromatase stimulation by FSH is amplified by the expression of constitutively activated Akt [57], whereas treatment with an inhibitor of the PI3K [58] or overexpression of dominant negative Akt [57] prevents aromatase up-regulation.

FSH stimulation of Akt seems to relieve aromatase from a repressive effect of forkhead box O1 (FOXO1) since a constitutively active FOXO1 protein prevents aromatase stimulation by FSH and activin [58]. Consistent with this hypothesis, both FSH and IGF-I, act post-translationally to phosphorylate FOXO1 [59], resulting in cytoplasmic localization and, presumably, loss of FOXO1 repression activity. Moreover, after PMSG treatment of immature rats, FOXO1 expression decreases [59] remaining expressed only in cumulus cells and antral granulosa cells which as mentioned before do not expressed aromatase [60].

The mechanism by which FOXO1 blocks aromatase expression is not known. It is known, however, that the DNA binding ability of FOXO1 is crucial to repress aromatase since overexpression of a constitutively active FOXO1 protein that does not bind DNA has no effect. Since no FOXO1 binding sites have been described in the aromatase proximal promoter, the effect of this transcription factor is probably indirect. It is also possible that the DNA-binding region of FOXO1 is necessary for the interaction of this factor with other regulatory proteins directly at the level of the aromatase promoter; in this case the effect of FOXO1, although independent of DNA binding, would be direct.

Estradiol enhances the expression of components of the IGF-I pathway such as the IGF-1 receptor; in turn, IGF-1 stimulates the expression of ER β [59]. Moreover, the stimulation of Akt by IGF-1 [61] is potentiated by estradiol in the rat [62]. These results suggest that the synergistic effect of estradiol, IGF-1, and FSH on the induction of aromatase could converge on Akt. On the other hand, synergism also exists between the ER β and the cAMP/PKA signaling pathways [63]. Therefore, the two signaling pathways activated by FSH (PI3K/Akt and cAMP/PKA) known to be involved in the regulation of aromatase may receive positive inputs from the estradiol/ER β and the IGF-1/PI3K/Akt pathways (Fig. 2).

2.3.c Kinetics of aromatase expression

One intriguing characteristic of the effect of FSH on aromatase expression is the relative long time (24 to 48 h) required for FSH to induce aromatase mRNA [32]. Since FSH stimulates cAMP production very rapidly, it has been proposed that an increase in the expression of the regulatory and catalytic units of protein kinase A (PKA) or proteins synthesized as a consequence of PKA activation may be required for aromatase induction [32]. Interestingly, FSH rapidly stimulates aromatase in the Sertoli cells of immature rats [64]. In these cells, the stimulatory action of FSH on aromatase expression also depends on the cAMP/PKA and PI3K/AKT1 pathways [64]. Studies aimed to compare the regulation of the aromatase gene in Sertoli and granulosa cells could provide information on the mechanisms by which the expression of this gene is delayed in granulosa cells.

In a human tumor granulosa cell line, ectopic expression of the transcription factors NURR1 (Nr4a2) or NGFI-B (Nr4a1) severely attenuates the stimulation of the aromatase promoter by stimulation of the adenylyl cyclase (forskolin treatment) [65]. The same effect was observed by overexpression of JunB [66]. Since these factors are transiently induced by forskolin, the authors suggested that they may be responsible for the delayed induction of aromatase. However, it is unlikely that these factors play any role since maximal levels of aromatase expression are reached only after 48 h of treatment with FSH [32] while NURR1 and JunB disappear completely 4 h after stimulation. Moreover, a high concentration of forskolin was used in this study, suggesting a pharmacological effect.

2.4 Pregnant rats

Aromatase is highly expressed in the corpora lutea of pregnant rats. Luteal cell function is greatly affected by locally produced estradiol, which stimulates both progesterone biosynthesis and luteal cell hypertrophy [67]. As shown in figure 3, luteal aromatase mRNA content is low

on day 4 of pregnancy, increases progressively to reach high levels of expression between days 14 and 19, and decreases from day 20 to reach undetectable levels on day 23, the day of parturition [68-70]. To facilitate the description of the mechanisms that may control luteal aromatase expression, three periods will be considered: i) luteinization, ii) pregnancy, and iii) before parturition.

2.4.a Luteinization

Aromatase expression reaches its zenith in preovulatory follicles. Preovulatory follicles ovulate and initiate the processes of luteinization in response to the LH surge. The LH surge also induces a rapid decrease in aromatase mRNA levels [52]. The inhibitory effect of LH contrasts with the stimulatory action of FSH in the sense that both hormones use adenylyl cyclase/cAMP as their main signaling pathway. Differences in the magnitude and duration of the cAMP signal that each receptor produces [32] and the generation of specific intracellular signals [57] have been proposed to explain the differential effects of FSH and LH on aromatase expression. In support of the latter mechanism, Ascoli and collaborators have proposed that the differential effects of FSH and LH may depend on the density of their receptors. Thus, the number of LH receptor molecules expressed in granulosa cells dictates the signaling pathway activated by this hormone [71]. By using an adenovirus to direct the expression of LH receptors in primary cultures of immature rat granulosa cells, Donadeu and Ascoli [71] demonstrated that only cells with a high density of receptors respond to LH by activating the inositol phosphate cascade in addition to the cAMP and Akt pathways. In this situation, the cAMP and Akt induction of aromatase is inhibited by activation of the inositol phosphate cascade [71]. At low receptor density, however, LH stimulates aromatase expression. Similar to aromatase, the LH receptor is highly expressed in preovulatory follicles [72] suggesting that only in preovulatory granulosa LH stimulates inositol phosphate production and intracellular calcium release. The intracellular increase of these second messengers leads to the activation of PKC. Accordingly, PKC activation is sufficient to inhibit the induction of aromatase by FSH [73]. Activation of PKC in rat granulosa cells results in decreased expression of the catalytic unit of PKA and of the transcription factor steroidogenic factor 1 (SF-1 or Nr5a1) [73]; however, if this two events mediate the effect of LH remains to be determined.

The inhibitory effect of PKC may be mediated by extracellular regulated kinase (ERK1/2 or MAPK) pathway. In granulosa cells, ERK1/2 phosphorylation is stimulated by LH [74-76]. Remarkably, LH causes a rapid and transient activation of ERK1/2 in granulosa cells expressing low levels of LH receptors; whereas, if high levels of LH receptor are present, a delayed and more sustained activation of ERK1/2 take place [77]. The early increase in ERK1/2 phosphorylation is PKA dependent and PKC independent [75]. In contrast the delayed effect is PKA and PKC dependent and seems to be mediated by an increase in epidermal growth-like factors (EGF-1) [77]. Moreover, an inhibitor of MEK, the upstream kinase of ERK1/2, overcomes the ability of PKC activation to antagonize the induction of aromatase. This results suggest that PKC-mediated phosphorylation of ERK1/2 may be ultimately responsible for the inhibition of aromatase in immature granulosa cells expressing a high density of LH receptors.

Inhibition of protein synthesis partially prevents the loss of aromatase mRNA induced by LH or PKC activation [32,73], suggesting that the expression of a repressor protein is needed. In fact, the transcription factors Cebpb (CCAAT/enhancer-binding protein β) and ICER (inducible cAMP early repressor), which are known to be rapidly induced by the LH surge [72,78], seem to participate in the silencing of the aromatase gene. For instance, in Cebpb-null mice, the decrease in aromatase expression observed after the LH surge does not occur [79], and overexpression of Cebpb in endometriotic stromal cells prevents cAMP stimulation of the aromatase promoter [80]. Moreover, downregulation of Cebpb in endometriotic stromal cells has been proposed as the cause of elevated aromatase expression in endometriosis [80].

ICER is an isoform of CREM (cAMP-responsive element modulator) that represses cAMP-induced transcription [72]. ICER has been shown to participate in the repression of the inhibin α -subunit gene that takes place after the surge of LH [81]. Overexpression of ICER decreases aromatase promoter activity induced by forskolin [82] and mediates the inhibition of aromatase expression by tumor necrosis factor- α (TNF- α) [83].

Whether the induction of Cebpb and ICER is due to PKC activation by the LH surge remains to be determined, but this seems to be unlikely because these genes do not respond to PKC [84,85]. Additional studies are needed to bring together the effects of LH/inositol, Cebpb, and ICER on the downregulation of aromatase in granulosa cells.

2.4.b Pregnancy

In newly formed corpora lutea, aromatase remains expressed at low levels, which are not affected by the elevation of intracellular cAMP [52,86]. Luteal aromatase expression starts to increase between days 6 and 8 of pregnancy [87]. As expected, luteinized granulosa cells also express very low levels of aromatase; interestingly, aromatase expression is recovered after 5 to 6 days of incubation [52,88]. The factors involved in the reactivation of the aromatase gene either *in vivo* or *in vitro* are not known.

During the first week of pregnancy, corpus luteum function is maintained by the actions of prolactin (PRL) and LH [89]. Prolactin is secreted from the pituitary gland until approximately days 9–10 of gestation [90]. Although PRL is essential to maintain luteal function, evidence suggests that this hormone inhibits luteal aromatase. For instance, PRL decreases aromatase expression in luteinized granulosa cells and in the corpora lutea of pseudopregnant rats [87]. In pregnant rats, treatment with a blocker of PRL secretion on day 5 of gestation leads to an increase in aromatase mRNA expression that can be prevented by treatment with PRL (Stocco unpublished).

Placental lactogens (PL) and estradiol are required to maintain rat luteal function during the second half of gestation [89]. These hormones have also been implicated in the regulation of aromatase expression. Placental lactogens removal after day 10 of pregnancy is followed by low expression and activity of luteal aromatase [91,92]. Placental lactogens signal through the same receptor that PRL uses [89]. Accordingly, in hysterectomized pregnant rats treated daily with PRL (or PRL plus either testosterone or estrogen), aromatase expression increases, reaching levels similar to those found in intact rats on day 15 [92]. Thus, in contrast to the inhibitory effect of PRL during the first part of gestation, placental lactogens are necessary to sustain luteal aromatase expression during the second part of gestation.

Administration of human chorionic gonadotropin (hCG), an LH analog, does not affect aromatase expression before (days 8–10) or after (days 13–19) midgestation; however, aromatase mRNA and protein increase in intact pregnant rats treated with hCG between days 10 and 12 [92]. In agreement with these results, the induction of aromatase on day 15 of gestation can be blocked by the administration of an anti-LH antibody on day 10 but not when given on day 12 [92].

In summary, luteal aromatase seems to be maintained by PRL at a low level during the first part of pregnancy and modulated by LH at midgestation. During the second half of pregnancy, aromatase becomes highly expressed by the stimulatory action of placental lactogens and testosterone in coordination with the stimulatory effect of ovarian estradiol itself (Fig. 3). The molecular and cellular mechanisms by which these hormones control the expression of the aromatase gene in the corpora lutea of pregnant rats are not known.

In humans, estrogen levels also undergo a transient decline after the gonadotropin surge and a later increase due to marked induction of aromatase mRNA [93]. In contrast, in cattle, this gene remains expressed at very low levels during pregnancy [94,95]. Thus, the pattern of luteal estrogen production differs between species.

2.4.c Before Parturition

Aromatase expression rapidly decreases just before partition. We have demonstrated that prostaglandin F₂ α (PGF₂ α), a well-known luteolytic hormone, represses luteal aromatase mRNA and protein levels when administered to rats on day 19 of pregnancy [70]. This inhibitory effect of PGF₂ α on aromatase receptor-knockout mice [70]. In these mutant animals, parturition does not occur and luteal expression of aromatase remains high. *In vitro* experiments demonstrated that PGF₂ α inhibits aromatase gene transcription in luteinized granulosa cells [70].

3. Regulation of the ovarian aromatase promoter

3.1 Structure of the aromatase gene

In the rat, two promoters for the aromatase gene have been found: a proximal promoter, which controls aromatase expression in granulosa and luteal cells [70,73] as well as in Leydig cells [96,97], and a distal promoter, which drives aromatase expression in the brain [98]. The transcripts produced by both promoters contain an identical open reading frame and encode the same protein. Transient transfection experiments have demonstrated that the first 160 bp of the ovarian promoter are sufficient to mediate cAMP [53,99] and PGE₂ [100] stimulation in rat granulosa cells.

Both the brain and the ovarian transcripts are produced in the rat corpus luteum [70], although transcripts produced by the brain promoter are expressed at very low levels when compared to the ovarian transcripts. Throughout pregnancy, only the expression of the ovarian transcript correlates with the changes observed in luteal aromatase protein levels. The low and constant levels of the brain transcript suggest that activation of the brain promoter drives the nominal and cAMP-independent expression of aromatase found at the beginning of pregnancy.

The region necessary for the activation of the proximal promoter was investigated in transgenic mice carrying 2700, 278, or 43 base pairs (bp) of the region upstream of the translation start site of the human aromatase gene linked to the human growth hormone coding region as reporter [101]. In mice carrying 2700 or 278 bp, the reporter is specifically expressed in the ovary whereas animals carrying the 43 bp construct show no expression of the reporter. This *in vivo* evidence suggests that the -278 to -43 region of the ovarian promoter contains the elements that drive aromatase expression in the ovary. However, the 278 bp reporter construct is active in ovarian stromal cells of adult animals, which do not express aromatase. Moreover, this construct is expressed throughout large antral follicles while aromatase expression is restricted to mural granulosa cells [Fig. 1b and ref. 14]. This indicates that the 278 region does not contain the elements that silence aromatase expression in cumulus granulosa cells and in stromal cells. It is also possible that chromatin modifications are important for the cell-specific expression of aromatase in the ovary. Since the reported construct could have been integrated anywhere in the genome, the activity of these constructs is not controlled by the same chromatin modification as the aromatase gene. As the author noticed, this abnormal expression could also be due to the presence of high level of growth hormone that was used as reporter.

3.2 Cis-elements in the ovarian promoter and their regulation

Nevertheless, there is no doubt that the first 300-bp region of the proximal promoter contains the element necessary for the expression of aromatase in mural granulosa cells. As shown in

Figure 4, within this region a cAMP-responsive element-like sequence (CLS) [52] and two binding sites for members of the nuclear receptors 5A family of transcription factors (NREa and NREb) are present [53,55,99,102-104]. The proximal promoter also contains one response element for members of the zinc finger family of transcription factors known as GATA [70, 105,106] and one AP-3 binding site [107]. These binding sites are highly conserved between species.

CLS (TG~~C~~ACGTCA)—This region differs from a consensus CRE binding site (TGACGTCA) by the insertion of a cytosine between the second and third nucleotides. Reporter experiments suggest that it plays a key role in the activation of the proximal promoter in granulosa cells [54,99]. However, mutation of this element greatly reduces but does not completely block the induction of promoter activity by cAMP. This region is recognized by the cAMP-responsive element binding (CREB) protein as demonstrated using gel shift assays [54,99]. CLS is also recognized by Cebpb, which has an inhibitory effect on aromatase promoter activity in human endometrial cells [80].

CREB is rapidly phosphorylated by treatment of granulosa cells with FSH, but the expression and subcellular localization of CREB do not change during granulosa cells differentiation, [54,108]. Overexpression of a nonphosphorylatable mutant of CREB in primary cultures of rat granulosa cells decreases estradiol production induced by FSH [109], suggesting that CREB activation is required for the expression of the aromatase gene. Noticeably, the FSH-induced increase in aromatase mRNA is detectable only 24 h after treatment [32], but FSH stimulation of CREB phosphorylation occurs within 1 h and declines to basal levels by 6 h [86,103,108]. There is no consensus as to whether phosphorylation of CREB is subsequently increased. For instance, no [108], low [86], or very high [103] levels of CREB phosphorylation have been reported 24 and 48 h after FSH treatment. Moreover, in forskolin-treated cells, phospho-CREB remains high in the presence of an inhibitor of phosphodiesterase 4 (PDE4), but no alterations in either the magnitude or the pattern of aromatase expression occur [86]. These results suggest that the effect of CREB on aromatase expression is complex and that CREB may have a direct effect via CLS and indirect effects by inducing the expression of other proteins involved in the activation of the aromatase gene.

The proximal promoter of the bovine aromatase gene has a one base pair deletion in the CLS element that destroys this element as a site for CREB binding [110]. Consequently, bovine aromatase reporter constructs do not respond to cAMP [110]. However, bovine granulosa cells do express aromatase in response to FSH [111,112]. Moreover, rat luteal cells, which expressed high levels of aromatase, do not contain proteins that bind to CLS [Fig. 5 and ref. 107]. In addition, in rat luteal cells, CREB resides in the cytoplasm [86], suggesting that neither CREB nor CLS activation is involved in the regulation of aromatase in the rat corpus luteum. This evidence supports the conclusion that activation of CLS is cell specific and that other proteins and elements are necessary to activate the proximal promoter in the ovary.

NREa (AGGTCA)—Because the aromatase promoter does not have a consensus cAMP response element, this nuclear receptor half-site was originally proposed to mediate the cAMP stimulation of aromatase [53]. Later, it was demonstrated that CLS and NREa interact in an additive manner to control aromatase expression in granulosa cells [103,113]. NREa is recognized by steroidogenic factor-1 (SF-1; officially known as Nr5a1) and liver receptor homolog-1 (LRH-1; officially known as Nr5a2). Although both SF-1 and LRH-1 are derived from different genes, these proteins share a high degree of identity, particularly in their DNA binding domains [114]. In fact, *in vitro* translated mouse SF-1 and LRH-1 proteins [104] or SF-1 expressed in bacteria [55] bind to oligonucleotides containing the NREa present in the aromatase gene. However, because both factors are expressed in granulosa cells [115,116], whether SF-1 or LRH-1 or both are important for aromatase expression in these cells remains

under debate (describe below), although evidence suggests that SF-1 has a more prominent role.

Exogenous expression of LRH-1 in bovine granulosa cells [104], mouse Leydig and Sertoli cell lines [117], or 3T3L1 mouse preadipocytes [118] increases aromatase promoter activity. However, overexpression of LRH-1 in rat granulosa cells does not enhance estrogen production or aromatase expression in either the presence or absence of FSH, although it did increase progesterone production [119]. This suggests that LRH-1 may not be crucial for aromatase expression in granulosa cells.

On the other hand, strong evidence suggests that SF-1 is a main player in the regulation of aromatase in the ovaries of rodents. Ovaries of granulosa-cell-specific SF-1 knockout mice contain hemorrhagic follicles [120]; similar defects were found in the ovaries of the aromatase knockout mice [19]. Moreover, exogenous expression of SF-1 in rat granulosa cells seems to stimulate aromatase expression. Thus, although Saxena et al. [121] demonstrated that FSH-stimulated estrogen production is only slightly increased by overexpression of SF-1, others have reported a strong augmentation of FSH-stimulated aromatase by SF-1 [122]. These differences could be due to the presence of testosterone in the experiments performed by Saxena et al. The synergistic effect of testosterone on the stimulation of aromatase by FSH could mask the stimulatory effect of SF-1. Noteworthy, both studies used serum to stimulate attachment of the cells to the plate. It is known that serum inhibits aromatase induction by FSH [32,123] and that even pre-incubation with serum blocks the response of granulosa cells to FSH [123]. Interestingly, in cells cultured with serum, the presence of testosterone in the medium restores the ability of FSH to stimulate aromatase [32]. Thus, SF-1 and testosterone block the inhibitory effect of serum, suggesting that the effects of testosterone on aromatase expression may be mediated by SF-1.

Recent evidence further supports a prominent role of SF-1 in aromatase expression in granulosa cells. For instance, it has been demonstrated that FSH- and cAMP-dependent regulation of aromatase in granulosa cells is enhanced by β -catenin (Ctnnb1) and that this stimulatory effect of β -catenin is mediated through its interactions with SF-1 [122]. Using a granulosa cell line, Parakh et al. demonstrated for the first time that SF-1 binds to the endogenous ovarian-specific aromatase promoter and that this finding is stimulated by cAMP [122]. Moreover, overexpression of constitutively active mutant FOXO1, which prevents aromatase stimulation by FSH and activin, also decreases SF-1 expression [58]. Levels of SF-1 protein do not exhibit major changes during follicular growth or in response to low levels of FSH in culture but decrease rapidly after the LH surge along with the downregulation of aromatase [115], further supporting the idea of an important role for SF-1 in the regulation of aromatase expression.

Recently, Hinshelwood et al. [104] described a second NRE upstream of CLS (Fig. 4). The authors demonstrated that NREb is able to bind *in vitro* transcribed SF-1 or LRH-1 proteins and that this response element is necessary for the stimulation of the human aromatase promoter by forskolin in bovine granulosa cells. We investigated the activation of the two NRE elements in the rat corpus luteum throughout pregnancy using gel shift analysis and found that their activation is developmentally regulated and correlates with luteal aromatase expression (Fig. 5).

GATA (TGATAA)—This element was described first in the human aromatase proximal promoter by Jin et al. in 2000 [105] as part of an effort to characterize a silencer element on this promoter. This GATA response element is conserved in several species, including the mouse, rat, and human [70]. Using a kidney cell line, it was later shown that mutation of this element decreases aromatase promoter activity [106]. In rat luteal and granulosa cells, this GATA binding site is recognized by GATA-4 [70,124]. GATA-4 binding is stimulated *in*

vitro and *in vivo* by FSH [70,124] and by PGE2 [100]. In addition, GATA-4 silencing blunts FSH induction of aromatase expression, suggesting that GATA-4 mediates at least in part the stimulatory effect of FSH in granulosa cells [124]. The activation of this GATA binding site in the corpus luteum follows a pattern similar to that of aromatase expression [Fig. 5 and ref. 107], suggesting that also in luteal cells GATA-4 participates in the regulation of aromatase.

AP-3 (TAACCACA)—We recently described the presence of an AP-3 binding site in the proximal promoter of the rat aromatase gene [107]. AP-3 binding sites are also present in the promoter of humans and mice, and at least in rats, it seems to be necessary for full activation of the aromatase promoter [107]. In rats, this AP-3 binding site interacts with luteal nuclear extracts obtained from pregnant rats but not with ovarian extracts of immature rats or of immature rats treated with PMSG (Fig. 5). In PMSG-treated immature rats, AP-3 binding activity increases after treatment with hCG, suggesting that AP-3 is active only in luteal cells [107]. In pregnant rats, luteal protein binding to AP-3 correlates with variations in aromatase expression (Fig. 5). These results suggest that AP-3 activation may play a role in the regulation of luteal aromatase expression. The protein that recognizes AP-3 has a molecular weight of approximately 48 kDa [107]; however, its identity is not known yet.

4. Signaling and Genomic Integration

In the rat ovary, aromatase expression results from the activation of the proximal promoter. The cAMP/PKA/CREB pathway is considered to be the primary signaling cascade through which this promoter is regulated. Very interestingly, in rat luteal cells, the proximal promoter is not controlled in a cAMP-dependent manner. Of the elements found in this region, GATA and NRE are used by both cell types. On the other hand, CLS is active only in granulosa cells whereas the AP-3 binding site is activated exclusively by luteal cells (Fig. 5). It is clear then that there is a change in the composition of the transcription complex formed on the aromatase promoter during the transformation of granulosa cells into luteal cells (Fig. 6).

The complex formed in granulosa cells has as principal component CREB. CREB transcriptional activity and binding to CLS is stimulated by the activation of PKA by FSH. FSH stimulation of aromatase expression is also mediated by activation of Akt. Akt seems to relieve aromatase from a repressive effect of FOXO1. The mechanism by which FOXO1 blocks aromatase expression is not known. FSH also stimulates binding of GATA-4 and SF-1 to the aromatase promoter. The stimulatory effect of FSH on aromatase expression is potentiated by IGF-1 and androstenedione probably by enhancing FSH intracellular signaling. IGF-I also seems to assist FSH in the repression of FOXO1. CREB interaction with cofactors such as CREB binding protein (CBP) leads to the assembly of the general transcription machinery and the recruitment of the TATA binding protein to the aromatase promoter. GATA-4 and SF-1 participate in the activation of the promoter by binding to NRE and GATA and by interacting with cofactors such as CBP and β -catenin, respectively.

In luteal cells, CLS is inactive and it seems that CREB does not participate in the regulation of the aromatase gene. This is a striking feature of the regulation of aromatase in luteal cells; especially if we consider that cAMP is the major regulator of the proximal promoter across species. It remains to be determined which intracellular signaling pathway is involved in the upregulation of aromatase in luteal cells during the second half of pregnancy in rats. Since activation of the prolactin receptor at this time stimulates luteal aromatase and because Akt can be activated by prolactin [125], it is possible that Akt participate in the regulation of aromatase in luteal cells. Binding assays suggest that luteal aromatase activation is controlled by the GATA, NRE and AP-3 response elements. Except for GATA, the proteins that recognize these elements in luteal cells are still under investigation. Because GATA is recognized by GATA-4 in luteal cells and because the activity of this transcription factor can be modulated

by Akt [126], it is possible to postulate that the PRL/PI3/Akt/GATA-4 pathway is involved in the activation of the aromatase gene in the corpus luteum.

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5. References

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Fig. 1. Aromatase expression in the ovary of immature and adult rats

A and B, *in situ* hybridization analysis of ovaries at 15 days postnatal (dpn) and first proestrus: At 15 dpn, aromatase is expressed in primary, preantral, and antral growing follicles. At first proestrus, aromatase is observed only in preovulatory follicles (PoF). Immunohistochemistry analysis of the ovaries of adult animals (C and D) demonstrated that levels of expression of aromatase are not uniform throughout the antral follicles, being highest in the mural granulosa cells at the periphery of the follicle (arrows) and absent from the granulosa cells surrounding the antrum and within the cumulus (arrowheads). The intensity of immunostaining increases in the periovulatory follicle (D, proestrus) compared with less mature antral follicles (C, diestrus). O: oocyte.

Panels A and B were adapted from Turner KJ, et al. (2002) Development and validation of a new monoclonal antibody to mammalian aromatase. *Journal of Endocrinology*;172:21–30. ©Society for Endocrinology (2002). Panels C and D were adapted from Guigon CJ, et al. (2003) Unaltered development of the initial follicular waves and normal pubertal onset in female rats after neonatal deletion of the follicular reserve; *Endocrinology*;144:3651–62. Reproduced by permission.

Fig. 2. Hormone interactions in the regulation of aromatase expression in follicles

FSH activates the cAMP/PKA and PI3K/PKB signaling pathways, which are known to mediate its stimulatory effect on aromatase expression. Androgens, IGF-1, and estradiol potentiate this effect of FSH. Androgens have direct effects probably mediated by the activation of androgen receptors and indirect effects throughout its conversion to estradiol. IGF-1 stimulates the expression of the FSH receptor and probably synergizes with FSH in the activation of PKB. Estradiol also enhances the stimulatory effect of FSH. The positive feed-back of estradiol may be mediated by a cooperative effect on the activation of the PI3K/PKB and cAMP/PKA pathways. On the other hand, the oocyte (O) produces factors (GDF-9 and BMP-15) that block the stimulatory effect of FSH in cumulus cells and in antral granulosa cells in contact with the follicular fluid, limiting the expression of aromatase to mural granulosa cells. BM: basal membranes.

Fig. 3. Hormonal regulation of luteal aromatase during pregnancy

Bars represent aromatase mRNA levels in corpora lutea of rats on different days of pregnancy. Aromatase and L19 mRNA levels were determined by real-time PCR and the results expressed as aromatase mRNA molecules per μg of total RNA was determined by using a standard curve generated from known quantities of aromatase cDNA. Values represent average $\pm$ SEM. Luteal aromatase seems to be maintained by prolactin (PRL) at a low level during the first part of pregnancy, is modulated by LH at midgestation, and becomes highly expressed by the stimulatory action of placental lactogens (PL) and testosterone in coordination with the stimulatory effect of ovarian estradiol (E2). Prostaglandin F_{2α} (PGF_{2α}) is involved in the rapid downregulation of aromatase toward the end of pregnancy.

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                                CLS
h  TGAGTTTATTTTCTTATAATTGGCAAGAAATTGGCTTCAATTGGGAATGCACGTCA
r  GAACTTCA-----GAACGTTGACTCTCAATTGAGTATGCACGTCA
m  *****CA-----GAGAGT-GACTCTCAATTGA----GCACGTCA

                                NREb      GATA
h  CTCTACCCACTCAAGGGCAAGATGATAAGGTTCTATCAGACCAAGCGTCTAAAGGAAC
r  CTCTACCCACTCAAGGGCAAGATGATAAGGTTCTATCAGACCAACCGCTGAACAGGAC
m  CTCTACTAACTCAAGGGCGAGATGATAAGGTTCTATCAGACCAACTGTTGAACAGAAC

                                NREa
h  CTGAGACTCTACCAAGGTCAGAAATGCTGCAATTCAAGCCAAAAGATCTTCTTGGGCTT
r  CTGAGTCTC--CCAAGGTCA-----T
m  CTGAGCCTC--CCAAGGTCA-----T

                                AP-3
h  CCTTGTTTGGACTTGTAACCATAAATTAGTCTTGCCCTAAATGTCTGATCACATATAAAA
r  CCTTGTTTGGACTTGTAACCACAAATTTGTCTTGCCCT---TG-----TCAC--TATAAAA
m  CCTTGTTTGGACTTGTAACCACAAATTTGTCTTGCTAAGTGTCCAATCAC--TATAAAA

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Fig. 4. Alignment of the nucleic acid sequences of the proximal promoter region of the human (h), rat (r), and mouse (m) aromatase genes

Bolded sequences indicate identical nucleotides among species. Solid lines indicate transcription factors' binding sequences. TATA binding sites are indicated by boxes.

Fig. 5. Dynamics of the activation of the aromatase promoter in ovarian cells

Aromatase mRNA levels and DNA protein binding in 26-day-old immature rats (d26), immature rats treated with PMSG (PMSG), and from corpora lutea of rats on days 4 (d4), 15 (d15), or 23 (d23) of pregnancy. Aromatase mRNA levels were determined as in figure 3 and expressed as the ratio between aromatase and ribosomal L19 mRNA. DNA protein binding was investigated using gel shift analyses with oligonucleotides spanning the cAMP response element-like sequence (CLS), the nuclear receptor elements a and b (NREa and NREb), the GATA binding sites, or the AP-3 binding site. Only shifted bands are shown.

Fig. 6. Scheme depicting hypothetical multiunit complexes on the aromatase promoter in granulosa and luteal cells

The participation and binding of CREB, GATA-4, β-catenin and AP-3 have been shown using *in vitro* approaches such as gel shift and gene reporter assays. The involvement of SF-1 has been inferred from knockout and over-expression experiments. Interactions between CREB, SF-1 and GATA-4 with CBP are deduced from experiments in cells other than granulosa or luteal cells. The spatial binding of transcription factors on the DNA does not necessarily represent an *in vivo* situation but highlights the complexity of this promoter and the differences between granulosa and luteal cells on the activation of the aromatase gene. GTM= general transcriptional machinery; TBP: TATA binding protein; RNApol: RNA polymerase. See the text for more details.

Table I

Compounds that modulate the induction of aromatase expression by FSH.

Compound	Effect	Species	Reference
Glucocorticoids	Inhibition	Rat	[127]
Prolactin	Inhibition	Rat	[128,129]
Progestins	Inhibition	Rat	[130,131]
TGF β	Enhanced	Rat	[132]
TNF α	Attenuates stimulates attenuates/stimulates	Rat Pig Human	[133–135] [136] [137]
Inhibin	Inhibition	Rat	[132]
T3 — T4	Inhibition	Pig Mouse	[138] [139]
EGF	Attenuates	Rat	[140]
Aminoglutetamide	Enhances	Rat	[141]
Cyclosporin A	Enhances	Rat	[142]
Transferrin	Attenuates	Rat	[143]
Gossypol	Attenuates	Pig	[144]
Mono-(2-ethylhexyl) phthalate	Attenuates	Rat	[145]
Antioxidants (SOD)	Attenuates	Rat	[146]
Leptin	Enhances inhibition	Human Rat	[147] [148]
NO/cGMP	Attenuates	Rat	[149]

TGF- β : Transforming growth factor- β ; TNF- α : Tumor necrosis factor- α ; T3 and T4: triiodothyronine and thyroxine; EGF: epidermal growth factor; SOD: superoxide dismutase; NO: Nitric Oxide