Regulation of Aromatase Expression in Estrogen-Responsive Breast and Uterine Disease:
From Bench to Treatment

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Abstract—A single gene encodes the key enzyme for estrogen biosynthesis termed aromatase, inhibition of which effectively eliminates estrogen production. Aromatase inhibitors successfully treat breast cancer and endometriosis, whereas their roles in endometrial cancer, uterine fibroids, and aromatase excess syndrome are less clear. Ovary, testis, adipose tissue, skin, hypothalamus, and placenta express aromatase normally, whereas breast and endometrial cancers, endometriosis, and uterine fibroids overexpress aromatase and produce local estrogen that exerts paracrine and intracrine effects. Tissue-specific promoters distributed over a 93-kilobase regulatory region upstream of a common coding region alternatively control aromatase expression. A distinct set of transcription factors regulates each promoter in a signaling pathway- and tissue-specific manner. Three mechanisms are responsible for aromatase overexpression in a pathologic tissue versus its normal counterpart. First, cellular composition is altered to increase aromatase-expressing cell types that use distinct promoters (breast cancer). Second, molecular alterations in stromal cells favor binding of transcriptional enhancers versus inhibitors to a normally quiescent aromatase promoter and initiate transcription (breast/endometrial cancer, endometriosis, and uterine fibroids). Third, heterozygous mutations, which cause the aromatase coding region to lie adjacent to constitutively active cryptic promoters that normally transcribe other genes, result in excessive estrogen formation owing to the overexpression of aromatase in many tissues.

I. Introduction

A. The Aromatase Enzyme

The initial entry of cytosolic cholesterol into the mitochondrion, which is facilitated by steroidalogenic acute regulatory protein (STAR1), represents a major step for steriodogenesis. Six enzymes encoded by at least five specific genes then catalyze the conversion of cholesterol to the biologically active estrogen estradiol. The aromatase enzyme catalyzes the final and key step, i.e., the conversion of C19 steroids to estrogens (Fig. 1).

The aromatase enzyme is localized in the endoplasmic reticulum of estrogen-producing cells (Sebastian and Bulun, 2001; Simpson et al., 2002). Aromatase enzyme complex is comprised of two polypeptides. The first of these is a specific cytochrome P450 (P450), namely aromatase cytochrome P450 (P450arom) (the product of the CYP19 gene) (Simpson et al., 2002). The second is a flavoprotein, NADPH-cytochrome P450 reductase and is ubiquitously distributed in most cells. Thus, cell-specific expression of P450arom determines the presence or absence of aromatase activity. For practical purposes, we will refer to “P450arom” as “aromatase” throughout this text. Since only a single gene (CYP19) encodes aromatase in mice and humans, targeted disruption of this gene or inhibition of its product effectively eliminates estrogen biosynthesis in these species (Simpson et al., 2002).

In the human, aromatase is expressed in a number of cells, including the ovarian granulosa cell, the placental syncytiotrophoblast, the testicular Leydig cell, and various extraglandular sites, including the brain and skin.
fibroblasts (Simpson et al., 1994). The principal product of the ovarian granulosa cells during the follicular phase is estradiol (E2). Additionally, aromatase is expressed in human adipose tissue. Whereas the highest levels of aromatase are in the ovarian granulosa cells in premenopausal women, the adipose tissue becomes the major aromatase-expressing body site after menopause (Fig. 2) (Grodin et al., 1973; Bulun and Simpson, 1994). Although aromatase level per adipose tissue fibroblast may be small, the sum of estrogen arising from billions of adipose tissue fibroblasts in the entire body makes a physiologic impact. The principal product of the ovary is the potent estrogen estradiol. In adipose tissue, estrogenically weak estrone is produced from androstenedione of adrenal origin in relatively large quantities. However, at least half of this peripherally produced estrone is eventually converted to estradiol in extraovarian tissues (Fig. 2) (MacDonald et al., 1979).

B. The Aromatase (CYP19) Gene

The recent availability of the Human Genome Project on the Internet permitted us to reconstruct the entire aromatase gene by computer-assisted analysis (Sebastian and Bulun, 2001) (Fig. 3A). Two overlapping bacterial artificial chromosome clones contained the entire gene, which spans approximately 123 kb (Sebastian and Bulun, 2001) (Fig. 3A). The gene is transcribed from the telomere to the centromere, and the region encoding the aromatase protein spans 30 kb of the 3′-end and contains nine exons (II-X) (Shozu et al., 2003b). The ATG translation start site is located in coding exon II. The upstream (telomeric) 93 kb of the gene contains a number of promoters (Simpson et al., 1994; Sebastian and Bulun, 2001). The most proximal gonad-specific promoter II and two other proximal promoters, I.3 (expressed in adipose tissue and breast cancer) and I.6 (expressed in bone), are found within the 1-kb region upstream of the ATG translation start site in exon II, as expected (Fig. 3A). Promoter I.2, the minor placenta-specific promoter, is located approximately 13 kb upstream of the ATG site in exon II. The promoters specific for the brain (I.f), endothelial cells (I.7), fetal tissues (I.5), adipose tissue (I.4), and placenta (I.1) are localized in tandem order at 33, 36, 43, 73, 78, and 93 kb, respectively, upstream of the first coding exon, the exon II (Fig. 3A) (Mahendroo et al., 1993; Sebastian and Bulun, 2001). In addition to promoter II-specific sequences, transcripts containing two other unique sequences, untranslated exons I.3 and I.4, are present in adipose tissue and in adipose tissue fibroblasts maintained in culture (Mahendroo et al., 1993). Transcription initiated by use of each promoter gives rise to a transcript with a unique 5′-untranslated end that contains the sequence encoded in the first exon immediately downstream of this particular promoter (Fig. 3B). Therefore, the 5′-untranslated region of aromatase mRNA is promoter-specific and may be viewed as a signature of the particular promoter used. It should be emphasized again that all of these 5′-ends are spliced onto a common junction 38 bp upstream of the ATG translation start site (Mahendroo et al., 1993). Consequently, the sequence encoding the open reading frame is identical in
CYP19 (aromatase) GENE

A TISSUE AROMATASE mRNA DETECTED

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The placental promoter I.1 is the most distally located promoter (93 kb), which gave rise to splicing of a 103-bp first exon onto the common splice junction immediately upstream of the ATG translational start site (Kamat et al., 1998; Jiang et al., 2000; Jiang and Mendelson, 2003) (Fig. 3A). The activity of promoter I.1 is the basis for 100 to 1000 times elevated levels of circulating estrogen in pregnant women (Kamat et al., 1998; Jiang et al., 2000; Jiang and Mendelson, 2003). Thus, recruitment of this promoter may have an evolutionary impact, since, of all species, humans are unique to acquire and maintain extraordinarily high levels of aromatase expression in placenta.

A transgene containing the human genomic region upstream of the placental exon I.1 was found to exhibit readily detectable promoter activity in the mouse placenta (Kamat et al., 2002). Thus, although mouse placental tissue does not express aromatase endogenously, it may contain the necessary transcriptional factors for human promoter I.1 expression. This suggests that the recruitment of aromatase expression in human placenta occurred via alterations in the mammalian genome throughout the evolution. One of the key mechanisms that permits the recruitment of such a large number of promoters seems to be the extremely promiscuous nature of the common splice acceptor site, since activation of each promoter gives rise splicing of an untranslated first exon onto this common junction immediately upstream of the translation start site in the coding region.

C. Physiologic Regulation of Aromatase Expression in Human Tissues

The primary site of aromatase expression in premenopausal women is the ovarian follicle, where FSH induces aromatase and thus estradiol production in a cyclic fashion (Simpson et al., 1994). Ovarian aromatase expression is mediated primarily by FSH receptors, cAMP production and activation of the proximal promoter II (Simpson et al., 1994) (Fig. 4). Men and postmenopausal also produce estrogen by aromatase that resides in extragonadal tissues such as adipose tissue and skin (Simpson et al., 1994) (Fig. 4). Estrogen produced in these extragonadal tissues are of paramount importance for the closure of bone plates and bone mineralization in both men and postmenopausal women, since the phenotype of men with defective genes of aromatase or estrogen receptor (ER)-α include severe osteoporosis and extremely tall stature with growth into adulthood (Bulun, 2000). A distal promoter (I.4) located 70 kb upstream of the coding region directs aromatase expression in adipose tissue and skin fibroblasts. Promoter I.4 in these tissues is regulated by combined action of a glucocorticoid and a member of the class I cytokine family [e.g., interleukin (IL)-6, IL-11, leukemia inhibitory factor, and oncostatin-M] (Fig. 4) (Zhao et al., 1995).

The alternative use of promoters comprises the basis for differential regulation of aromatase expression by
studies showed that prostaglandin (PG) E₂ function of hormonal treatments. For example, in vitro (Zhao et al., 1995). Transcription (STAT)-3 pathway mediate this induction of adipose and skin fibroblasts via promoter I.4 located 73 kb upstream of the coding region. Binding of STAT-3 and glucocorticoid receptor (GR) upstream of promoter I cytokine family induces aromatase expression in skin and adenose tissue fibroblasts. Adipose tissue fibroblasts express promoter I.4 cytokine family switches promoter use to I.4 (Zhao et al., 1995; Zhou et al., 2001). At the same time, a separate set of promoters II and I.3, whereas treatment with a glucocorticoid plus a member of the class I cytokine family maintains constitutively in the syncytiotrophoblast (Kamat et al., 1998; Jiang et al., 1999, 2003b). This gives rise to increased circulating estrogen and both local and systemic (endocrine) effects of estrogen such as gynecomastia and hypogonadism in males and premature breast development and anovulation in females.

II. Aromatase and Breast Cancer

Paracrine interactions between malignant breast epithelial cells, proximal adipose fibroblasts, and vascular endothelial cells are responsible for estrogen biosynthesis and the lack of adipogenic differentiation in breast cancer tissue. It seems that malignant epithelial cells secrete factors that inhibit the differentiation of surrounding adipose fibroblasts to mature adipocytes and stimulate aromatase expression in these undifferentiated adipose fibroblasts (Meng et al., 2001). The in vivo presence of malignant epithelial cells also enhances aromatase expression in endothelial cells in breast tissue (Zhou et al., 2001). We developed a model in breast cancer, which reconciles the inhibition of adipogenic differentiation and estrogen biosynthesis in a positive feedback cycle (Fig. 5).

The desmoplastic reaction (formation of the dense fibroblast layer surrounding malignant epithelial cells) is essential for structural and biochemical support for tumor growth. In fact, the pathologists refer to 70% of breast carcinomas as “scirrhous”-type, indicating the rock-like consistency of these tumors (Haagensen, 1986). This consistency comes from the tightly packed undifferentiated adipose fibroblasts around malignant epithelial cells. Malignant epithelial cells achieve this by secreting large quantities of TNF and IL-11 that inhibit the differentiation of fibroblasts to mature adipocytes. Thus, large numbers of these estrogen-producing cells are maintained proximal to malignant cells (Meng et al., 2001; Zhou et al., 2001). At the same time, a separate set of factors secreted by malignant epithelial cells activates aromatase expression in surrounding adipose fibroblasts (Meng et al., 2001; Zhou et al., 2001) (Fig. 5).
Malignant epithelial cells induce aromatase via activation of aberrant promoters in breast cancer tissue and adipose fibroblasts proximal to tumor (Fig. 6). The breast adipose tissue in disease-free women maintains low levels of aromatase expression primarily via promoter I.4 that lies 73 kb upstream of the common coding region (Mahendroo et al., 1993) (Fig. 6). The proximal promoters I.3 and II are used only minimally in normal breast adipose tissue (Mahendroo et al., 1993). Transcription via activity of promoters II and I.3 in the breast tumor fibroblasts and malignant epithelial cells, however, is strikingly increased (Harada et al., 1993; Agarwal et al., 1996; Utsumi et al., 1996; Zhou et al., 1996) (Fig. 6). Additionally, the endothelial-type promoter I.7 is also up-regulated in breast tumor tissue (Sebastian et al., 2002) (Fig. 6). Thus, it seems that the prototype estrogen-dependent malignancy breast cancer takes advantage of four promoters used in various cell types for aromatase expression (Fig. 6). The sum of aromatase mRNA species arising from these four promoters markedly increases total aromatase mRNA levels in breast cancer compared with the normal breast that uses almost exclusively promoter I.4 (Fig. 6).

A. Estrogen Formation in Breast Cancer

There are two sources of estrogen for breast cancer. First, estrogen that arises from extraovarian body sites such as subcutaneous adipose tissue and skin reaches breast cancer by way of circulation in an endocrine manner. Second, estrogen locally produced in breast cancer tissue makes an impact via paracrine or intracrine mechanisms.

1. Aromatase in Adipose Tissue and Skin: Endocrine Effect. The potential roles of extraovarian aromatase in human physiology and pathology were also recognized initially in the 1960s (MacDonald et al., 1968). These studies demonstrated that the conversion rate of plasma androstenedione to estrone in humans increased as a function of obesity and aging (Grodin et al., 1973; Hemsell et al., 1974) (Fig. 2). These studies also revealed the importance of extraovarian tissues (primarily adipose tissue and skin) as the origin of estrogen in postmenopausal women (Figs. 2 and 4). Extraovarian estrogen formation was shown to be correlated positively with excess body weight in both pre- and postmenopausal women and may be increased as much as 10-fold in morbidly obese postmenopausal women (Grodin et al., 1973; Hemsell et al., 1974). This elevation in association with both obesity and aging bears a striking relationship to the incidence of endometrial hyperplasia and cancer, which are more commonly observed in elderly obese women (MacDonald et al., 1978). It is now recognized that the continuous production of estrogen by the adipose tissue in these women is one of the risk factors of endometrial hyperplasia and cancer.

Evidence also suggests a role of estrogens produced by adipose tissue in the pathogenesis of the breast cancer. For example, breast cancer incidence correlates positively with the body fat content or serum estradiol levels in postmenopausal women, suggesting that estrogen collectively produced in all extraovarian sites reach the
breast tissue by circulation in an endocrine fashion and stimulate tumor growth (Huang et al., 1997; Hankinson et al., 1999). A role for adipose tissue estrogen biosynthesis in promoting the growth of breast cancer is implied because of the palliative effects of adrenalectomy in the past. Because estrone production by adipose tissue depends on plasma androstenedione secreted by the adrenal cortex as substrate, the role of adrenalectomy is explicable in terms of the denial of substrate precursor for adipose tissue estrogen biosynthesis. Today, reduction of estrogen biosynthesis in the adipose tissue is accomplished by the use of aromatase inhibitors in the treatment of breast cancer (Brodie et al., 1999a,b) (Fig. 7).

2. Local Aromatase in Breast Cancer Tissue: Paracrine/Intracrine Effect. Mechanisms giving rise to increased local concentrations of estrogen in breast cancer via aromatase overexpression within the tumor tissue have been demonstrated by a number of investigators (Van Landeghem et al., 1985; Chetrite et al., 2000; Geisler et al., 2000). These studies showed strikingly increased local levels of estrone, estrone sulfate, and estradiol in breast tumor tissue compared with circulating estrogen levels (Van Landeghem et al., 1985; Chetrite et al., 2000; Geisler et al., 2000).

A series of paracrine interactions between malignant breast epithelial cells and surrounding adipose stroma were uncovered and explained increased local estrogen levels in the breast bearing a cancer. For example, independent studies from at least six different laboratories indicated striking increases in aromatase enzyme activity and mRNA levels in breast fat adjacent to cancer compared with those in distal fat or disease-free breast adipose tissue (O’Neill et al., 1988; Bulun et al., 1993; Harada et al., 1993; Reed et al., 1993; Sasano et al., 1994; Utsumi et al., 1996; Zhou et al., 1996) (Fig. 7). We also found that an elevation in aromatase expression in adipose stroma surrounding malignant epithelial cells is regulated by complex cellular, molecular, and genomic mechanisms (Bulun et al., 1993; Agarwal et al., 1996; Zhou et al., 2001). Interestingly, the overall aromatase expression in breast adipose tissue in mastectomy specimens bearing a breast tumor was significantly higher than that in benign breast tissue removed for reduction mammoplasty (Agarwal et al., 1995, 1996, 1997).

Estrogens can act both directly or indirectly on human breast cancer cells to promote proliferation. Breast cancer cells in culture elaborate a number of growth stimulants in response to estrogen, which can act in an autocrine and paracrine manner to stimulate their growth; however, there is also evidence that estrogens can directly induce proliferation of breast cancer cells. The pathologic significance of local aromatase activity in breast cancer was recognized based on the following in vitro data. MCF7 breast cancer cells, which were stably transfected to express an mouse mammary tumor virus-promoter-driven human aromatase cDNA and inoculated into oophorectomized nude mice, remained dependent on circulating androstenedione for their rapid growth (Yue et al., 1994). Further evidence for the importance of local aromatase expression in the breast tissue came from an in vivo mouse model demonstrating that aromatase overexpression in breast tissue is sufficient for maintaining hyperplasia in the absence of circulating estrogen and that aromatase inhibitors abrogated hyperplasia (Tekmal et al., 1999). These transgenic mice with mouse mammary tumor virus-promoter-driven local aromatase in breast tissue are more prone for breast cancer development (Kovacic et al., 2004).

B. Cellular Mechanisms That Regulate Aromatase Expression in Normal and Malignant Breast Tissue

1. Cellular Localization of Aromatase in Breast Cancer. Breast adipose tissue is primarily composed of mature lipid-containing cells and other stromal elements. This latter group of cells in the breast adipose tissue was characterized using immunohistochemical methods (Price et al., 1992). Ninety percent of these resident cells of adipose tissue are fibroblasts, i.e., the potential precursors of mature adipocytes, and another 7% represented endothelial cells. Most (80–90%) aromatase transcripts in adipose tissue were demonstrated to reside in fibroblasts compared with mature adipocytes (Price et
Moreover, aromatase enzyme activity was found to reside primarily in the fibroblast component of the adipose tissue in a previous study from the same laboratory (Ackerman et al., 1981). Immunoreactive aromatase was localized to both the malignant epithelial cells and surrounding fibroblasts in breast tumor tissues (Santen et al., 1994; Sasano et al., 1996; Brodie et al., 1998). Different antibodies, however, showed variable affinity to malignant epithelial cells versus fibroblasts (Sasano et al., 2003). Immunoreactive aromatase was also observed in endothelial cells in normal breast tissue and breast tumors. Recently published data using RNA from laser-captured breast tumor cells showed aromatase mRNA in both stromal and malignant epithelial cells in tumor tissues from three patients (Sasano et al., 2003). Markedly high levels of aromatase enzyme activity have been consistently detected in breast adipose fibroblasts freshly isolated from breast tissue with or without cancer (Ackerman et al., 1981; Price et al., 1992). Aromatase enzyme activity in malignant breast epithelial cells, on the other hand, is either undetectable or extremely low (Pauley et al., 2000).

Adjacent adipose tissue including the dense fibroblast layer seems to account for most aromatase expression in breast tumors for the following reasons. First, the quantity of adjacent adipose tissue surrounding a clinically detectable breast tumor is comparatively very large; e.g., the volume of adipose tissue within a 1-inch radius of a 1-ml breast tumor is 129 ml. Second, the most intense aromatase immunostaining was observed in the adipose tissue fibroblasts located in and around the fibrous capsule (i.e., desmoplastic reaction) surrounding malignant cells (Sasano et al., 1994). Third, levels of aromatase expression and activity in fibroblasts isolated from breast adipose tissue or tumor are 10 to 15 times higher than those found in malignant epithelial cells or cell lines (Pauley et al., 2000).

2. Inhibition of Adipogenic Differentiation in Breast Cancer: Link to Aromatase Overexpression. Extraordinarily large quantities of TNF and IL-11 are produced and secreted by malignant breast epithelial cells (Meng et al., 2001) (Fig. 8). These two cytokines mediate one of the most commonly observed biological phenomena in breast tumors, the desmoplastic reaction. Desmoplastic reaction or accumulation of fibroblasts around malignant epithelial cells serves to maintain the strikingly hard consistency in many of these tumors (i.e., the traditional macroscopic description of malignant breast tumors as “scirrhous cancer”) and increased local concentrations of estrogen via aromatase overexpression localized to these undifferentiated fibroblasts. The inhibition of differentiation of fibroblasts to mature adipocytes mediated by TNF and IL-11 is the key event responsible for desmoplastic reaction, because neither malignant cell-conditioned media nor these cytokines caused the proliferation of adipose tissue fibroblasts (Meng et al., 2001). Moreover, blocking both TNF and IL-11 in cancer cell-conditioned media using neutralizing antibodies is sufficient to reverse this antidifferentiative effect of cancer cells completely (Fig. 8) (Meng et al., 2001). In summary, desmoplastic reaction primarily occurs via the action of cytokines (TNF and IL-11) secreted by the malignant epithelial cells to inhibit the differentiation of adipose tissue fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of expression of the essential adipogenic transcription factors C/EBPα and PPARγ (Meng et al., 2001) (Fig. 8).

Estrogen per se seems to potentiate this antiadipogenic action via indirect mechanisms. For example, treatment of T47D breast cancer cells with estradiol increased the mRNA levels of IL-11 by 3-fold (Crichton et al., 1996). Moreover, the cellular actions of TNF are mediated by two distinct receptors: TNF receptor type 1 (TNFR1) (also known as p60 in humans) and TNFR2 (p80). TNFR1 but not TNFR2 was found to be responsible for the inhibition of adipocyte differentiation using mutants of TNF specific for the stimulation of either receptor type (Huber and Hauner, 2000). We recently demonstrated that TNFR1 is responsible for inhibition of adipocyte differentiation in breast cancer (Deb et al., 2004). Interestingly, estradiol enhances this antiadipogenic effect by inducing TNFR1 levels in adipose fibroblasts (Deb et al., 2004) (Fig. 8).

Thus, large amounts of antiadipogenic cytokines (e.g., TNF and IL-11) secreted by malignant epithelial cells serve to maintain increased numbers of the aromatase-
expressing cell type, i.e., undifferentiated adipose fibroblast, in breast tumor tissue. This is further enhanced by stimulatory effects of estrogen on IL-11 production in cancer cells and on the TNF receptor type that mediates adipogenic inhibition (Fig. 8).

C. Molecular Mechanisms Responsible for Elevated Aromatase Expression in Breast Cancer

Alternative promoter use is a major mechanism that mediates increased aromatase expression in breast cancer. The normal breast adipose tissue maintains low levels of aromatase expression primarily via promoter I.4 that lies 73 kb upstream of the common coding region (Fig. 6). The proximally located promoters I.3 and II are used only minimally in normal breast adipose tissue. Promoter II and I.3 activities in the breast cancer, however, are strikingly increased (Harada et al., 1993; Agarwal et al., 1996; Utsumi et al., 1996; Zhou et al., 1996). Additionally, the endothelial-type promoter I.7 is also up-regulated in breast cancer (Sebastian et al., 2002). Thus, it seems that the prototype estrogen-dependent malignancy breast cancer takes advantage of four promoters (II, I.3, I.7, and I.4) for aromatase expression (Fig. 6). The sum of aromatase mRNA species arising from these four promoters markedly increases total aromatase mRNA levels in breast cancer compared with the normal breast that uses almost exclusively promoter I.4 (Fig. 6).

1. Up-Regulation of Promoters I.3 and II. Using an in vivo approach, we and two other groups demonstrated by quantitative exon-specific RT-PCR that the use of the proximal promoters II/I.3 are strikingly up-regulated in adipose tissue adjacent to breast cancer and in breast cancer tissue per se (Agarwal et al., 1996; Utsumi et al., 1996; Zhou et al., 1996). As noted earlier, promoters II and I.3 are located within 215 bp from each other and are coordinately induced by cAMP-dependent or -independent mechanisms in adipose fibroblasts in breast tumors. These promoters possibly share common regulatory DNA motifs.

Increased promoter I.3/II activity is, in part, the basis for increased aromatase expression in peri- and intratumoral adipose tissue fibroblasts (Fig. 6) (Agarwal et al., 1996). Over the past several years, we and others sought to elucidate the mechanisms underlying this cancer-induced increase in promoters I.3/II activity in adipose tissue fibroblasts.

There are two experimental models, which are not mutually exclusive, for the regulation of promoters I.3 and II in breast adipose fibroblasts in tumor tissue. In the first model, PGE$_2$ induces aromatase via promoters I.3 and II employing both cAMP/protein kinase A- and protein kinase C-dependent pathways (Zhao et al., 1996a). In this model, PGE$_2$ stimulates binding activity of an orphan nuclear receptor, LRH-1, to a nuclear receptor half-site (~136/~124 bp) upstream of promoter II (Clyne et al., 2002; Karuppu et al., 2002). Treatment with PGE$_2$ strikingly increased both LRH-1 expression and its binding activity to the aromatase promoter II in cultured adipose fibroblasts. LRH-1 overexpression significantly increased aromatase promoter II activity and aromatase enzyme activity in cultured adipose fibroblasts (Fig. 9). From an in vivo perspective, LRH-1 was up-regulated in undifferentiated fibroblasts in breast tumor tissue compared with those in disease-free breast tissue (C. Clyne, personal communication). It was suggested that a corepressor of LRH-1, short heterodimer partner (SHP), may inhibit aromatase expression in fibroblasts of normal breast tissue (Kovacic et al., 2004) (Fig. 9). The recently reported increases in cyclooxygenase (COX)-2 expression and beneficial effects of nonsteroidal anti-inflammatory in breast cancer support this model (Brueggemeier et al., 1999; Richards et al., 2002). In vivo evidence for increased PGE$_2$ formation in breast cancer, however, is still lacking at this time.

In an alternative experimental model, we found that conditioned medium from malignant breast epithelial cells (MCF7 or T47D) markedly induces aromatase expression in adipose tissue fibroblasts via promoters I.3 and II (Zhou et al., 2001) (Fig. 9). We hypothesize that a hormonal cocktail secreted from malignant epithelial cells induces aromatase in undifferentiated adipose fibroblasts via redundant pathways. For example, the addition of a COX-2 inhibitor or an adenylyl cyclase inhibitor or mutation of the LRH-1 binding site of the aromatase promoter II does not reverse induction of aromatase expression by malignant epithelial cell-conditioned medium. We isolated two cis-acting elements...
that stimulated promoter I.3/II use in response to cancer cell-conditioned medium (Zhou et al., 2001). Two critical elements were determined as a C/EBP site (−317/−304) and a CRE (−211/−197), since mutation of either element abolished conditioned medium-induced promoter activity (Fig. 9). Malignant epithelial cell-conditioned medium strikingly induced the expression of C/EBPβ, which binds to the C/EBP site in promoter I.3/II region and increases its activity (Zhou et al., 2001) (Fig. 9). In contrast, promoter I.3 and II are inhibited by transcription factors C/EBPα and δ that bind to the same site in adipose fibroblasts treated with benign epithelial cell-conditioned medium.

Malignant epithelial cell-conditioned medium also induced phosphorylation of ATF-2 and its binding to the CRE (Fig. 9). This CRE is occupied by nonphosphorylated ATF-2 in fibroblasts treated with benign epithelial cell-conditioned medium associated with inactivation of promoters I.3 and II. Moreover, chromatin immunoprecipitation PCR showed that the activator transcriptional complex in a malignant environment contains C/EBPβ, phosphorylated ATF-2, and p300/cAMP response element-binding protein-binding protein (CBP), whereas the inactivator complex (benign environment) contained nonphosphorylated ATF-2 and histone deacetylase-1 (HDAC1) (Fig. 9). Although this second model reflects in vivo conditions, the individual factors in malignant cell-conditioned medium, which contribute to up-regulation of aromatase, are not currently known. Cytokines in malignant cell-conditioned medium probably do not account for this up-regulation, since the TNF and IL-6 group of cytokines selectively induce only promoter I.4, which is not up-regulated either in vivo or in vitro in breast tumors (Zhao et al., 1995, 1996b; Agarwal et al., 1996; Zhou et al., 2001).

A unified model for promoter II/I.3 activation in breast cancer therefore predicts that malignant epithelial cells secrete a number of factors including PGE₂ (Fig. 9). These factors induce a number of signaling pathways in a redundant fashion to activate the transcription of the aromatase gene via promoter I.3/II in adipose fibroblasts. PGE₂ possibly arising from malignant epithelial cells is a candidate factor for activation of promoters I.3 and II in breast cancer. This, however, has not been demonstrated in vivo. Neither PGE₂ nor its downstream regulators cAMP or LRH-1-binding site in promoter II were found to be essential for activation of promoters II in adipose fibroblasts treated with malignant cell-conditioned medium (Zhou et al., 2001). In disease-free breast tissue, the incorporation of a number of transcriptional repressors into the multimeric complex that occupies promoter I.3/II region is associated with inhibition of transcription. Malignant epithelial cell-conditioned medium, on the other hand, gives rise to replacement of this inhibitory complex by an activator transcriptional complex composed of distinct factors such as phosphorylated ATF-2, C/EBPβ, p300/CBP, and possibly LRH-1 (our unpublished observations) (Fig. 9).

In summary, the proximal promoters II and I.3 clustered within a 215-bp region are coordinately regulated. They remain quiescent in fibroblasts of normal breast tissue via redundant binding of multiple transcriptional inhibitors (Fig. 9). In a malignant breast environment, however, these promoter regions are occupied by multiple transcriptional enhancers as a result of activation of multiple signaling pathways in a fail-safe fashion to increase aromatase expression in breast fibroblasts (Fig. 9).

2. Regulation of Promoters II and I.3 in MCF7 Cells. Chen et al. (2002) studied the regulation of a number of gene reporter constructs of the promoter II/I.3 region. They found that estrogen receptor-related α-1 and CREB1 up-regulate and v-erbA-related factor-2, chicken ovalbumin upstream transcription factor (COUP-TF) inhibitor, retinoic acid receptor γ, Snail, and Slug proteins down-regulate this promoter region (Chen et al., 2002). The in vivo relevance of these findings will become clearer in the future once the relative significance of aromatase enzyme activity and estrogen biosynthesis is demonstrated in malignant epithelial cells.

3. Up-Regulation of Promoter I.7 in Breast Cancer. Studies summarized above employed exon-specific RT-PCR analysis of 5′-untranslated ends of aromatase mRNA in breast cancer tissues. This limited strategy permitted only the detection of promoters previously identified from healthy tissues (Agarwal et al., 1996; Utsumi et al., 1996; Zhou et al., 1996). Discovery-driven approaches designed to identify novel promoter regions in breast cancer or adjacent adipose tissues, however, have not been published until recently. To identify novel promoter regions in cancer tissues and proximal fat, we employed the 5′-rapid amplification of cDNA ends (RACE) procedure using total RNA isolated from breast cancer and proximal adipose tissue samples. We cloned a novel 101-bp untranslated first exon (I.7) that comprises the 5′-end of 29 to 54% of aromatase mRNA isolated from breast cancer tissues (Sebastian et al., 2002) (Fig. 10). The levels of aromatase mRNA with exon I.7 were significantly increased in breast cancer tissues and adipose tissue adjacent to tumors (Fig. 10). We identified a promoter immediately upstream of exon I.7 and mapped this to about 36 kb upstream of the ATG translation start site of the aromatase gene (Sebastian et al., 2002) (Fig. 10). Promoter I.7 is a TATA-less promoter containing cis-regulatory elements found in megakaryocytic and endothelial type promoters (Fig. 10). Maximal promoter activity could be demonstrated in human microvascular endothelial cells (Fig. 10). Binding of the transcription factor GATA-2 to a specific GATA cis-regulatory element in this promoter was critical for its regulation in endothelial cells (Sebastian et al., 2002). In conclusion, promoter I.7 is a GATA-2-regulated endothelial-type promoter of the human aro-
matase gene and may increase estrogen biosynthesis in vascular endothelial cells of breast cancer. The activity of this promoter may also be important for intracrine and paracrine effects of estrogen on blood vessel physiology (Fig. 10).

D. Summary of Regulation of Aromatase Expression in Breast Cancer

Several alternative cellular and molecular mechanisms serve to maintain excessive levels of aromatase activity in breast stroma proximal to malignant epithelial cells. First, malignant epithelial cell-derived factors induce aromatase overexpression via the transcription factors LRH-1, C/EBPβ, and phosphorylated ATF-2 (Fig. 5). These factors are incorporated into a multimeric transcriptional complex that occupies the aromatase promoter. Second, aromatase is overexpressed in vascular endothelial cells of tumor tissue via binding of GATA-2 and other endothelial-type transcription factors to promoter I.7. These factors may also mediate angiogenesis in tumor tissue. Moreover, estrogen is known to induce the angiogenic factor VEGF in cancer cells (Nakamura et al., 1996, 1999; Ruohola et al., 1999; Shekhar et al., 2000) (Fig. 5). Third, we demonstrated recently that antiadipogenic cytokines IL-11 and TNF secreted by malignant epithelial cells block the differentiation of the aromatase-expressing cells (fibroblasts) to mature adipocytes that do not express aromatase (Fig. 5). These cytokines thereby secreted abundantly by malignant epithelial cells serve to maintain a dense layer of aromatase-expressing fibroblasts proximal to malignant epithelial cells to provide structural and hormonal support. Fourth, the expression of IL-11 in malignant epithelial cells and antiadipogenic-type TNF receptors in adjacent adipose tissue fibroblasts are up-regulated by estrogen produced as a consequence of elevated aromatase activity in breast tumors. This positive feedback involving complex epithelial-stromal interactions favor higher numbers of undifferentiated fibroblasts, angiogenesis, and increased local estrogen concentrations in breast tumors (Fig. 5). These four mechanisms interact to maintain high levels of estrogen production in a breast tumor.

E. Aromatase Inhibitors in the Treatment of Breast Cancer

Today, aromatase inhibitors are the most effective endocrine treatment of estrogen-responsive breast cancer (Santen, 2002) (Fig. 7). Six recent head-to-head randomized clinical trials published since 2000 demonstrated the superiority of aromatase inhibitors to tamoxifen in the treatment of breast cancer (Bonneterre et al., 2000; Mouridsen et al., 2001; Baum et al., 2002, 2003; Goss et al., 2003; Milla-Santos et al., 2003; Paridaens et al., 2003; Buzdar et al., 2004). Long-term side effect profiles of these agents will determine whether aromatase inhibitors will replace tamoxifen or other selective estrogen receptor modulators in the long run. There are two intriguing implications of these results. First, it is pharmacologically more efficacious to block estrogen formation rather than its action at least by currently approved estrogen antagonists or selective estrogen receptor modulators. Second, the local effect of aromatase inhibitors at the target tissue level to block local estrogen formation possibly represents the most critical mechanism for the superior therapeutic potential of aromatase inhibitors (Fig. 7). Targeting aromatase in breast cancer as a therapeutic strategy was first conceptualized in the 1960s (Santen, 2002). Aminoglutethimide was the first aromatase inhibitor tested for this purpose. Although the first-generation aromatase inhibitor aminoglutethimide was as efficacious as tamoxifen in the treatment of breast cancer, its adverse side effects precluded widespread use (Santen, 2002). Tamoxifen was introduced in the 1970s and became the gold standard for hormonal treatment of breast cancer (Santen, 2002). Second-generation aromatase inhibitors were tested in Europe in the 1980s and were found to be as efficacious as tamoxifen (Santen, 2002). Finally, the third-generation aromatase inhibitors were approved in the United States to treat postmenopausal breast cancer in the 1990s and proven to be superior to tamoxifen (Lu et al., 1998; Bonneterre et al., 2000; Dixon et al., 2000; Mouridsen et al., 2001; Baum et al., 2002, 2003; Goss et al., 2003; Milla-Santos et al., 2003; Paridaens et al., 2003; Buzdar et al., 2004). These new inhibitors have a benign side-effect profile...
and suppress estrogen production in extraovarian tissues and within the breast cancer tissue itself. This effectively blocks estrogenic action, reduces recurrences, and prolongs disease-free survival in postmenopausal women with breast cancer (Baum et al., 2002, 2003). Aromatase inhibitors are also effective in the treatment of breast cancer that becomes resistant to treatment with tamoxifen (Goss et al., 2003). In these studies, tumors that express ER were more responsive to aromatase inhibitors compared with the tumors with an unknown receptor status (Baum et al., 2002, 2003). Future studies are required to determine whether aromatase inhibitors might be beneficial in ER-negative tumors via ER-independent mechanisms.

III. Aromatase and Endometriosis

Endometriosis is an estrogen-dependent disease that affects 6 to 10% of American women of reproductive age (approximately 4–6 million) and is the most common cause of chronic pelvic pain (Giudice and Kao, 2004). Endometriosis is a systemic disorder that is characterized by the presence of endometrium-like tissue in ectopic sites outside the uterus, primarily on pelvic peritoneum and ovaries, and is linked to chronic pelvic pain, pain during sex, and infertility (Giudice and Kao, 2004). In the United States, endometriosis is the third most common gynecologic disorder that requires hospitalization and is a leading cause of hysterectomy. Only 50% of women with endometriosis achieve pain relief in response to existing hormonal treatments or conservative surgery (Vercellini et al., 1997). Thus, there is a clear need to develop novel and effective therapies for endometriosis.

The clinical significance of estrogen biosynthesis in endometriosis is exemplified by the clinical observations that estrogen is essential for growth of endometriosis. We and others demonstrated abundant aromatase expression and local estrogen production in endometriotic tissue (Kitawaki et al., 1997; Zeitoun et al., 1999; Bulun et al., 2001; Fang et al., 2002; Gurates et al., 2002; Yang et al., 2002). The subsequent introduction of aromatase inhibitors in the treatment of endometriosis successfully underscored the presence of aromatase in endometriotic tissue (Takayama et al., 1998; Ailawadi et al., 2004). These recent results suggested that aromatase inhibitors might treat endometriosis more effectively than GnRH analogs via suppression of local estrogen formation in endometriotic tissue. Thus, as in breast cancer, aromatase is a critical therapeutic target in endometriosis.

A. Mechanisms of Growth and Inflammation in Endometriosis

Two basic pathologic processes, growth and inflammation, are responsible for chronic pelvic pain and infertility, which are the primary devastating symptoms of endometriosis. Estrogen, growth factors, and metalloproteinases enhance the growth and invasion of endometriotic tissue, whereas prostaglandins and cytokines mediate pain, inflammation, and infertility (Bruner et al., 1997; Ryan and Taylor, 1997). Research work from our laboratory and other investigators over the past 10 years uncovered a molecular link between inflammation and estrogen production in endometriosis (Bulun et al., 2001). This is mediated by a positive-feedback cycle that favors expression of key steroidogenic genes, most notably StAR and aromatase, expression of COX-2, and continuous local production of estradiol and PGE2 in endometriotic tissue (Noble et al., 1997; Tsai et al., 2001; Sun et al., 2003) (Fig. 11). We find that the aberrantly expressed transcription factor steroidogenic factor-1 (SF-1) mediates PGE2-cAMP-dependent coactivation of multiple steroidogenic genes, most notably StAR and aromatase, in endometriosis (our unpublished observations) (Zeitoun et al., 1999).

B. Definitions of Experimental Models and Abnormal Tissues in Women with Endometriosis

We and others demonstrated a number of molecular abnormalities in endometriosis (Fig. 12). The prototype...
abnormality was the presence of significant levels of StAR and aromatase activity and expression of protein and mRNA in the stromal cell component of endometriosis, whereas StAR or aromatase expression was either absent or barely detectable in the eutopic endometrium of disease-free women (Noble et al., 1996; Noble et al., 1997; Tsai et al., 2001; Gurates et al., 2002; Sun et al., 2003) (Fig. 12). The eutopic endometrium of women with endometriosis contains low but significant levels of aromatase mRNA and enzyme activity and represents an intermediate state of this disease. It seems that upon retrograde menstruation and implantation of this inherently abnormal tissue on pelvic peritoneal surfaces, aromatase expression and enzyme activity are amplified by up to 400 times (Noble et al., 1996, 1997). COX-2 expression, which is important for PGE$_2$ synthesis, is increased markedly in both eutopic endometrium and endometriotic tissue of women with endometriosis (Ota et al., 2001; Wu et al., 2004) (Fig. 12).

We define herein the terminology to be used in reference to the tissues and cells that are used as model systems. The terms “endometriotic tissue”, “endometriotic cell”, and “endometriosis” refer to the pathologic ectopic endometrium-like tissues isolated from the pelvic peritoneum or ovaries during surgery. The term “endometrium” refers to eutopic endometrial tissue that lines the uterine cavity. “Normal endometrium” and “normal endometrial cells and tissues” refer to the eutopic endometrium from disease-free women. The term “endometrium from endometriosis patient” refers to eutopic endometrial tissue or cells from patients with endometriosis. “Endometriotic stromal cells” are isolated from the walls of cystic endometriosis lesions (endometriomas) in the ovaries (Ryan et al., 1994). These cells in primary culture have been characterized extensively in Robert Taylor’s and our laboratories (Ryan et al., 1994; Noble et al., 1997). Endometriotic stromal cells display the differentiation markers observed in eutopic endometrial stromal cells in culture. For example, progestin-treated endometriotic stromal cells express prolactin mRNA (readily detectable by Northern analysis), albeit in significantly lower levels compared with eutopic endometrial cells, whereas control ovarian granulosa cells do not show detectable prolactin mRNA.

The key molecular abnormalities described in this article were first observed in vivo as differential gene expression in endometrial and endometriotic tissues (Fig. 12). The molecular mechanisms responsible for these abnormalities were then determined in primary endometriotic and endometrial stromal cells in culture. We found that most of these molecular mechanisms were altered expression and/or DNA-binding activity of transcription factors that regulate steroidogenic genes. Finally, we went back to human tissues and verified these novel molecular mechanisms by demonstrating altered levels of transcription factors in vivo.

C. Estrogen Formation in Endometriosis

The biologically active estrogen estradiol is produced from cholesterol through six serial enzymatic conversions in two ovarian cell types that cooperate in a paracrine fashion. The two rate-limiting steps include the entry of cholesterol into the mitochondrion facilitated by StAR and the conversion of androstenedione to estrone by aromatase (Figs. 1 and 11). We and others recently showed that StAR, aromatase, and all other steroidogenic enzymes are expressed in vivo in endometriosis, enabling this tissue to synthesize estradiol from cholesterol de novo (Bulun et al., 2001; Tsai et al., 2001; our unpublished data). Additionally, PGE$_2$ induces expression of the steroidogenic genes StAR, side-chain cleavage (P450sc), 3β-hydroxysteroid dehydrogenase (HSD) type 2, 17-hydroxylase/17-20-lyase (P450c17), and aromatase (P450arom) in endometriotic stromal cells (Figs. 11 and 12). The PGE$_2$-dependent inductions of StAR and aromatase mRNA levels were the highest compared with other steroidogenic genes (our unpublished observations). In contrast, normal endometrium does not biosynthesize estradiol. Both endometriosis and normal endometrium contain the enzyme 17β-HSD-1 that catalyzes the final step, which is the conversion of estrone to estradiol (Fig. 11). What separates normal endometrium from endometriosis, however, is the in vivo lack of StAR and aromatase (Fig. 12). Physiologically significant levels of these gene products are not detected in normal endometrial tissue or PGE$_2$-stimulated endometrial stromal cells (our unpublished observations).
Until recently, we focused our investigation on aromatase expression in endometriosis and hypothesized that the substrate androstenedione for this enzyme originated primarily from the adrenal and/or ovary and arrive at the target tissue (i.e., endometriosis) via circulation. The recent demonstration of StAR and a complete set of steroidogenic enzymes, including aromatase within the endometriotic stromal cell, however, implies that estrogen is synthesized de novo from cholesterol and that endometriotic aromatase is not solely dependent for substrate on adrenal or ovarian secretion. These new findings revised our view of the pathogenesis of estrogen biosynthesis in endometriosis (Figs. 11 and 12).

D. Cellular Mechanisms That Regulate Aromatase Expression in Eutopic Endometrium and Endometriosis

Both StAR and aromatase are expressed primarily in the stromal cells of endometriosis (Fig. 13). This has been supported by a number of in vivo and in vitro observations. We and others demonstrated immunoreactive aromatase in stromal cells of extraovarian and ovarian endometriotic tissue (Kitawaki et al., 1997; our unpublished observations). Abundant StAR activity and aromatase enzyme activity are present in stromal cells isolated and cultured from ovarian endometriotic cysts (endometriomas) (Noble et al., 1997; Zeitoun et al., 1999; Tsai et al., 2001; Sun et al., 2003) (Fig. 13). StAR and aromatase mRNAs are present only in the stromal but not epithelial cell compartment of endometriosis (Noble et al., 1997; Zeitoun et al., 1999; Tsai et al., 2001; Sun et al., 2003). PGE2 or a cAMP analog stimulates StAR mRNA and protein levels and aromatase mRNA in endometriotic stromal cells in a time- and concentration-dependent fashion (Tsai et al., 2001) (Fig. 13). PGE2 stimulates the production of progesterone, 17-hydroxyprogesterone, androstenedione, and estradiol primarily via induction of StAR and aromatase in endometriotic cells (Noble et al., 1997; Tsai et al., 2001) (Fig. 13).

StAR or aromatase expression or steroid hormone production is virtually undetectable in eutopic endometrial stromal cells from disease-free women (Fig. 12). High number of cycles of PCR may amplify aromatase mRNA in endometrial biopsies, but we could not detect aromatase enzyme activity in cultured endometrial stromal cells of disease-free women (Noble et al., 1997) (Fig. 12). Aromatase activity or mRNA could not be induced by PGE2 or cAMP analogs in stromal cells from disease-free women (Noble et al., 1997).

On the other hand, small but significant levels of aromatase enzyme activity and mRNA were detected in eutopic endometrial tissue and stromal cells of women with endometriosis (Noble et al., 1996, 1997) (Fig. 12). This is consistent with the observations of many other investigators that the eutopic endometrial tissue of women with endometriosis exhibits multiple molecular abnormalities that may be linked to aberrant production of estrogen, prostaglandins, cytokines, and decreased production of molecules important for differentiation and implantation (Lessey et al., 1994; Tseng et al., 1996; Kao et al., 2003; Wu et al., 2004).

E. Prostaglandin E2 Biosynthesis and Action in Endometriosis

1. Prostaglandin E2 Biosynthesis in Endometrial Disease. PGE2 plays a key role in the estrogen biosynthetic pathway in endometriotic tissue because it is a potent stimulator of key steroidogenic genes (Noble et al., 1997; Zeitoun et al., 1999; Tsai et al., 2001; Sun et al., 2003). PGE2 or a cAMP analog stimulates StAR mRNA and protein levels and aromatase mRNA in endometriotic stromal cells in a time- and concentration-dependent fashion (Tsai et al., 2001) (Fig. 13). PGE2 stimulates the production of progesterone, 17-hydroxyprogesterone, androstenedione, and estradiol primarily via induction of

![Fig. 13. Effects of PGE2 on StAR and aromatase expression in endometriotic stromal cells.](image-url)
IL-1β in a variety of fibroblasts (Miller et al., 2000; Frasor et al., 2003).

Most importantly, COX-2 is strikingly up-regulated in stromal cells of endometriosis, whereas its expression is also significantly higher in endometrium of patients with endometriosis compared with endometrium of disease-free patients (Ota et al., 2001; Wu et al., 2004). We and others and demonstrated that IL-1β and PGE₂ themselves induce COX-2 in endometriotic and endometrial stromal cells, whereas VEGF and PGE₂ rapidly induced COX-2 in uterine endothelial cells (Tamura et al., 2002a,b,c, 2003, 2004; Wu et al., 2004) (Figs. 14 and 15). Thus, a large number of pathways in endometriosis induce COX-2 to increase PGE₂ formation in this tissue (Figs. 14 and 15). The regulation of PGE₂ synthase in endometriosis is not known.


Both endometriotic and eutopic endometrial stromal cells express similar mRNA and protein levels of the known PGE₂ receptor subtypes, including EP₁, EP₂, EP₃, and EP₄ (our unpublished observations). Use of receptor-selective agonists, however, showed that only the EP₂ receptor is responsible for PGE₂-mediated StAR and aromatase expression in endometriotic stromal cells (Zeitoun and Bulun, 1999). Stimulation of the EP₂ receptor rapidly increases intracellular cAMP (Zhao et al., 1996a; Noble et al., 1997) (Fig. 13). Treatment with PGE₂ or a cAMP analog gives rise to comparable increases in StAR and aromatase activity, protein, and mRNA levels (Noble et al., 1997) (Fig. 13). On the other hand, neither PGE₂ (despite the presence of EP₂ receptors) nor cAMP analogs can induce StAR or P₄₅₀arom in normal eutopic endometrial stromal cells (Noble et al., 1997). Thus, the block for PGE₂-dependent steroidogenesis in eutopic endometrial stromal cells is mediated by inhibitory mechanisms downstream of cAMP.

F. Molecular Mechanisms Responsible for Increased Expression of Steroidogenic Genes in Endometriosis

1. Up-Regulation of Promoter II for Increased Aromatase Expression. Transcription of the aromatase gene in human tissues is regulated by at least 10 distinct promoters, each giving rise to aromatase mRNA species with variable 5-untranslated ends (UTRs) but an identical coding region (Sebastian and Bulun, 2001; Sebastian et al., 2002) (Fig. 3). Extraovarian endometriotic tissue and ovarian endometrioma-derived cells use almost exclusively promoter II, which is the PGE₂/cAMP-responsive proximal promoter, for aromatase expression in vivo (Noble et al., 1996, 1997; Zeitoun et al., 1999). Thus, aberrant aromatase expression in endometriosis is primarily mediated by activation of promoter II (Fig. 16).

2. Transcriptional Mechanisms Responsible for Increased Expression of the Aromatase Gene in Endometriosis. We uncovered a number of molecular abnormalities that are responsible for PGE₂/cAMP-dependent aromatase expression in endometriosis. One critical mechanism is mediated by aberrantly expressed key transcriptional enhancers (e.g., SF-1) in biopsied endometriotic tissues (in vivo) and cultured endometriotic stromal cells (in vitro) (Figs. 12 and 16).
A flip side of this enhancer-mediated mechanism serves as a second mechanism to suppress steroidogenic gene expression in normal eutopic endometrial stromal cells exposed to PGE$_2$ or cAMP analogs (Fig. 16). This involves the redundant presence and steroidogenic promoter-binding activity of multiple transcriptional inhibitors (COUP-TF) and corepressors of SF-1 [e.g., Wilms' tumor-1 (WT-1)] serving as fail-safe mechanisms (Figs. 12 and 16).

The key regulatory elements that regulate aromatase in endometriosis are within the 500-bp region that lies upstream of promoter II (Fig. 16). We found a dose-dependent direct competition between the orphan nuclear receptors SF-1 (enhancer) and COUP-TF (inhibitor) for occupancy of a nuclear receptor half-site (−136/−124 bp) to which SF-1 bound with a higher affinity (Zeitoun et al., 1999). COUP-TF is expressed in both eutopic endometrial and endometriotic cells, whereas SF-1 is expressed in endometriotic but not normal eutopic endometrial cells (Figs. 12 and 16). COUP-TF is part of the transcriptional system that inhibits aromatase in eutopic endometrial stromal cells; in contrast, aberrantly expressed SF-1 in endometriotic stromal cells overrides this inhibition by competing for the same regulatory element. Thus, the mechanism here is differential expression of an enhancer in endometriosis but not in eutopic endometrium favoring aromatase expression (Zeitoun et al., 1999) (Fig. 16).

Additional redundant mechanisms serve to inhibit aromatase in normal endometrium and stimulate it in endometriosis (Yang et al., 2002). For example, we demonstrated that overexpression of C/EBP$_\alpha$ stimulated whereas C/EBP$_\beta$ inhibited P450arom promoter in endometriotic and eutopic endometrial cells (Yang et al., 2002) (Fig. 16). Unexpectedly, C/EBP$_\alpha$ or $\beta$ was found to bind to the −211/−197 CRE but not the nuclear factor-IL-6 sites in the aromatase promoter II (Fig. 16). Moreover, C/EBP$_\alpha$ was selectively down-regulated in vivo in endometriosis but not in eutopic endometrium indicating that differential down-regulation of a transcriptional inhibitor in endometriosis is an additional mechanism for aromatase expression in this pathologic tissue (Figs. 16).

Dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X gene 1 (DAX-1), a corepressor of SF-1, inhibits SF-1-dependent expression of aromatase in endometriotic and endometrial cells (Gurates et al., 2003). Moreover, we demonstrated for the first time that WT-1, another coregulator of SF-1, also acts as a co-repressor and inhibits SF-1-dependent activity of the aromatase promoter II in endometriotic/endometrial stromal cells (Fig. 16) (Gurates et al., 2002, 2003). Intriguingly, WT-1 but not DAX-1 is selectively down-regulated in vivo in endometriotic stromal cells (Gurates et al., 2002) (Figs. 12 and 16). Thus, WT-1 seems to be a physiologically significant corepressor for the inhibition of steroidogenesis in eutopic endometrium (Fig. 16).

On the other hand, p300/CREB, which is a coactivator of SF-1, enhances promoter II activity in endometriotic and endometrial stromal cells (our unpublished observations; Fig. 16). These findings together suggest that SF-1 acts as a master switch to activate the promoters of aromatase and other key steroidogenic genes. Corepressors or coactivators of SF-1 in endometrial versus endometriotic cells may further modify SF-1-dependent transcriptional activity.

G. Activation of Multiple Steroidogenic Promoters in Endometriosis

As indicated above, endometriotic tissue or stromal cells in primary culture express the complete cascade of steroidogenic genes and thus can potentially make estrogen from cholesterol. In contrast to normal endometrium, SF-1,StAR, and aromatase are specifically and distinctly up-regulated in endometriotic tissues and cultured cells. The regulatory elements in StAR and aromatase promoters are very similar (Fig. 17). Additionally, similar transcription factors (SF-1, C/EBPs, and CREB) mediate PGE$_2$-dependent regulation of both promoters (Christenson and Strauss, 2001; Manna et al., 2002, 2003; Yang et al., 2002) (Fig. 17). These promoters remain quiescent under the influence of inhibitory tran-
scription factors (e.g., COUP-TF) and corepressors (e.g., WT-1) in normal endometrial stromal cells (Fig. 17). Our unpublished observations demonstrated that there are no differences between endometrium and endometriosis with respect to LRH-1 levels. SF-1, however, is readily detectable in endometriosis, whereas it is absent in endometrium. These data strongly suggest that SF-1 expression in endometriosis is primarily responsible for PGE2-mediated estrogen formation, in particular via induction of StAR and aromatase promoters (Fig. 17).

H. Summary of Regulation of Promoter II in Endometriotic Stromal Cells and Breast Adipose Fibroblasts

Both endometriotic stromal cells and adipose fibroblasts in breast tumors overexpress aromatase via promoter II using apparently similar transcriptional mechanisms. There are, however, several cell-specific differences. First, PGE2 or a cAMP analog coordinately stimulates two very closely located promoters, II and I.3, in adipose fibroblasts but only promoter II in endometriosis (Figs. 6 and 13). Second, the nuclear receptor half-site in promoter II is occupied by LRH-1 in adipose fibroblasts that do not contain SF-1. Coact (2); second hypothetical coactivator; Enh, hypothetical enhancer; Inh, hypothetical transcription factor.

I. Aromatase Inhibitors in the Treatment of Endometriosis

Aromatase expression and local estrogen biosynthesis in endometriotic implants prompted us and other investigators to target aromatase in endometriosis using its third-generation inhibitors. Among these inhibitors, anastrozole and letrozole were used successfully to treat endometriosis in both postmenopausal and premenopausal women (Takayama et al., 1998; Amsterdam et al., 2003; Ailawadi et al., 2004; Razzi et al., 2004; Shippen and West, 2004; Soysal et al., 2004).

We introduced aromatase inhibitors into the treatment of endometriosis in 1998 by a case report of postmenopausal endometriosis (Takayama et al., 1998) (Fig. 18). This patient had her uterus and both ovaries surgically removed and was therefore postmenopausal but continued to have a persistent pelvic mass and severe pain (Takayama et al., 1998). An aromatase inhibitor effectively eradicated the mass and eliminated her pain (Takayama et al., 1998). This was followed by another case report confirming that an aromatase inhibitor is the medical treatment of choice in postmenopausal endometriosis that is a relatively rare condition (Razzi et al., 2004).

In the much frequently encountered form of premenopausal endometriosis, some form of ovarian suppression needs to be added to aromatase inhibition; otherwise, estrogen depletion in the hypothalamus causes FSH secretion and ovarian stimulation (Fig. 18). Thus, an aromatase inhibitor was administered together with a
GnRH agonist, a progestin, progesterone, or a combination oral contraceptive in four phase II trials (Amsterdam et al., 2003; Ailawadi et al., 2004; Shippen and West, 2004; Soysal et al., 2004) (Fig. 18). All four studies showed a significant benefit of an aromatase inhibitor in reducing pelvic pain. One study showed laparoscopic evidence of eradicating visible pelvic endometriotic implants and significantly decreasing pain (Ailawadi et al., 2004). One randomized study (n = 80) that combined an aromatase inhibitor with a GnRH agonist showed recurrence of pain in only 10% of patients in the GnRH-agonist-plus-aromatase-inhibitor arm versus 55% in the GnRH-agonist-only arm within 24 months from the completion of the treatment (Soysal et al., 2004) (Fig. 18). The combination of an aromatase inhibitor with an oral progestin or an oral contraceptive gave rise to similar results, and we predict that many patients and physicians will prefer these simpler oral regimens (Amsterdam et al., 2003; Ailawadi et al., 2004; Shippen and West, 2004).

These reports suggest the following. 1) Aromatase inhibitors effectively treat pelvic pain associated with endometriosis, which is resistant to existing therapeutic modalities. 2) An aromatase inhibitor is the medical treatment of choice for persistent postmenopausal endometriosis. 3) Use of aromatase inhibitors in premenopausal women with endometriosis requires ovarian suppression via the addition of a GnRH analog, progestin, or combination oral contraceptive. 4) Side-effect profiles were quite favorable and did not include bone loss in most of these regimens. Thus, aromatase inhibitors represent one of the most promising new treatments of pain associated with endometriosis.

IV. Aromatase and Endometrial Cancer

The role of aromatase expression and the therapeutic use of aromatase inhibitors have been well defined for breast cancer endometriosis. Aromatase is also expressed in endometrial cancer tissue, and aromatase inhibitors have been used to treat endometrial cancer (Bulun et al., 1994a, 2001; Rose et al., 2000; Burnett et al., 2004). The pathologic significance of local estrogen biosynthesis via aromatase expression in endometrial cancer tissue or the therapeutic value of aromatase inhibitors in its management, however, is not clear at the moment.

Endometrial cancer is the most common gynecological malignancy found in women (Inoue et al., 1994). These tumors have high concentrations of estrogen receptors, their growth is clearly enhanced by estrogen, and unopposed estrogen exposure (in the absence of progesterone) predisposes women to development of endometrial cancer (Inoue et al., 1994). Although there is no consistent evidence of increased concentrations of circulating estrogen in women with endometrial cancer, the local concentration of estradiol in endometrial cancer tissues was reported to be higher than that in blood and in the endometrium of cancer-free women (Naitoh et al., 1984; Vermeulen-Meiners et al., 1986; Nagasako et al., 1988; Potischman et al., 1996; Sherman et al., 1997; Berstein et al., 2003). It is therefore conceivable that endometrial cancer itself synthesizes estradiol in situ, which then contributes to growth and possibly carcinogenesis.

A conversion study demonstrated significant conversion of androstenedione to estrone in endometrial cancer tissue (Yamamoto et al., 1993). Aromatase protein and mRNA were detected in endometrial cancer using immunohistochemistry and RT-PCR, whereas aromatase expression was low or undetectable in endometrial hyperplasia (a precursor lesion of endometrial cancer) (Bulun et al., 1994a; Watanabe et al., 1995). These observations suggest that intratumoral aromatase may play a role in the pathology of endometrial cancer. Immunoreactive aromatase was found in malignant epithelial, endometrial stromal, and myometrial cells. Aromatase in stromal but not epithelial cells correlated positively with advanced surgical stage and poor survival (M. Shozu, personal communication).

Currently available third-generation aromatase inhibitors may be used for endocrine treatment of endometrial cancer (Berstein et al., 2002). Treatment of endometrial cancer tissues in vitro with aromatase inhibitors demonstrated that in situ depletion of estrogen results in decreased cell proliferation of tumor cells (Sasano et al., 1999). Treatment of women with endometrial cancer with aromatase inhibitors blocked estrogen production in vivo in tumor tissue (Yamamoto et al., 1990). Safety data from the clinical trials of postmenopausal women with breast cancer indicated a preventive role of aromatase inhibitors in that an aromatase inhibitor reduced the risk of endometrial cancer (Duffy and Greenwood, 2003).

On the other hand, the therapeutic efficacy of aromatase inhibitors in advanced endometrial cancer is not clear. The Gynecologic Oncology Group performed a phase II trial of anastrozole in advanced, recurrent, or persistent endometrial cancer (Rose et al., 2000). Twenty-three patients were entered, all with grade 2 or 3 cancers. Two partial responses were noted (Rose et al., 2000). In a recent report, two cases of reproductive-aged women with grade 1 endometrial cancer who were treated with medroxyprogesterone acetate and anastrozole daily for 3 and 6 months subsequently reverted to normal endometrium. A progestin combined with the elimination of production of estrogen may be an effective therapy in well differentiated endometrial cancer in the obese premenopausal woman (Burnett et al., 2004). Thus, there are some early encouraging results. It is, however, too early to predict whether aromatase inhibitors will be used widely in the treatment of endometrial cancer.
V. Aromatase and Uterine Fibroids (Leiomyomata)

Uterine fibroids (leiomyomata), benign tumors of myometrial origin, are the most common neoplasms of the uterus. Symptomatic leiomyomata are diagnosed in 30% of women over 30 years of age and represent the leading cause of hysterectomy (Andersen and Barbieri, 1995). Each leiomyoma is composed of monoclonal proliferation of a transformed myocyte derived from myometrium. The pathogenesis of this transformation remains unknown, although cytogenetic studies have implicated the involvement of several genes that may function as tumor suppressor genes in some leiomyomata, such as high mobility group protein I-C on chromosome 12q15 (Hu and Surti, 1991; Sreekantaiah et al., 1994; Ozisik et al., 1995; Hennig et al., 1997; Van de Ven, 1998; van der Heijden et al., 1998; Gattas et al., 1999; Schoenmakers et al., 1999). On the other hand, it is widely accepted that the growth of uterine leiomyomata is up-regulated by sex steroids, namely estrogen and progesterone secreted from the ovary (Stewart, 2001). Leiomyomata develop only after the commencement of menstrual cycles and becomes symptomatic usually in the 30s or 40s. At the time of menopause, leiomyomata in a portion of patients start to regress. Thus, the ovary is thought to be the major source of sex steroids for leiomyoma growth.

In addition to endocrine estrogen from the ovary, we and others reported the possible contribution of in situ estrogen to leiomyoma growth, namely estrogen synthesized in leiomyoma cells. Leiomyomata per se express aromatase at strikingly higher levels than the surrounding myometrium and can synthesize estrogen (Folkerd et al., 1984; Yamamoto et al., 1985; Bulun et al., 1994b; Sumitani et al., 2000). In fact, the tissue concentrations of estrogen are elevated in leiomyoma nodules compared with levels in surrounding myometrium (Otubu et al., 1982; Pasqualini et al., 1990). Moreover, it was shown in vitro that estrogen synthesized in leiomyoma smooth muscle cells in culture is sufficient to promote proliferation in an intracrine fashion, since the stimulation of aromatase activity increased cellular proliferation, whereas the addition of an aromatase inhibitor inhibited proliferation (Sumitani et al., 2000). A recent report provided limited but promising evidence that uterine leiomyomata shrink in response treatment with an aromatase inhibitor (Shozu et al., 2003a).

A. Molecular Basis for Estrogen Dependence of Uterine Leiomyoma for Growth

A large body of experimental and clinical evidence shows that estrogen stimulates the growth of uterine leiomyomata (Li and McLachlan, 2001; De Leo et al., 2002; Walker, 2002). Estrogen may directly increase proliferation of leiomyoma cells or indirectly increase growth by enhancing progesterone action in human leiomyomata (Andersen and Barbieri, 1995; Rein et al., 1995; Palomba et al., 2002; Walker, 2002). In an in vivo rat model, uterine leiomyomata were shown to be dependent on estrogen for growth (Houston et al., 2001; Walker, 2002). In this model, progestins were inhibitory for estrogen-dependent growth (Hodges et al., 2002). In human leiomyoma cells, however, both estradiol and progesterone enhanced proliferation in vitro. Estrogen induces leiomyoma growth possibly by altering the expression of a large number of genes (Li and McLachlan, 2001; Walker, 2002). These include but are not limited to estradiol-dependent increases in epidermal growth factor and epidermal growth factor receptor expression in leiomyoma cells (Li and McLachlan, 2001). Additionally, human ER-α polymorphisms have recently been reported to be associated with uterine leiomyomata. Recently, a negative cross-talk was uncovered between ER and PPAR signaling pathways (Houston et al., 2003). These data indicate a strong relationship between estrogen and pathogenesis of leiomyomata.

B. Regulation of Estrogen Biosynthesis in Leiomyoma Smooth Muscle Cells

We and others demonstrated local estrogen biosynthesis in intact leiomyoma tissue explants as well as cultured leiomyoma smooth muscle cells (Bulun et al., 1994b). The incubation of these cells with labeled androstenedione gives rise to significant conversion of this precursor steroid to estrone. Estrone is weakly estrogenic and needs to be converted to estradiol to exert full biologic activity. Estrone is further reduced to the biologically active estrogen estradiol by 17β-hydroxysteroid dehydrogenase activity in leiomyoma tissue (Sumitani et al., 2000). This is verified by significant stimulation of proliferation of leiomyoma smooth muscle cells by androstenedione to the same extent as estradiol (Sumitani et al., 2000). The addition of an aromatase inhibitor blocked the stimulatory effect of androstenedione (Sumitani et al., 2000) (Fig. 19). These results indicate that androstenedione is locally converted to biologically significant quantities of estrone and further to estradiol by leiomyoma smooth muscle cells (Fig. 19). Moreover, aromatase seems to be the key enzyme in this process, since the inhibition of aromatase activity blocked proliferation. Aromatase activity was significantly stimulated by a cAMP analog, PGE2, or a combination of a glucocorticoid and a cytokine (IL-1β) (Bulun et al., 1994b; Shozu et al., 2002) (Fig. 19).

C. Aromatase Expression in Uterine Leiomyoma Tissues

Aromatase mRNA was detectable by quantitative RT-PCR in 91% of 35 leiomyomata from 26 women (Bulun et al., 1994b; Sumitani et al., 2000). Aromatase mRNA was also detectable in 75% of normal-seeming myometrial tissues (n = 24) biopsied 2 cm from a leiomyoma. Aromatase mRNA was not detectable in myometrial tissues from disease-free uteri (n = 8). To provide a perspective, leiomyoma aromatase mRNA levels were similar to those ordi-
narily found in breast adipose tissue and were 1.5- to 25-fold higher than those in adjacent myometrial tissues.

There was no correlation between aromatase mRNA levels and leiomyoma size, uterine weight, or patient age, although there was a positive trend between advancing age and aromatase mRNA levels. For example, the highest mRNA levels were detected in patients above age 45, and leiomyoma tissue from a 65-year-old patient contained moderately high aromatase mRNA levels (Bulun et al., 1994b; Sumitani et al., 2000). These data suggest that local aromatase expression may be important for the availability of estrogen to leiomyoma tissue despite severely decreased circulating estrogen levels in perimenopausal or postmenopausal women.

D. Regulation of Aromatase Expression in Leiomyoma Tissues and Smooth Muscle Cells

We and others used leiomyoma tissue explants and smooth muscle cells in monolayer culture as model systems to study the regulation of aromatase mRNA levels (Bulun et al., 1994b; Shozu et al., 2002). Myometrial smooth muscle cells isolated from normal uteri did not contain aromatase activity or aromatase mRNA (Bulun et al., 1994b; Sumitani et al., 2000; Shozu et al., 2001). In our hands, a cAMP analog gave rise to maximum induction, whereas glucocorticoids alone or together with serum moderately increased aromatase mRNA levels (Bulun et al., 1994b). The alterations in aromatase enzyme activity induced by hormonal treatments were accompanied by comparable changes in the levels of aromatase mRNA (Bulun et al., 1994b) (Fig. 19).

Our published and unpublished observations on 14 samples from 13 patients indicated that cAMP-responsive promoters I.3 and II (86%) are primarily used for aromatase expression, whereas promoter I.4 (14%) plays a less important role (Bulun et al., 1994b) (Fig. 19). A Japanese group of investigators recently reported that promoter I.4-specific aromatase mRNA species were predominantly present in most leiomyoma tissues (n = 6) (Shozu et al., 2002). A glucocorticoid plus IL-1β stimulated aromatase enzyme activity and mRNA levels maximally in cells isolated from these specimens (Shozu et al., 2002) (Fig. 19). These investigators also demonstrated that glucocorticoids and IL-1β regulated aromatase expression via promoter I.4 (Fig. 19). The discrepancy between their results and ours may be attributed to race-dependent differences, since our samples were obtained from African American, white, and Hispanic patients, whereas all patients in the Japanese study were of Asian origin (Bulun et al., 1994b; Shozu et al., 2002). Studies are underway to understand the basis of these different findings from two different laboratories.

E. Treatment of Uterine Leiomyomata with Aromatase Inhibitors

A preliminary but promising piece of clinical evidence for the significance of local aromatase expression was published recently (Shozu et al., 2003a). These authors described the shrinkage of a large leiomyoma in a 53-year-old menopausal woman using an aromatase inhibitor (Shozu et al., 2003a). Because the patient did not have ovarian function, the therapeutic effect was postulated to be mediated by blocking local aromatase activity in the leiomyoma (Fig. 19). This case report provides limited but promising evidence for a role of aromatase in the biology of uterine leiomyomata and the future use of aromatase inhibitors to treat these tumors. To our knowledge, several pilot clinical trials are in progress and should provide a better insight to this question.

VI. Aromatase Excess Syndrome

A. Background and Clinical Manifestations

Wilson and McPhaul and their colleagues described a genetically inherited form of estrogen excess in henny-feathered Sebright roosters (George and Wilson, 1980; McPhaul et al., 1988; Matsumine et al., 1990). In birds, aromatase is normally expressed in the ovaries and brain but not in skin fibroblasts (McPhaul et al., 1988; Simpson et al., 2002). In Sebright chickens with the autosomal dominant henny-feathered trait, aromatase is overexpressed in skin fibroblasts, leading to inappropriate estrogen formation in skin and a female pattern of feather development in roosters (George and Wilson, 1980; Matsumine et al., 1990). Furthermore, the 5′-untranslated region of aromatase mRNA in skin fibroblasts suggested that a unique promoter regulated the expression of aromatase (Matsumine et al., 1990). We published two distinct rearrangements in chromosome 15, which may be responsible for the human counterpart of this disorder, i.e., aromatase excess syndrome (Shozu et al., 2003b) (Fig. 20).
Boys affected by this syndrome present with growth of breast tissue (gynecomastia), whereas girls present with premature breast development (thelarche). Both sexes exhibit a premature growth spurt, early fusion of epiphyses, and decreased adult height. Adult men are undervirilized, whereas adult women experience irregular uterine bleeding. Increased conversion of steroid precursors to estrogens in extraglandular tissues is responsible for excessive estrogen formation. MacDonald and his colleagues described originally a feminized, prepubertal adopted boy in whom large amounts of estrone and estradiol were produced by extraglandular aromatization of plasma androstenedione (Hemsell et al., 1977). Five families were subsequently described in which several members had estrogen excess as a result of increased extraglandular aromatization inherited in an autosomal dominant fashion (Berkovitz et al., 1985; Leiberman and Zachmann, 1992; Stratakis et al., 1998; Martin et al., 2003; Shozu et al., 2003b).

**B. Genetic Basis for Familial Aromatase Excess Syndrome**

We evaluated for genetic abnormalities a 36-year-old man, his 7-year-old son, and an unrelated 17-year-old boy with severe gynecomastia of prepubertal onset and hypogonadotropic hypogonadism caused by elevated estrogen levels (Shozu et al., 2003b). Aromatase activity and mRNA levels in fat and skin and conversion of circulating androstenedione to estrone were severely elevated. Treatment with an aromatase inhibitor decreased serum estrogen levels and normalized gonadotropin and testosterone levels. The 5’-untranslated regions of aromatase mRNA contained the same sequence (FLJ) in the father and son and another sequence (TMOD3) in the unrelated boy; neither sequence was found in control subjects nor had they been previously described (Fig. 20). These 5’-untranslated regions normally make up the first exons of two ubiquitously expressed genes clustered in chromosome 15q21.2 to 3 in the following order (from telomere to centromere): FLJ, TMOD3, and aromatase. The aromatase gene is normally transcribed in the direction opposite to that of TMOD3 and FLJ. Two distinct heterozygous inversions reversed the direction of the TMOD3 or FLJ promoter in the patients (Shozu et al., 2003b) (Fig. 20).

We found that overproduction of estrogen arose from gain-of-function mutations in chromosome 15, giving rise to the formation of cryptic promoters that regulate the aromatase gene. These constitutively active promoters normally serve to transcribe two ubiquitously expressed genes—FLJ and TMOD3—that encode products homologous to muscle proteins in many human tissues. The functions of FLJ and TMOD3 are not known (Cox and Zoghbi, 2000; Nagase et al., 2000) (Fig. 20). An identical mutation involving the FLJ promoter was transmitted from a father to his son in an autosomal-dominant manner (Fig. 20). The cryptic promoter in a third unrelated patient was distinct and arose from the TMOD3 gene (Fig. 20). The relatives of this third patient were not affected. Therefore, this rearrangement seemed to be the result of a new mutation.
These mutations are somewhat analogous to transgenic mice, in which a gene product fused to a constitutively active promoter is expressed in multiple tissues after random insertion. The tissue selectivity (or nonselectivity) and level of activity of this aberrant promoter determines the tissue distribution and levels of mRNA encoded by this gene. In human peripheral tissues and gonads, basal levels of aromatase mRNA is extremely low. Only FSH induces aromatase expression physiologically to very high levels in the ovaries of premenopausal women during cycle days 5 to 14. Thus, in general, low basal expression of aromatase is tightly controlled in the peripheral tissues because comparatively low levels of aromatase expression and formation of estrogen (measured in picomoles per liter in the serum) are sufficient for many estrogenic effects. The mutations described in aromatase excess syndrome represent a major deviation from this physiology and are caused by constitutively active cryptic promoters that take control over aromatase expression.

C. Discussion on Gain-of-Function Mutations That Affect the Aromatase Gene

The upstream region of the aromatase gene may represent a “hot spot” for mutations. Occasional mutations, such as those that we described, caused extremely high aromatase activity and striking clinical consequences. More common rearrangements, however, may go clinically unrecognized and cause subtle degrees of estrogen excess, which may increase the risks of estrogen-dependent disease, such as cancers of the breast and endometrium, endometriosis, and uterine fibroids.

Adult men with this syndrome had hypogonadotropic hypogonadism. During treatment with an aromatase inhibitor, their estrogen levels declined and testosterone, luteinizing hormone, and follicle-stimulating hormone levels rose to normal. This response suggests a crucial role of estrogen in the suppression of both gonadotropins in men. Despite low testosterone levels in these patients, luteinizing hormone remained suppressed, possibly owing to the high levels of circulating estrogen. These mutations may have given rise to the overexpression of aromatase in the brain and thus to increased local estrogen production, which might also have contributed to the suppression of gonadotropins.

Aromatase inhibitors may potentially be used to prevent or halt the progression of gynecomastia in young boys (Plourde et al., 2004). The potential objective of long-term treatment of adult men with an aromatase inhibitor is to restore gonadal function, but this approach is not clearly justified for several reasons (Shozu et al., 2003b). First, men with this condition are not at risk for osteoporosis. Second, although men with estrogen excess may be subfertile, infertility is not a uniform feature of the syndrome. Third, the consequences of long-term exposure to high levels of estrogen in men are not known. We suggest that these men should periodically be evaluated for breast and prostate disease, given the potentially deleterious effects of estrogen on these tissues (Shozu et al., 2003b; Plourde et al., 2004).

There are no published reports regarding the use of the new-generation aromatase inhibitors in women with aromatase excess syndrome. It is conceivable that these women are at increased risk for developing breast cancer, endometrial cancer, macromastia, endometriosis, and uterine fibroids. Thus, aromatase inhibitors may potentially be used for prevention or treatment. Answers to these questions require clinical trials in carefully selected populations.

VII. Conclusions Regarding Aromatase Overexpression in Estrogen-Dependent Human Disease

Several principles emerge from the data summarized in this review. First, human aromatase encoded by a single gene acts as a master switch in physiological and pathological systems to turn on estrogen formation or serves as a therapeutic target to turn off estrogen biosynthesis. By the same token, estradiol acts as a master switch in many biological systems to regulate hundreds to thousands of diverse genes at one time point, which makes it extremely difficult to therapeutically target any of these downstream genes. Second, biologically active quantities of aromatase mRNA and protein and their product estrogen are relatively small. For example, circulating or tissue estradiol is measured in picomole quantities that are 100 to 1000 orders of magnitude smaller than those used for testosterone measured in nanomolar quantities. Thus, comparatively small levels of aromatase expression have physiologically or pathologically critical consequences. Third, local aromatase expression and estrogen biosynthesis seems to be a far more critical therapeutic target compared with circulating estrogen. Fourth, complex and redundant epigenetic mechanisms are responsible for aromatase expression in the estrogen-dependent pathologic tissues. These range from accumulation of aromatase-expressing cell types to aberrant expression of transcriptional enhancers and permit the use of additional promoters in pathological tissues, which are normally silenced in disease-free tissue counterparts. Most notably, the two proximal promoters, I.3 and II, which are located within 215 bp from each other and coordinately regulated by PGE2 or protein kinase A- and C-dependent pathways, are aberrantly activated in breast cancer, endometriosis, endometrial cancer, and uterine fibroids. Finally, gain-of-function-type germ cell mutations give rise to cryptic promoters that overexpress the aromatase gene ubiquitously in many tissues. We speculate that the promiscuous nature of the splice acceptor site immediately upstream of the coding region is in part responsible for regulation of the aromatase gene by multiple alternatively used physiological or pathological promoters.
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REFERENCES


Agarwal VR, Bulun SE, and Simpson ER (1995) Quantitative detection of alternatively spliced transcripts of the aromatase cytochrome P450 (CYP19) gene in aromatase


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