Aromatase expression and regulation in breast and endometrial cancer

Hong Zhao, Ling Zhou, Anna Junjie Shangguan and **Serdar E Bulun**

Division of Reproductive Science in Medicine, Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA

Correspondence should be addressed to H Zhao
Email
h-zhao@northwestern.edu

Abstract

Long-term exposure to excess estrogen increases the risk of breast cancer and type 1 endometrial cancer. Most of the estrogen in premenopausal women is synthesized by the ovaries, while extraovarian subcutaneous adipose tissue is the predominant tissue source of estrogen after menopause. Estrogen and its metabolites can cause hyperproliferation and neoplastic transformation of breast and endometrial cells via increased proliferation and DNA damage. Several genetically modified mouse models have been generated to help understand the physiological and pathophysiological roles of aromatase and estrogen in the normal breast and in the development of breast cancers. Aromatase, the key enzyme for estrogen production, is comprised of at least ten partially tissue-selective and alternatively used promoters. These promoters are regulated by distinct signaling pathways to control aromatase expression and estrogen formation via recruitment of various transcription factors to their *cis*-regulatory elements. A shift in aromatase promoter use from I.4 to I.3/II is responsible for the excess estrogen production seen in fibroblasts surrounding malignant epithelial cells in breast cancers. Targeting these distinct pathways and/or transcription factors to modify aromatase activity may lead to the development of novel therapeutic remedies that inhibit estrogen production in a tissue-specific manner.

Key Words

- aromatase
- estrogen
- breast cancer
- endometrial cancer

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Introduction

Aromatase is the rate-limiting enzyme in estrogen biosynthesis (Simpson & Santen 2015). The biologically active estrogen, 17β-estradiol (E2), exerts its actions by binding to its receptors, estrogen receptor-α (ERα) (Lubahn *et al*. 1993, Couse *et al*. 1999, Dupont *et al*. 2000) and estrogen receptor-β (Krege *et al*. 1998). Beyond its essential role in reproductive function (Bulun *et al*. 2005), estrogen is also involved in vascular biology (O'Lone *et al*. 2007), lipid and carbohydrate metabolism (Jones *et al*. 2001), bone mineralization (Nakamura *et al*. 2007), and cognitive and other brain-related functions (Arevalo *et al*. 2015). Estrogen also plays an important role in initializing development and further growth of a number of benign and malignant hormone-dependent disorders, including breast and endometrial cancers (Bulun *et al*. 2005, Cavalieri *et al*. 2006, Kim *et al*. 2013, Santen *et al*. 2015).

Breast cancer is the most common cancer among women in the United States (other than skin cancer). It can be divided into four main molecular subtypes based on the presence or absence of routinely evaluated biological markers: hormone (estrogen or progesterone) receptors (HR+/HR–) and excess levels of human epidermal growth factor receptor 2 (HER2+/HER2–), a protein promoting breast epithelial cell growth (Perou *et al*. 2000, Cancer Genome Atlas 2012). These four subtypes are luminal A

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(HR+/HER2–, 74%); luminal B (HR+/HER2+, 10%); HER2-enriched (HR–/HER2+, 4%); and triple negative (HR–/HER2–, 12%) (Anderson *et al*. 2014, Kohler *et al*. 2015). The majority of breast cancers (84%) express estrogen and progesterone receptors, indicating the essential role of estrogen in breast cancer development. Endometrial cancer is the sixth most common cancer in women worldwide and is divided into two types. Type 1 endometrial cancer is thought to be caused by excess estrogen (Jarzabek *et al*. 2013). Risk factors that affect a woman's estrogen balance play a critical role in this type of endometrial cancer. Type 2 endometrial cancer is not related to estrogen (Morice *et al*. 2016). This review describes the molecular basis of tissue-specific estrogen production and its role in breast and endometrial cancers. Understanding the mechanisms that control the levels of estrogen in specific tissues may lead to the development of tissue-targeted therapies for estrogen-dependent diseases, such as breast and type 1 endometrial cancer.

Estrogen synthesis and deactivation

Estrogen is synthesized in the gonads and in several extragonadal organs (Simpson 2003, Bulun *et al*. 2009). In premenopausal women, six enzymes encoded by five specific genes synthesize estrogen *de novo* from cholesterol, primarily in the granulosa cells and corpus luteum of ovaries and in the placenta (Simpson 2003). The entry of cytosolic cholesterol into mitochondria initiates estrogen synthesis. In postmenopausal women, estrogen is produced in many extragonadal organs (skin, adipose tissues, liver, heart and brain) (Bulun *et al*. 2005).

There are three major forms of physiological estrogens in women: estrone (E1), estradiol (E2, or 17β-estradiol), and estriol (E3) (Fig. 1) (Cui *et al*. 2013). E1 is synthesized in skin and adipose tissues from circulating androstenedione of adrenal origin and is the major form of estrogen produced in postmenopausal women (Shozu *et al*. 2003, Fujisawa & Castellot 2014). E2 is the most potent estrogen and is the major estrogen product synthesized in the premenopausal ovaries. As in premenopausal women, E2 is also the biologically active estrogen in postmenopausal women even if the circulating E2 levels are low. It is synthesized either by reduction of E1 in extragonadal sites including skin and adipose tissue or alternatively by direct aromatization of circulating testosterone. E3 is the least potent estrogen and is synthesized in large quantities in the placenta.

Estrogen deactivation occurs via several mechanisms of metabolism and conjugation. The first metabolic pathway of estrogen involves the conversion of E2 to the less active

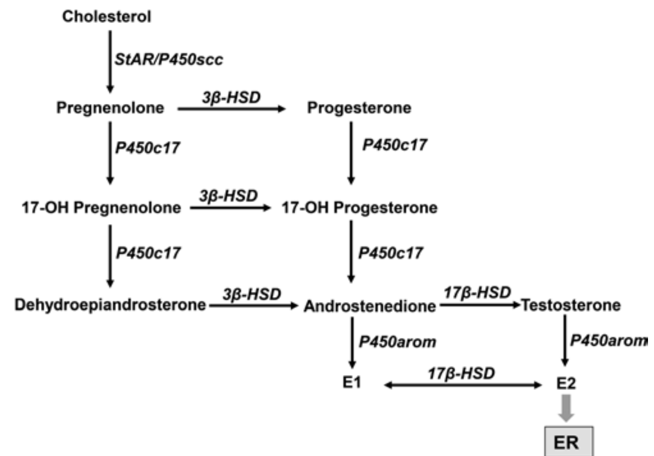

Figure 1
The estrogen biosynthetic pathway involving the conversion of the substrate cholesterol to progestogens, androgens and finally estrogens. The conversion of androgen to estrone (E1) and estradiol (E2) catalyzed by aromatase (P450arom) is the last and key step for production of estrogen, which binds to estrogen receptor (ER). STAR, steroidogenic acute regulatory protein; P450scc, cholesterol side-chain cleavage enzyme; P450c17, steroid 17α-hydroxylase/17,20 lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

E1 by 17β-hydroxysteroid dehydrogenase (17β-HSD) and the subsequent sulfation via estrogen sulfotransferase (EST) to form estrone sulfate (E1S), which is a storage form of estrogen that does not interact with ERs. These sulfated E1 can also be reverted to E2 first through deconjugation by steroid sulfatase and then further reduction. The ability of EST to form E1S at physiologic concentrations is critical in regulating the activation of the ER in estrogen-responsive cells (Suzuki *et al*. 2005, Cos *et al*. 2014). Conjugation of lipophilic estrogens with sulfate is thus a main pathway for estrogen inactivation in estrogen target tissue (Fujisawa & Castellot 2014).

In addition to sulfation, E1 and E2 can be metabolized by several other pathways. E1 and E2 can be oxidized by cytochrome P450 enzymes to generate 2- and 4-hydroxy-catecholestrogen, which can be further methoxylated by catechol-*O*-methyltransferase to form 2/4-methoxy-estrogen. In addition, E2 and E1 as well as their metabolites can also be conjugated to glucuronic acid via UDP-glucuronosyltransferases. The resulting estrogen glucuronides are completely devoid of biologic activities because they have significantly altered chemical structures that prevent interaction with ERs. The glucuronic acid moiety also increases the aqueous solubility of estrogen and eventually facilitates the excretion of estrogen metabolites in the urine. Thus, while a small amount of estrogen is sulfated and detected in circulation and

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in the bile, the majority of parent estrogens and their metabolites (80–90%) are glucuronidated and excreted through the urine (Kotov *et al*. 1999, Guillemette *et al*. 2004, Tong *et al*. 2005). The balance between estrogen synthesis and deactivation maintains physiological estrogen homeostasis. Aromatase catalyzes the last and rate-limiting step in E2 synthesis, and aromatase regulation is a major mechanism for controlling estrogen synthesis (Bulun *et al*. 2005, 2009).

Aromatase: the key estrogen synthase

The *CYP19A1* gene that encodes aromatase protein in humans spans approximately 123 kb on chromosome 15q21.2 and consists of a 93 kb 5′-untranslated region (UTR), 30 kb of coding region, and the 3′-end (Bulun *et al*. 2005, Boon *et al*. 2010). The coding region of aromatase contains nine exons (II–X) with the ATG translational start site located in exon II. The 5′-UTR contains a number of alternative untranslated first exons that are regulated by tissue-specific promoters (Fig. 2A). Thus far, ten alternative tissue-specific promoters have been found in humans, including promoters I.1, I.2 and I.2a in placenta; I.4 in adipose tissue and skin; I.5 in fetal tissues; I.f in brain; I.7 in endothelial cells; I.6 in bone; I.3 in adipose tissue; and PII in gonads and adipose tissue (Bulun *et al*. 2005, 2012). These promoters differentially regulate aromatase expression in gonads, adipose tissue, bone, brain, skin, fetal liver and placenta.

In mice, aromatase is encoded by a single gene named *Cyp19a1* that extends to approximately 103 kb in chromosome 9. The ATG translation start site location (exon II) and the number of coding exons (II–X) are similar to that of the human aromatase gene (Golovine *et al*. 2003);

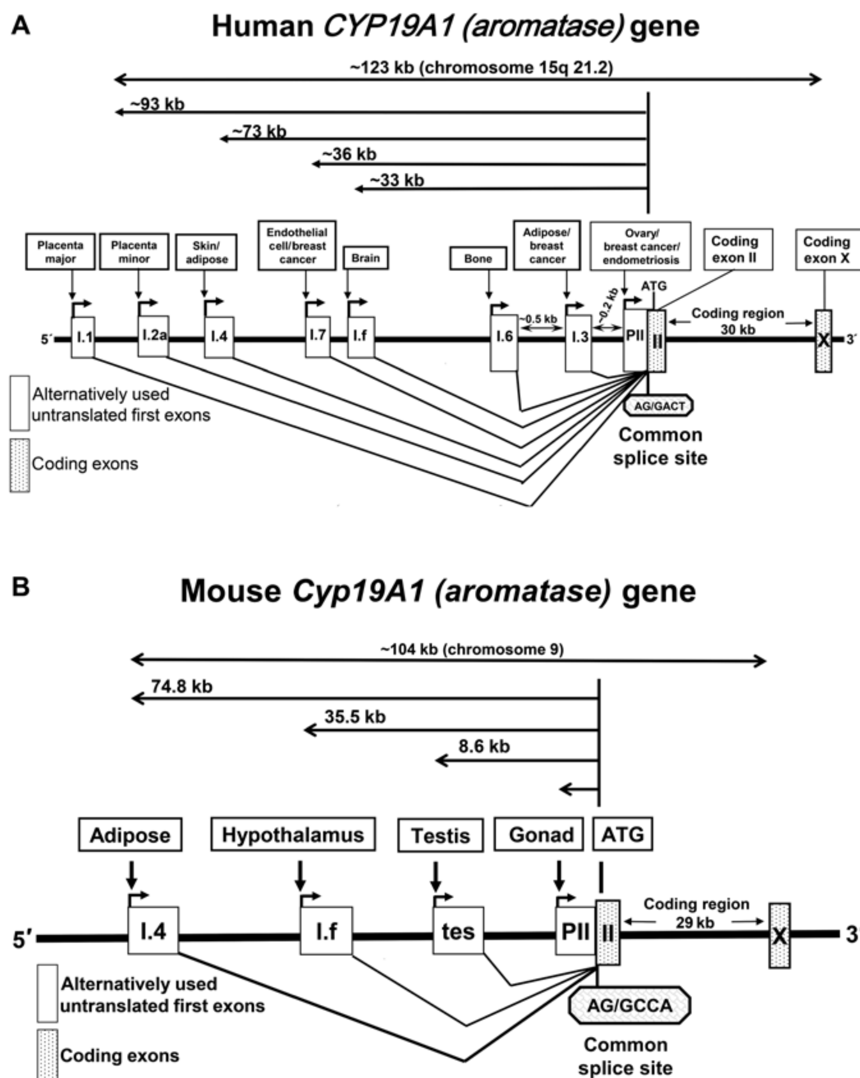

Figure 2
(A) Human aromatase (*CYP19A1*) and (B) mouse aromatase (*Cyp19a1*) genes. Expression of the aromatase gene is regulated by the tissue-specific activation of a number of promoters via alternative splicing. Aromatase mRNA species contain promoter-specific 5′-UTRs. The coding region and encoded protein, however, are identical regardless of the promoter used.

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however, the lengths of the 5′-UTR and the coding region are 73 and 29 kb, respectively, which are shorter than their human counterparts. Moreover, the aromatase gene in the male mouse contains only four tissue-specific promoters, which are promoters II (gonad), tes (a testis-specific promoter), I.f (brain) and I.4 (gonadal fat) (Fig. 2B). The female mouse contains two tissue-specific promoters (II and I.f) (Zhao *et al*. 2009, 2012). The mouse gonad-, brain- and fat-specific first exons share 100%, 93% and 37% homology with the human exons PII, I.f and I.4, while the testis-specific first exon is unique to mice (Honda *et al*. 1994, 1996, Chow *et al*. 2009, Zhao *et al*. 2012). In both humans and mice, all of the 5′-untranslated tissue-specific first exons are spliced onto a common junction located 38 bp upstream of the ATG translational start site. Consequently, the aromatase protein is the same regardless of the splicing pattern (Bulun *et al*. 2005, Zhao *et al*. 2009). Mouse aromatase is expressed in fewer tissues than human aromatase; thus, mouse models do not mirror estrogen production in humans. For this reason, we generated a humanized aromatase (Arom^{hum}) mouse model that contains the full human aromatase gene containing the 5′-UTR, the coding region, and the 3′-end, mimicking human aromatase expression pattern in the mouse model (Zhao *et al*. 2012). This and other mouse models are described later in this review.

The expression and regulation of the aromatase gene are complicated processes. Tissue-specific aromatase expression depends on three major factors: (1) activation of tissue-specific promoters and transcription of promoter-related first exons, (2) alternative splicing, and (3) the availability of various transcription factors (Bulun *et al*. 2005, Cui *et al*. 2013). As described above, the human aromatase gene contains 10 first exons in its 5′-UTR. Each exon 1 is tissue-specific, untranslated and driven by its upstream cognate promoter. Activation of each promoter leads to an alternatively spliced form of mature mRNA with identical coding regions but distinct first untranslated exons. Therefore, the aromatase protein itself is identical across all tissue types regardless of the promoter used. Each promoter is regulated by distinct sets of hormones, cytokines and second messenger signaling pathways, which recruit different transcription factors to regulate tissue-specific aromatase expression and estrogen biosynthesis under physiological or pathological conditions such as breast cancer and endometrial cancer.

Estrogen and breast cancer

There are three sources of estrogen that support the development and growth of breast cancers (Simpson 2003, Bulun *et al*. 2005). First, estrogen synthesized in the ovaries by granulosa cells reaches the breast tissue to act in an endocrine manner. Secondly, estrogen synthesized by aromatase in extraovarian body sites such as subcutaneous adipose tissue and skin also acts on the breast in an endocrine manner. Thirdly, aromatase overexpression within the tumor tissue or in the surrounding stroma increases local levels of estrogen, which acts at the site of synthesis in a paracrine and/or intracrine manner. Tissues involved in estrogen production differ between pre- and post-menopausal women. In postmenopausal breast cancer patients, extraovarian sites and breast cancer tissue are the major estrogen sources, whereas ovaries are the major source of estrogen in premenopausal women with breast cancer. While local estrogen synthesis may be upregulated in postmenopausal benign breast tissue or malignant breast tumors, others have argued that the relative role of circulating pool of estrogens may be more important for breast carcinogenesis (Dunbier *et al*. 2010, Haynes *et al*. 2010, Lonning *et al*. 2011). In this review, we underscore the tumor microenvironment and potential impact of local estrogen produced by aromatase in adipose fibroblasts surrounding the benign or malignant breast epithelial cells.

E2 synthesis in the ovary

In premenopausal women, circulating E2 is mainly synthesized in the ovaries, which secrete E2 into the bloodstream for endocrine transport to target tissues to carry out its biological functions. Ovarian follicles are the basic units of female reproductive biology. Each follicle is composed of an oocyte and surrounding granulosa cells. Theca cells form a layer around the granulosa cells as the follicle matures. After ovulation, theca cells luteinize to form the corpus luteum. The theca and granulosa cells collaborate in tissue-specific estrogen synthesis in the ovary (Liu & Hsueh 1986, Yada *et al*. 1999). Theca cells lack aromatase, but are able to produce androgen from progesterone, which is then released from theca cells and diffuses into granulosa cells. In contrast, granulosa cells, which lack enzymes for androgen synthesis, express aromatase, which converts androgen from theca cells to estrogen (Cui *et al*. 2013). Androgen released from adrenal glands also provides a substrate for aromatase to produce estrogen in granulosa cells (Fig. 3A). Circulating E2 levels fluctuate monthly due to the menstrual cycle, with the highest levels immediately before ovulation, intermediate levels in the luteal phase and lowest levels in the

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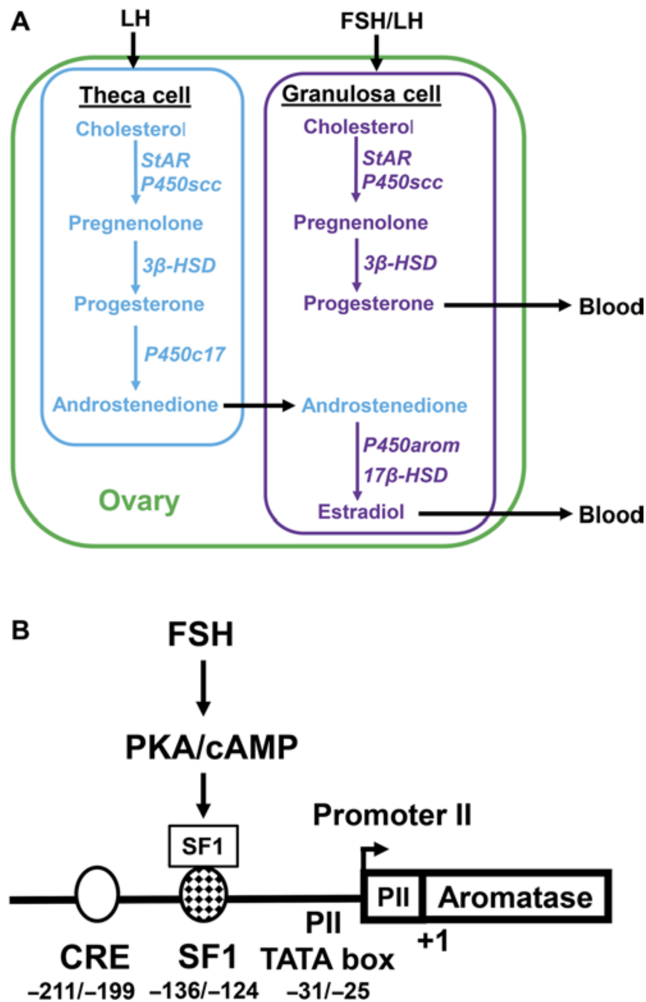

Figure 3

Estrogen synthesis and aromatase promoter II use in the ovary. (A) Gonadotropins (FSH and LH) from the pituitary induce estrogen production in ovary. Progesterone is synthesized from cholesterol via the steroidogenic acute regulatory protein (STAR), the cholesterol side-chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in both theca and granulosa cells and is converted to androstenedione via steroid 17α-hydroxylase/17, 20 lyase (P450c17) only in theca cells. Theca cell androstenedione is transported into granulosa cells, where it is converted to estrogen by aromatase (P450arom) and 17β-hydroxysteroid dehydrogenase (17β-HSD). (B) FSH induces aromatase expression via a PKA/cAMP-dependent pathway in ovarian granulosa cells via promoter II. SF-1 mediates this action of FSH.

follicular phase. Over the course of a woman's life, the level of estrogen synthesis is highest during the reproductive years and declines during the transition stage, reaching its lowest levels in the postmenopausal period. Ovarian aromatase expression is mediated primarily by follicle-stimulating hormone (FSH) signaling, cyclic AMP (cAMP) production, protein kinase A (PKA) phosphorylation and activation of the aromatase proximal promoter II (Fig. 3B) (Simpson *et al*. 1994).

E2 synthesis in skin and adipose tissue

The estrogen precursor androstenedione is primarily secreted by the adrenal glands in postmenopausal women. Aromatase expression and enzyme activity in skin and subcutaneous adipose fibroblasts give rise to formation of systemically available E1 and, to a lesser extent E2, after menopause. The potential roles of adipose tissue and skin aromatase in human physiology and pathology were initially recognized in the 1960s, specifically the role of adipose tissue-derived estrogen in obese postmenopausal women with breast cancer (MacDonald *et al*. 1968, Calle *et al*. 2003). Estrogen formation in skin and fat is positively correlated with increased body weight in postmenopausal women and may be increased by as much as ten-fold in morbidly obese postmenopausal women (Grodin *et al*. 1973, Hemsell *et al*. 1974). Later studies in female Arom^{hum} mice also found that mice fed a high-fat diet gained more weight and had a larger mammary gland mass with elevated total human aromatase mRNA levels (Chen *et al*. 2012). Overweight or obese postmenopausal women exhibit a three-fold higher risk for developing breast cancer compared with normal-weight postmenopausal women (Ziegler *et al*. 1996, Morimoto *et al*. 2002, Gunter *et al*. 2009), suggesting that estrogen produced in skin and adipose tissue reaches breast tissue via the circulation to stimulate tumor growth (Huang *et al*. 1997, Hankinson *et al*. 1998). In addition, surgical ablation of pituitary or adrenal glands was shown to have a palliative effect on breast cancer. E1 or E2 production in adipose tissue uses plasma androstenedione secreted by the adrenal cortex or ovarian testosterone as substrates, suggesting that the palliative effects of adrenalectomy or hypophysectomy may be due to aromatase substrate depletion and a decrease in aromatase-catalyzed adipose tissue estrogen biosynthesis (Santen 1981). The clinical relevance of these findings is underscored by the observed efficacy of aromatase inhibitors in breast cancer treatment in postmenopausal women, which causes a reduction in estrogen biosynthesis in adipose tissue (Brodie *et al*. 1999*a*,*b*). However, for premenopausal women, both prospective cohort and case–control studies consistently report a modest (20–40%) decreased risk of breast cancer in obese women compared with normal-weight women (Harris *et al*. 2011, John *et al*. 2011, Anderson & Neuhouser 2012, White *et al*. 2012).

E2 synthesis in normal breast and breast cancer tissues

In the breast, benign or malignant epithelial cells lie in close contact with endothelial cell-lined capillaries,

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mesenchymal stromal cells (undifferentiated adipose fibroblasts also known as preadipocytes), and lipid-filled mature adipocytes (Bulun *et al*. 1993). In breast adipose tissue, most aromatase (80–90%) expression is found in adipose fibroblasts rather than in mature adipocytes (Price *et al*. 1992). Normal breast adipose tissue maintains low levels of aromatase expression primarily via distal promoter I.4 and uses the proximally located promoters I.3 and II only minimally (Fig. 4). In breast cancer, malignant epithelial cells enrich the population of adipose fibroblasts by secreting large amounts of cytokines such as tumor necrosis factor (TNF) α and interleukin 11 (IL-11) to inhibit differentiation of preadipocytes into mature adipocytes; thus, creating a dense fibroblast layer surrounding malignant epithelial cells in a process called the desmoplastic reaction (Meng *et al*. 2001). As a result, the total amount of promoter I.4-specific aromatase transcript is increased in breast cancer tissue (Harada 1997). More importantly, malignant breast epithelial cells secrete prostaglandin E2 (PGE2) and other unknown factors to cause aromatase promoter switching from I.4 to the more potent I.3 and II promoters in adipose fibroblasts, leading to increased production of aromatase (Fig. 4) (Zhao *et al*. 1996*a*, Zhou *et al*. 2001, Diaz-Cruz *et al*. 2005). In addition to breast adipose fibroblasts, breast tumors produce high levels of aromatase, especially via promoter I.3/II (Agarwal *et al*. 1996). Finally, breast endothelial cells, which proliferate in the pro-angiogenic environment of breast cancer, appear to be a significant site of aromatase expression via promoter I.7 (Fig. 4) (Sebastian *et al*. 2002). Thus, the prototypical estrogen-dependent breast cancer takes advantage of four promoters (II, I.3, I.7 and I.4) to drive aromatase expression (Fig. 4). The sum of the aromatase mRNA species arising from these four promoters markedly increases total aromatase mRNA levels in breast cancer compared with the normal breast tissue, which almost exclusively uses promoter I.4. Thus, the paracrine interaction between malignant epithelial cells and adipose stromal cells affects adipogenic differentiation and activates a subset of aromatase promoters to drive local estrogen production (Bulun *et al*. 2005).

Aromatase promoter usage in normal and malignant breast tissues

Promoter I.4 Aromatase promoter I.4 does not have a TATA or CAAT box upstream of the transcription start site for the untranslated exon I.4 (Fig. 5A) (Zhao *et al*. 1995*a*, Chen *et al*. 2009). The 5′-UTR of exon I.4 contains a putative silencer and several positive *cis*-acting elements, including an AP1 site (−500/−494), an interferon γ activation site (GAS, −282/−272), a glucocorticoid response element (GRE, −133/−119), and an Sp1-binding site (+151/+158), which can recruit and bind the transcriptional factors c-Fos/c-Jun, phosphorylated signal transducer and activator of

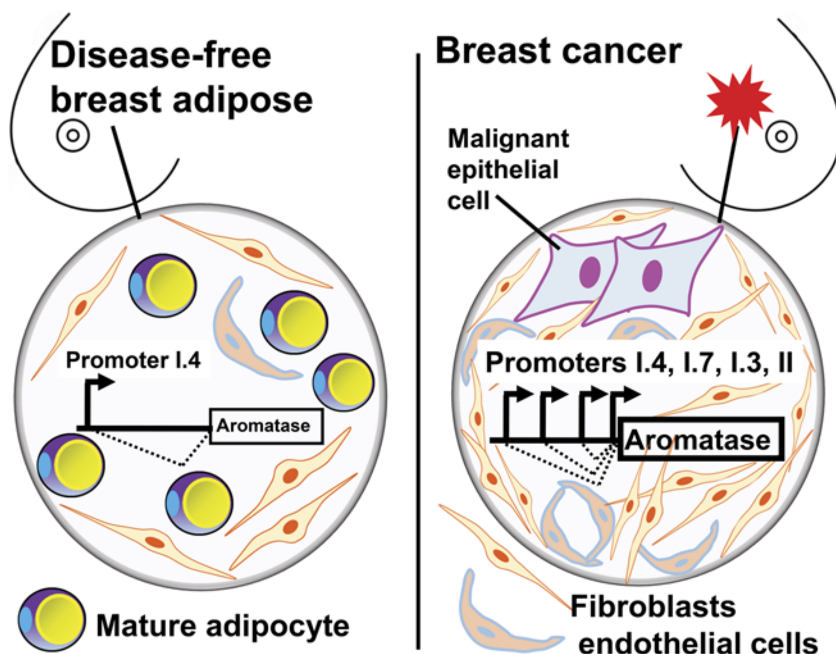

Figure 4
Alternative promoter use for aromatase expression in normal and malignant breast tissues. Normal breast adipose tissue maintains low levels of aromatase expression primarily via promoter I.4. Promoters I.3 and II are used only minimally in normal breast adipose tissue, whereas promoter I.3 and II activity in breast cancer are strikingly increased. Additionally, the endothelial-type promoter I.7 is upregulated in breast cancer. Thus, the levels of total aromatase mRNA levels from four promoters (II, I.3, I.7 and I.4) in breast cancer tissue are strikingly higher than normal breast tissue.

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transcription 3 (phospho-STAT3), glucocorticoid receptor (GR), and Sp1, respectively (Zhao *et al*. 1995*a*). Promoter I.4 is predominantly used and minimally activated in normal breast adipose tissue to maintain basal levels of aromatase expression (Bulun *et al*. 2005). Dexamethasone (DEX) is essential for promoter I.4 activation through stimulation of GR to bind to GRE (−133/−119). We found that TNFα and IL-11 are primarily present in malignant epithelial cells of mastectomy specimens (Meng *et al*. 2001); however, TNFα and type I cytokines (IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), and IL-6) in the presence of DEX stimulate promoter I.4 by different mechanisms. TNFα+DEX together induce expression of c-Fos and c-Jun, which can heterodimerize and bind to AP1 (−500/−494) (Zhao *et al*. 1996*b*). By contrast, type I cytokines+DEX stimulate tyrosine phosphorylation of Janus kinase 1 (JAK1), which in turn activate STAT3 through tyrosine phosphorylation. Phospho-STAT3 binds to GAS (−282/−272) as a part of I.4 activation (Zhao *et al*. 1995*b*). Promoter I.4 activation also requires binding of Sp1 protein to its binding site (+151/+158) (Zhao *et al*. 1995*a*). Moreover, TNFα and IL-11 inhibit adipogenic differentiation of undifferentiated fibroblasts, increase the number of aromatase-expressing fibroblasts and increase estrogen production, which in turn prompts IL-11 formation and increases TNFα receptor expression in malignant breast fibroblasts. Thus, promoter I.4-derived aromatase expression and estrogen formation are increased in breast cancer specimens, likely due to both cytokine-stimulated promoter I.4 activity and cytokine-induced desmoplastic reaction (Fig. 5A) (Harada 1997).

Promoter I.7 We identified a novel 101 bp untranslated first exon (I.7), a promoter immediately upstream of exon I.7 located approximately 36 kb upstream of the ATG translation start site of the aromatase gene in breast cancer tissues (Sebastian *et al*. 2002). Promoter I.7 is a TATA-less promoter with maximal promoter activity in human microvascular endothelial cells. Binding of the transcription factor GATA2 to a specific GATA *cis*-regulatory element in promoter I.7 is critical for aromatase regulation in endothelial cells (Sebastian *et al*. 2002). The level of aromatase mRNA with exon I.7 is significantly increased in breast cancer tissues and adipose tissue adjacent to tumors (Fig. 5B). Thus, promoter I.7 is a GATA2-regulated endothelial-type promoter of the human aromatase gene and may increase estrogen biosynthesis in vascular endothelial cells in breast cancer. Promoter I.7 activity may also be important for intracrine and paracrine effects of estrogen on blood vessel physiology.

Promoters I.3/II Promoters I.3 and II are located within 215 bp from each other. Because of their proximity, they may share some common *cis*-regulatory elements (Zhou & Chen 1999, Zhou *et al*. 2001). Thus, we will discuss the usage and regulation of these two promoters together. Unlike promoters I.4 and I.7, promoters I.3 and II have TATA boxes that are also 215 bp from each other (Zhou *et al*. 1997). The 5'-UTR of promoters I.3/II contains many positive *cis*-acting elements, of which the cAMP-responsive element (CRE, −522/−515), AP1 (−498/−492), CCAAT/enhancer-binding protein (C/EBP, −350/−337), C/EBP (−317/−304), CRE (−292/−285), CRE (−211/−199), and steroidogenic factor-1 (SF-1, −136/−124) are critical for promoter I.3/II activity. All identified *cis*-acting elements are located upstream of the promoter II TATA box. For promoter I.3, most of the *cis*-acting elements are upstream of its TATA box except for CRE (−211/−199) and SF-1 (−136/−124), which reside downstream of promoter I.3 and inside exon I.3 [24]. These two *cis*-acting elements are likely involved in regulation of both promoters I.3 and II (Fig. 5C) (Zhou & Chen 1999, Chen *et al*. 2001, Zhou *et al*. 2001, Clyne *et al*. 2002).

Aromatase promoter switching from I.4 to I.3/II is a major mechanism that mediates increased aromatase expression and local estrogen formation in adipose tissue adjacent to breast cancer and within breast cancer tissue (Agarwal *et al*. 1996, Utsumi *et al*. 1996, Zhou *et al*. 1996). Treatment of undifferentiated adipose fibroblasts with conditioned media from malignant breast epithelia induces aromatase expression (Zhou *et al*. 2001). This effect can be mimicked by PGE2, which is present in high concentrations in media conditioned with malignant epithelial cells but not in media conditioned with benign breast epithelial cells (Zhou *et al*. 2001). The biological actions of PGE2 are mediated via protein kinase C (PKC) and the adenylate cyclase/cAMP/PKA pathways (Zhao *et al*. 1996*a*, Brueggemeier *et al*. 2003). In fact, this PGE2 effect can be imitated using a combination of PKA activator (dibutyryl (Bt2) cAMP) and PKC activator (phorbol diacetate (PDA)) (Chen *et al*. 2007). Other stimulating factors present in breast cancer cell-conditioned medium can activate promoter I.3/II, independent of the PGE2/PKA/PKC pathway. Unknown factor(s) in the conditioned medium markedly induce expression of C/EBPβ, which binds to C/EBP (−317/−304) and stimulates promoter I.3/II. In contrast, C/EBP (−350/−337) is dispensable for promoter activation (Zhou *et al*. 2001).

The PGE2/PKA/PKC pathway regulates various transcription factors that bind to different

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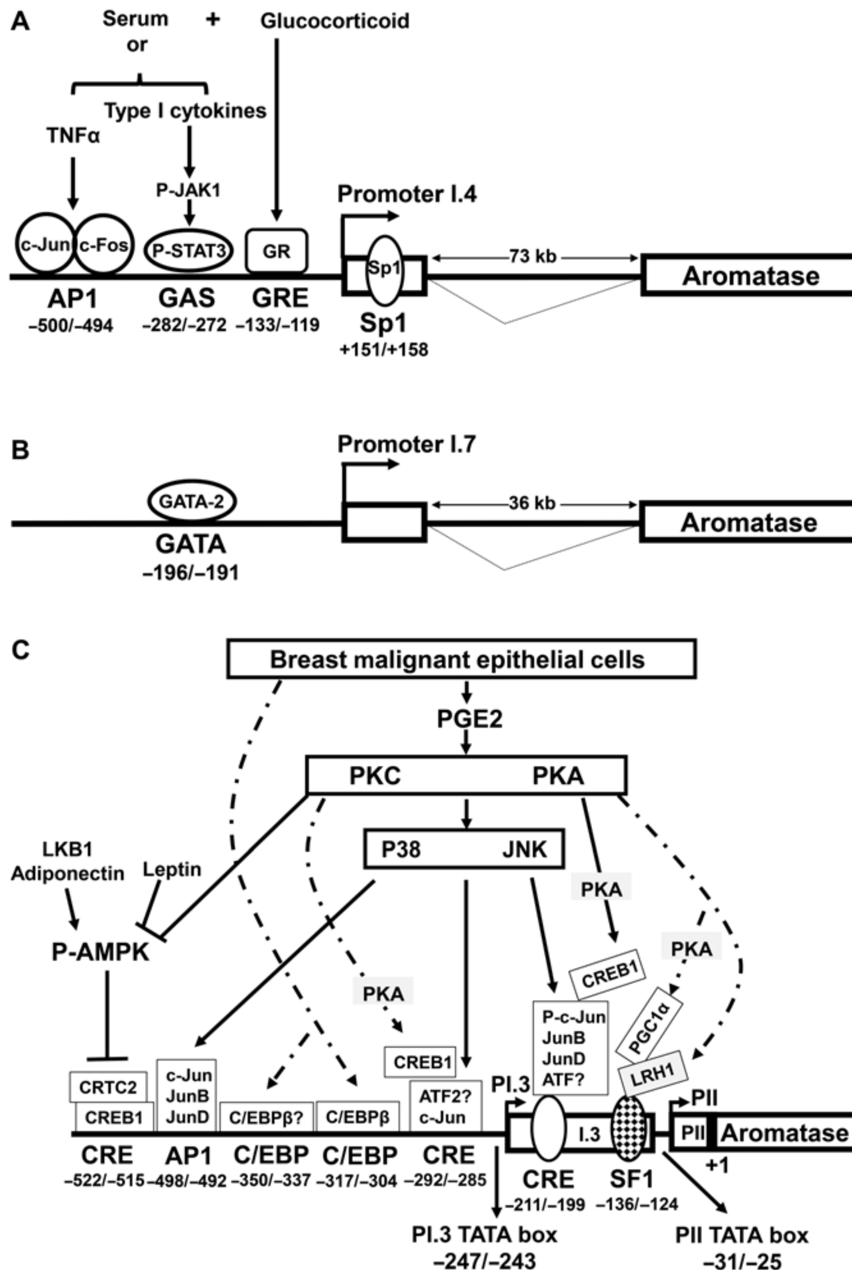

Figure 5

Activation of aromatase promoter I.4 and promoters I.3/II in breast adipose fibroblasts and promoter I.7 in breast endothelial cells. (A) Glucocorticoid plus serum stimulates aromatase promoter I.4. Serum can be substituted with TNFα or one of the type I cytokines. Glucocorticoid is obligatory for promoter I.4 stimulation by binding to and activating the glucocorticoid receptor (GR), which interacts with the glucocorticoid response element (GRE) in promoter I.4. TNFα plus glucocorticoid induces expression of c-Jun and c-Fos, which heterodimerize and bind to the AP1 site in promoter I.4. Type I cytokines plus glucocorticoid, on the other hand, activate the JAK1/STAT3 pathway, resulting in binding of tyrosine phosphorylated STAT3 to the interferon γ activation site (GAS) in promoter I.4. Sp1 protein binding to its binding site is also essential for promoter I.4 stimulation. (B) Promoter I.7 is a TATA-less promoter that directs expression of 29–54% of aromatase mRNA species in breast cancer. The −299/−35 regulatory region confers maximum basal activity in endothelial cells and contains at least three critical endothelial-type motifs, including Ets, GATA and E47. The binding of GATA2 protein to the −196/−191 bp site is important for baseline promoter activity in endothelial cells. (C) Breast cancer-conditioned medium and PGE2 can activate aromatase PI.3/II via the activation and binding of various transcription factors to their *cis*-acting elements, driven by distinct signaling pathways. This is accompanied by binding of LRH-1, CREB1, phosphorylated ATF-2 and c-Jun, JunB, JunD, and C/EBPβ to the promoter I.3/II regulatory region.

cis-acting elements. First, PGE2 strikingly increases both liver receptor homolog-1 (LRH-1) expression and its binding activity to the SF-1-binding site (–136/–124) in the aromatase promoter II in cultured adipose fibroblasts (Clyne *et al*. 2002). Secondly, CRE-binding protein 1 (CREB1) is a transcription factor directly phosphorylated and activated by PKA, which enhances CREB1 binding to both CRE (–292/–285) and CRE (–211/–199) in promoter I.3/II in breast adipose fibroblasts (Sofi *et al*. 2003, Chen *et al*. 2009). CREB1 mRNA levels are significantly higher in breast adipose tissue bearing a tumor than in normal breast adipose tissue, supporting the role of CREB1 in aromatase overexpression in breast cancer (Sofi *et al*. 2003). Thirdly, PGE2 activation of the p38 and JNK1 MAP kinases via both the PKA and PKC pathways is necessary for stimulation of aromatase expression via promoter I.3/II. Transcription factors ATF2 and c-Jun, which are *in vivo* substrates of p38 and/or JNK, are also phosphorylated and activated to interact with promoter I.3/II, but at different binding sites (Chen *et al*. 2007 , 2009, 2011). c-Jun binds to AP1 (–498/–492), CRE (–292/–285) and CRE (–211/–199) (Chen *et al*. 2011). p38- and/or

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JNK-phosphorylated ATF-2 may bind to CRE (−292/−285) and/or CRE (−211/−199). Malignant epithelial cell-conditioned medium is found to induce phosphorylation of ATF-2 and its binding to the CRE (−211/−199). However, we cannot exclude the possibility that phosphorylated ATF-2 induced by PGE2 does not bind to CRE (−292/−285) in the aromatase promoter II (Zhou *et al*. 2001, Bulun *et al*. 2005, Chen *et al*. 2007). PGE2 also increases the expression of transcription factors JunB and JunD via the PKA/PKC pathway and prompts their binding to AP1 (−498/−492) and CRE (−211/−199). Fourthly, AMP-activated protein kinase (AMPK) plays an important role in energy homeostasis (Brown & Simpson 2010). Both AMPK and its kinase LKB1 are involved in regulation of aromatase expression in human adipose fibroblasts. Treatment with activators of PKA and PKC, to mimic PGE2, results in nuclear translocation of CREB-regulated transcription coactivator 2 (CRTC2), increased CRTC2 binding to CRE (−522/−515) in promoter I.3/II, and increased aromatase activity via inhibition of the LKB1/AMPK pathway (Brown *et al*. 2009, 2010). Leptin treatment can mimic PGE2 action, whereas adiponectin exerts the opposite action (Brown *et al*. 2009). In summary, malignant breast epithelial cells secrete significant quantities of PGE2 that activates PKA and PKC pathways, which in turn induce aromatase expression by binding various transcriptional factors to the proximal I.3/II promoter region in adjacent adipose fibroblasts (Fig. 5).

Estrogen formation in various transgenic murine models

Aromatase is only present in gonads, brain and male gonadal fat in mice, whereas it is expressed in many more tissues, including breast tissue, in humans. Thus, wild-type murine models cannot mimic *in vivo* human aromatase expression patterns and estrogen formation in breast tissue. To circumvent this obstacle, several genetically modified mouse models have been generated to help understand the physiological and pathophysiological roles of aromatase and estrogen in normal breast tissue and the development of breast cancers (Table 1).

Tekmal and coworkers (1996) generated the first aromatase-overexpressing transgenic mouse model (Int5/aromatase) in 1996, which showed two-fold increased E2 levels in mammary tissue but no increase in circulating E2 levels. The transgene is driven by mouse mammary tumor virus (MMTV) promoter and gives rise to aromatase overexpression in mammary epithelial cells, leading to mammary hyperplasia, dysphasia and fibroadenoma in females and gynecomastia in 3-month-old males in a paracrine and intracrine manner (Tekmal *et al*. 1996, Kirma *et al*. 2001, Li & Rahman 2008). However, overexpressing aromatase in mammary epithelial cells of Int5/aromatase mice cannot mimic human aromatase expression, which occurs predominantly in adipose fibroblasts.

AROM+ mice, bearing the human aromatase gene driven by the human ubiquitin C promoter, have aromatase expression in many tissues (Li *et al*. 2001). AROM+ male mice show an extreme imbalance in sex hormone levels with highly elevated serum E2 concentrations and significantly reduced testosterone levels compared with wild-type littermates. In addition, serum levels of prolactin and corticosterone are increased and serum FSH levels are decreased (Li *et al*. 2001). AROM+ mice exhibit a low incidence of hyperplasia (10%) in females of advanced age (15 months) and gynecomastia in males at young age (20 days) (Li *et al*. 2001, 2002, Li & Rahman 2008). The aromatase-expressing cell types in mammary tissue are unknown. As the AROM+ mice have abnormal levels of several hormones, these mice cannot be used to pinpoint estrogen-specific biological functions. Moreover, AROM+ female mice with a milder phenotype indicate that breast *in situ* estrogen, but not systemic estrogen, may be more important for breast cancer development.

A doxycycline-inducible, breast epithelial cell-specific aromatase-expressing transgenic mouse (Arom) model was developed to investigate the molecular pathways involved in the development of mammary preneoplasia and carcinoma (Diaz-Cruz *et al*. 2011). These Arom mice exhibit increased preneoplasia and carcinoma. Increased prevalence of pathologic changes in Arom mouse mammary tissue correlate with increased cyclin E and cyclin-dependent kinase 2 expression. Arom mice have significantly higher aromatase activity in mammary tissue while the serum estrogen levels are not different, indicating that estrogen produced in epithelial cells induce breast cancer development in a paracrine and intracrine manner. Again, overexpressing aromatase in mammary epithelial cells does not mimic human aromatase expression, which occurs predominantly in adipose fibroblasts.

We generated a transgenic humanized aromatase ($Arom^{hum}$) mouse line containing a single copy of the human aromatase gene to study the link between aromatase expression in mammary adipose tissue and breast pathology (Chen *et al*. 2012, Zhao *et al*. 2012). $Arom^{hum}$ mice express human aromatase, driven by the proximal human promoters II and I.3 and the distal promoter I.4, in breast adipose fibroblasts and myoepithelial cells. Estrogen levels in the breast tissue of $Arom^{hum}$ mice are higher than in wild-type mice, whereas circulating levels

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Table 1 Comparison of the various aromatase transgenic mouse models.

Mouse models	Promoter used	Serum E2 levels	Mammary E2 levels	Mammary phenotypes
Int5/aromatase	MMTV (epithelial cells)	No change	two-fold increase	Hyperplasia, dysphasia and fibroadenoma in females and gynecomastia in males
Arom+	Ubiquitin C (ubiquitous expression)	~10- to 22-fold increase	Unknown	Hyperplasia in females and gynecomastia in males
Arom	MMTV (epithelial cells)	Unknown	No change	Preneoplasia and carcinoma
Arom^hum	Native (myoepithelial cells and adipose fibroblasts)	No change	~two-fold increase	Hyperplasia and carcinoma in females and gynecomastia in males

are similar. Arom^hum mice exhibit accelerated mammary duct elongation (puberty), and an increased incidence of lobuloalveolar breast hyperplasia (middle age) and mammary tumors (aging mice, our unpublished data). Hyperplastic epithelial cells have remarkably increased proliferative activity. With this model, we demonstrated that the human aromatase gene can be expressed via its native promoters in a wide variety of mouse tissues and in a distribution pattern nearly identical to that of humans. Locally increased tissue levels, but not circulating levels, of estrogen appeared to exert hyperplastic effects on the mammary gland in a paracrine manner. This novel mouse model will be valuable for developing tissue-specific aromatase inhibition strategies.

In summary, studies with these animal models have demonstrated that increased estrogen synthesis in mammary epithelial cells, adipose fibroblasts or in multiple organs with strikingly higher systemic estrogen leads to benign mammary hyperplasia and fibroadenoma in females and gynecomastia in males. Moreover, local mammary aromatase expression and estrogen formation increase breast cancer risk in a paracrine and/or intracrine manner. However, none of these murine models reveals the role of only increased circulating E2 in breast cancer development.

Estrogen and endometrial cancer

Endometrial cancer is the most common gynecological malignancy in US women (Morice *et al*. 2016). Type 1 endometrial cancer is the most common type, thought to be caused by excess estrogen, usually not very aggressive, and slow to spread to other tissues; however, type 2 endometrial cancer is not related to estrogen stimulation and usually presents as a higher-grade cancer with a poorer prognosis (Bokhman 1983, Rizner 2013). Type 1 endometrial adenocarcinoma occurs in the context of chronic exposure to excess estrogen (endogenous and exogenous) with insufficient opposing progesterone. High levels of endogenous estrogen arise as a consequence of various disease states, including anovulation, polycystic ovarian syndrome and obesity (Creasman 1997, Morice *et al*. 2016). Exogenous estrogen-only hormone replacement therapy (HRT) can promote the development of endometrial cancer compared with an estrogen plus progestin regimen (Beresford *et al*. 1997, Rossouw *et al*. 2002). With this regimen, progestin serves as an estrogen antagonist in the endometrium. Progestins have been used clinically to treat endometrial neoplasias. Tamoxifen exerts anti-estrogen effects in breast but acts as an E2 agonist in the uterus. Thus, treatment of breast cancer with tamoxifen increases the risk of developing endometrial cancer (Swerdlow *et al*. 2005, Chen *et al*. 2014). Although the pro-proliferative (Siiteri 1978, Key & Pike 1988) and DNA damaging (Roy & Liehr 1999, Shibutani *et al*. 2000) effects of estrogen and its metabolites lead to the hyperproliferation and transformation of cells, the underlying mechanisms involved in endometrial carcinogenesis from chronic estrogen exposure are unclear.

Three major sites in the body produce estrogen in women with endometrial cancers (Bulun *et al*. 2005, Bulun 2009). First, E2 secreted by the ovary reaches endometrial cancer tissue through the systemic circulation. Secondly, aromatase in adipose tissue and skin catalyzes the conversion of circulating androstenedione to E1 that is subsequently converted to E2, and both E1 and E2 can enter the circulation and reach sites of endometrial cancer. Finally, the third source is estrogen that is synthesized locally in endometrial cancer tissues.

As indicated above, a cascade of steroidogenic genes is needed to synthesize estrogen from cholesterol, including aromatase – the key enzyme for the last step of estrogen formation (Bulun *et al*. 2005). In premenopausal women, ovarian estrogen is the main source of estrogen for endometrial tissue or endometrial cancer, as disease-free endometrium lacks aromatase and thus does not produce estrogen locally (Bulun 2009). Although aromatase mRNA is absent in tissue homogenates of both proliferative-phase

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endometrium and secretory-phase endometrium, it is present in first-trimester decidua (Gibson *et al*. 2013). During the luteal phase, the progesterone-dependent 17β-HSD 2 enzyme catalyzes the conversion of the biologically active E2 to the less estrogenic E1, which partially inhibits systemic estrogen action.

Quantitative PCR with high cycle numbers amplifies aromatase mRNA in endometrial biopsies, but we cannot detect aromatase enzyme activity in cultured endometrial stromal cells from disease-free women (Noble *et al*. 1996). Aromatase activity or mRNA cannot be induced by PGE2 or cAMP analogs in stromal cells from disease-free women, which is the main molecular mechanism responsible for increased expression of steroidogenic genes in stromal cells from diseased tissue such as endometriosis (Noble *et al*. 1996, Bulun *et al*. 2001, Sebastian & Bulun 2001, Sebastian *et al*. 2002). In postmenopausal women, extraovarian adipose tissues and skin may be the main sites of estrogen formation, which exerts biological effects on the endometrium or endometrial cancer tissues in an endocrine manner. The local concentration of E2 in endometrial cancer tissues is also reported to be higher than in blood or in the endometrium of cancer-free women (Nagasako *et al*. 1988, Potischman *et al*. 1996, Sherman *et al*. 1997, Berstein *et al*. 2002). It is, therefore, conceivable that endometrial cancer synthesizes E2 *in situ*, which then contributes to cancer development. One study demonstrated significant conversion of androstenedione to E1 in endometrial cancer tissue (Yamamoto *et al*. 1993). Aromatase protein and mRNA are detected in endometrial cancer using immunohistochemistry and RT-PCR, whereas aromatase expression is low or undetectable in endometrial hyperplasia (a precursor lesion of endometrial cancer) (Bulun *et al*. 1994, Watanabe *et al*. 1995). Immunoreactive aromatase is also found in malignant epithelial, endometrial stromal and myometrial cells. These observations suggest that intratumoral aromatase may play a role in the pathology of endometrial cancer. Taken together, as supported by aromatase availability, tissues from ovaries, skin, adipose and endometrial cancer are the major sources of estrogen for premenopausal endometrial cancers; extraovarian skin and adipose tissue and endometrial cancer tissue are predominant sites of estrogen formation in postmenopausal endometrial cancers.

Conclusion and future directions

Estrogen plays a critical role in tumor formation and growth, and provides a therapeutic target for the prevention and treatment of breast and endometrial cancers. Aromatase is the rate-limiting enzyme in estrogen biosynthesis, with expression driven by multiple tissue-specific, untranslated and alternative promoters. Aromatase inhibitors have been used successfully to treat breast cancer in postmenopausal women but they reduce aromatase activity indiscriminately throughout the body, resulting in severe estrogen deprivation and major side effects such as bone loss and abnormal lipid metabolism. The shift of aromatase promoter usage in malignant tissues is a major mechanism driving abnormal aromatase overexpression and estrogen excess. Targeting these partially tissue-selective promoters in breast and endometrial cancers provides a potential opportunity to inhibit aromatase activity specifically in breast and endometrial tissue without the common side effects of aromatase inhibitors in other organs, such as the bone. Future basic research will focus on the mechanism of aromatase gene regulation in breast and endometrial cancer to identify ideal drug targets for developing selective aromatase modulators or inhibitors and for blocking estrogen production in targeted tissues.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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