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Title: **Extra-gonadal sites of estrogen biosynthesis and function**

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Abstract

Estrogens are the key hormones regulating the development and function of reproductive organs in all vertebrates. Recent evidence indicates that estrogens play important roles in the immune system, cancer development, and other critical biological processes related to human well-being [1-4]. Obviously, the gonads (ovary and testis) are the primary sites of estrogen synthesis, but estrogens synthesized in extra-gonadal sites play an equally important role in controlling biological activities [5-7]. Understanding non-gonadal sites of estrogen synthesis and function is crucial and will lead to therapeutic interventions targeting estrogen signaling in disease prevention and treatment. Developing a rationale targeting strategy remains challenging because knowledge of extra-gonadal biosynthesis of estrogens, and the mechanism by which estrogen activity is exerted, is very limited. In this review, we will summarize recent discoveries of extra-gonadal sites of estrogen biosynthesis and their local functions and discuss the significance of the most recent novel discovery of intestinal estrogen biosynthesis.

Introduction. Estrogens are a class of steroid hormones that regulate the development and function of male and female reproductive organs. In the ovary, estrogen synthesis begins in theca cells with androgen synthesis and ends with conversion of androgens to estrogens in granulosa cells by the enzyme aromatase. In the male gonad, estrogens are synthesized in the Leydig cells, Sertoli cells, and mature spermatocytes [8]. Like other steroid hormones, estrogens enter passively into the cells and bind to the estrogen receptors, which then regulate the transcription of downstream estrogen-responsive genes. Among the number of different forms of estrogens, 17β-estradiol (estradiol) is the most common and potent form of estrogen in mammals. Estradiol is also produced in a number of extra-gonadal organs, including the adrenal glands, brain, adipose tissue, skin, pancreas [5-7], and other sites yet to be identified. The discoveries of extra-gonadal sites of estradiol synthesis greatly expands our knowledge of the novel roles of estrogens beyond the reproductive system.

Extra-gonadal sites of estrogen synthesis and its local roles. The first discovery of extra-gonadal estrogen synthesis was made in 1974 by Hemsell and his colleagues when they made an unexpected observation that androgens were converted to estrogens in adipose tissue [9]. Since then, a number of other extra-gonadal sites of estrogen synthesis have been discovered. Adipose tissues are considered to be the major source of circulating estrogen after the gonads in both men and women, and the contribution made by the adipose tissues to the total circulating estrogens increases with advancing age [9]. The chemical structure and biological activity of the estrogens synthesized in the extra-gonadal sites are not different from those that are produced by the gonads. However, there are unique features that make the extra-gonadal estrogen synthesis differ from the gonadal synthesis. A major difference is in the biochemical pathway of estrogen synthesis. The tissues and cells of the extra-gonadal sites of estrogen synthesis are

unable to synthesize C19 steroids, the precursors of estrogen synthesis, but are able to convert C19 steroids to estrogens, a critical and rate-limiting step mediated by Cyp19 aromatase. Hence, extra-gonadal estrogen synthesis is dependent on an external source of C19 precursors [7] and the level of aromatase expression. Because C19 steroids can be supplied to a local tissue via circulation and are converted to estrogens in any tissue where aromatase is expressed, the presence of aromatase expression in a local tissue confirms extra-gonadal estrogen synthesis. **Table 1** lists the peripheral tissues that express aromatase and are therefore able to convert C19 precursors to estrogens. These extra-gonadally synthesized estrogens are thought to act and be metabolized locally, which limits their systemic effects [10]. Another unique feature of extra-gonadal estrogen synthesis is that while the total amount of estrogen synthesized in each tissue may be small, the local tissue concentrations of estrogens could be high enough to exert biological impact locally. The functional roles of estrogens are mediated mostly by estrogen receptors that are nuclear receptor transcription factors. Therefore, a tissue that expresses one or more estrogen receptors is considered to be a target of estrogenic regulation. **Table 2** lists key organs and tissues that express estrogen receptors.

1. Extra-gonadal sites of estrogen synthesis

Sites	Evidence of 17β-estradiol synthesis			References
	Cyp19 mRNA	Cyp19 protein	17β-estradiol	
Brain	Astrocyte (rat, mouse, human), Hippocampus and hypothalamus (rat, mouse, monkey, human)	Astrocyte (mouse), GnRH (rat), Dentate gyrus/ pyramidal cell (rat, mouse, human, monkey), Interneurons(human), Granular cell (human, monkey), Purkinje cell (human, mouse), Ependymal and subependymal cell (human).	Astrocyte (rat, monkey).	[11-20]
Fat	Stromal cell (human), Adipocyte (human)	Stromal cell (human), Adipocyte, mesenchymal cell (human)		[17-20]
Bone	Osteoblast (human)	Osteoblast (human)	Osteoblast (human)	[21-23]
Liver	HepG2 hepatoma and hepatocellular carcinoma (human), Hepatocyte (porcine).	HepG2 hepatoma and hepatocellular carcinoma (human)	HepG2 hepatoma and hepatocellular carcinoma (human)	[24-26]
Adrenal gland	Adrenocortical cell (human, porcine, rat)	Adrenocortical cell (human)	Adrenocortical cell (rat)	[26-28]
Intestine	Parietal cell (rat)	Parietal cell (rat)	Parietal cell (rat)	[29]
Skin	Fibroblast (human). Keratinocyte (human).	Fibroblast (chicken, human), Keratinocyte (human)	Fibroblast (chicken)	[30-32]
Blood vessel	Smooth muscle cell (human, rat, bovine)	Smooth muscle cell (human, rat, bovine)		[33-35]
Spleen	T cell (mouse)	T cell (mouse)		[36]

2. Extra-gonadal sites of estrogen receptor expression

Sites	Receptor subtypes			References
	ERα	**ERβ**	**Other receptors**	
Brain	Cholinergic neuron (rat), GABAergic neuron (rat), Pro-opiomelanocortin neuron (mouse).	GnRH neurons (mouse), Subiculum neuron (monkey), Ammon's horn neuron (monkey)	GPER1 (glial cell, rat), GPER1 (GABAergic neuron, rat).	[37-41]
Fat	Adipocyte (human)	Adipocyte (human)	GPER (adipocyte, mouse)	[42-44]
Bone	Osteoblast (mouse), Osteocyte (mouse).	Osteoblast (human), Osteocyte (human), Osteoclast (rat, human).		[45-50]
Liver	Hepatocyte (rat)			[29]
Blood vessel	Smooth muscle cell (human), Vascular endothelial cell (human)	Endothelial cell (human)	GPR30 (endothelial cell, rat)	[51-54]
Intestine	Epithelial cell (rat), Parietal cell (rat), Myenteric neuron (rat)	Epithelial cell (rat), Parietal cell (rat)	GPER (colonic epithelia, human)	[55, 56]
Skin	Keratinocyte (human), Mast cell (human) Sebocyte (human)	Keratinocyte (human), Mast cell (human)		[57-59]
Adrenal gland	Adrenal cortex (rat)	Adrenal cortex (rat)	GPER1 (rat)	[60]
Muscle	Satellite cell (rat)	Satellite cell (rat)		[61]
Kidney	Mesangial cells (human, mouse)	Mesangial cells (human, mouse)		[62]
Pancreas	β-cell (mouse)	β-cell (mouse)		[63, 64]

Adipose tissues. Adipose tissues, where estradiol stimulates the production of high density lipoprotein cholesterol (HDL) and triglycerides while decreasing LDL production and and fat deposition [65, 66], are the most extensively studied sites of extra-gonadal estrogen synthesis. Both male and female aromatase-

deficient (Cyp19KO) mice exhibit obesity and dyslipidemia [65, 66], proving that estradiol plays a beneficial role in the lipogenesis. However, an adverse effect of adipose tissue-driven estradiol is also indicated in the pathogenesis of breast cancer. For instance, in a breast with a tumor, adipose tissues proximal to the tumor exhibit higher aromatase activity than those distal to the tumor [67].

Bone. Aromatase expression in human bone has been demonstrated in osteoblasts, chondrocytes, and fibroblasts (**Table 1**), where they convert circulating androgens into estrogens [68]. In the bone of prepubertal children, the locally synthesized estradiol stimulates epiphyseal maturation during the growth phase [69]. However, in both males and females, the massive pubertal increase of estradiol leads to increased apoptosis of chondrocytes in the epiphyseal plate, causing chondrocyte depletion and hence, ossification and growth slow-down [70]. In adults, estradiol increases bone formation and mineralization and reduces bone resorption, thus reducing the risk of osteoporosis (68). Therefore, it is not surprising that the incidence of osteoporosis increases in postmenopausal women as their ovaries lose estradiol synthetic capacity.

Skin. Aromatase expression in the skin occurs mainly in hair follicles and sebaceous glands [71]. Glucocorticoids, cAMP analogs, growth factors, and cytokines modulate aromatase expression in these cells and therefore, local estrogene synthesis [72]. Estradiol enhances collagen synthesis, increases skin thickness, and stimulates blood flow in the skin. Therefore, in situ estrogen synthesis in the skin is vital for maintaining healthy skin [73]. Estradiol also prolongs the anagen phase of the hair cycle and therefore enhances hair growth by increasing the synthesis of essential growth factors stimulating the proliferation of hair follicle cells [74].

Liver. In the liver, estradiol regulates protein synthesis, including lipoprotein and proteins responsible for blood clotting (factors II, VII, IX, X, plasminogen) [75]. Estrogen signaling is also essential in regulating glucose homeostasis, thus improving glucose tolerance and insulin sensitivity [76]. Recent research has explored the possibility that postmenopausal women with nonalcoholic fatty liver disease and with long durations of estrogen deficiency could have a higher risk of having severe fibrosis than premenopausal women [77]. Estrogen receptor beta (ERβ) is implicated in mediating the protective role that estradiol plays under pathogenic condition in the liver as it shows potent anti-proliferative and anti-inflammatory properties. As such, chronic disease is linked to elevated ERβ expression in the liver [78]. ERβ is also known to mediate the anti-tumor action of estrogens in intrahepatic cholangiocarcinoma [79].

Brain. High levels of estrogen receptors are expressed during brain development. During this period, sex hormones determine apoptosis, neuronal migration, neurogenesis, axonal guidance, and synaptogenesis. Estradiol induces sexual differentiation in the developing brain. Aromatase mRNA expression in the hypothalamus of males peaks before and after birth, inducing sexual differentiation of the brain [80]. In the brains of both males and females, estradiol provides a neuroprotective effect. Estradiol's prevention of neurodegeneration in brain tissues is proven in both the Cyp19KO mouse model and the aromatase

inhibitor-treated mouse [12]. Inhibition or null mutation of aromatase, a key enzyme for estradiol synthesis, results in accelerated neurodegeneration [12]. Estradiol effects in the brain also include regulating mood, pain sensitivity, motor control, and cognitive behavior [17-20]. Estradiol regulates neuronal metabolism by modulating the expression of metabolic enzymes such as GLUT (glucose-transporter), glycolytic enzyme hexokinase, pyruvate dehydrogenase (PDH), aconitase, and ATP synthase [81].

Adrenal gland. Estrogens stimulate adrenal cortex growth during development by promoting cell proliferation and enhancing steroidogenic activity by increasing StAR and SF-1 expression in the adrenal gland [34]. In the fetal adrenal gland, estradiol and ACTH form as a positive regulatory loop in which estradiol increases ACTH secretion from adrenal cortex while ACTH increase estradiol in the ovary [82].

Pancreas. Estradiol increases insulin gene expression and insulin content in β-cells [63, 83], increases β-cell proliferation during pancreatic development and recovery from injury [84], and prevents apoptosis of β-cells upon inflammatory insult [63] via ERα– and ERβ-mediated pathways.

Others. In the blood vessel, estradiol positively impacts vascular function by preventing the oxidation of LDL cholesterol, stimulating nitric oxide synthesis and release, and inhibiting fibroblast transition to myofibroblast, preventing cardiac fibrosis [85-87] and atherosclerosis development. In the muscle, estradiol increases muscle mass and strength, alleviating disuse-induced muscle atrophy and promoting regrowth after reloading. It also stimulates muscle repair by stimulating satellite cell proliferation [88, 89]. Estradiol replacement on ovariectomized mice shows that estradiol can reduce stiffness in muscle as well as stimulate muscle regeneration [43]. In the kidney, estradiol has a role of protecting kidney functions during progressive glomerulosclerosis in the female rat remnant kidney model [90]. In the intestine, to maintein the intestinal epithelium, estrogens are necessary. Estrogens improve epithelial barriers and reduce intestinal permeability [91], preventing chronic mucosal inflammation in animals and humans.[92]

Inflammation. Estrogens play an important role in the inflammatory response by regulating development, proliferation, migration, and apoptosis of immune cells [93]. Lymphocytes have been shown to express estrogen receptors (ERα and ERβ), but the expression levels of both receptors vary among cell types. CD4+ T-lymphocytes express ERα whereas B-lymphocytes express ERβ [94]. In contrast, CD8+ T-lymphocytes express both receptors at low but equivalent levels [94]. Regardless of subcellular differences, estrogens appear to exert a suppressive effect on both B- and T-lymphopoiesis. In support, B-lymphocyte formation is selectively reduced with estradiol treatment [95], and ovariectomy results in increased B-lymphopoiesis [96, 97]. In addition to the inhibitory effect on lymphopoiesis, estradiol has been shown to influence T helper (Th) responses; inhibit the production of Th1 cytokines such as IL-12, TNF-α and IFN–γ; and stimulate Th2 anti-inflammatory cytokine production such as IL-10, IL-4, and TGF-β [98]. Estradiol has also been shown to modulate the main activities (maturation, differentiation, and migration) of myeloid cells, including monocytes, macrophages, and dendritic cells [99-102]. Thus, estradiol has an

important impact on immune cells and affects both the innate and the adaptive immune systems, which may account for its contribution in diseases associated with immune disorder.

Estrogen and estrogen receptors in the gut

In an effort to identify extra-gonadal sites of *de novo* estradiol synthesis, we generated a double transgenic mouse line in which a transgenic aromatase (*cyp19*) promoter induces the expression of a red fluorescent protein (RFP) (un-published). In this animal, RFP signal is strongly expressed in the Peyer's patch (Pp), a secondary lymphoid organ in the intestine. Pp have an organizational structure similar to lymph nodes consisting of multiple follicles and interfollicular areas. A follicle is made of a germinal center that is filled with proliferating B-lymphocytes, follicular dendritic cells, and macrophages; the interfollicular area is populated with T-lymphocytes as well as B-lymphocytes, macrophages, and dendritic cells. As part of the gut-associated lymphoid tissue, Pp are known as inductive sites of intestinal immune responses [103]. The induction process in the Pp starts with sensing antigens or microbes in the gut lumen by M-cells located in a monolayer of specialized intestinal epithelial cells known as the follicle-associated epithelium**.** M-cells transport antigens to antigen-presenting cells, specifically dendritic cells (DCs), within the underlying sub-epithelial dome through transcytosis. Dendritic cells then further present antigens to T-and B-lymphocytes, triggering priming and proliferation of lymphocytes to complete the immune response. A well-known effect of the Pp's induction function is generating antigen-specific intestinal IgA responses, which is critical for maintaining host-microbiota interaction, generating immune tolerance, and preventing infection [104-106]. Interestingly, estrogens plays a significant role in the gastrointestinal tract. In this section, we will describe some of the lesser known roles for estrogen in the gastrointestinal system.

Napoleon Bonaparte was not aware of the true importance of his words when he said "*An army marches on its stomach.*" Technically, an army marches on its intestines. The gastrointestinal tract (GIT) is a unique environment colonized by a remarkable variety of bacteria as well as other organisms including fungi and viruses. This superorganism, the microbiome, is not a simple spectator in biological processes but is an active component of the biochemical and metabolic health of the host [107]. The microbiome is capable of digesting large molecules into simpler ones that can be efficiently reabsorbed by the host. The importance of a healthy microbiome has been well published [108-112], and multiple pathologies have been correlated with poor diversity of the microbiome, including irritable bowel (IBS)[113], osteoporosis [114, 115], and gluten intolerance [116]. Therefore, controlling the microbiome is paramount to maintaining an optimally functioning GIT. The mucosal epithelium is perfectly adapted to monitor both microbial and nutrient composition. The release of antimicrobial peptides [117] or anti-inflammatory molecules maintains the optimal microbial ecology depending on the current GIT contents.

Appetite. Researchers have noted a correlation between estradiol levels and appetite. Food intake is significantly decreased during the preovulatory period when estradiol levels are increasing [118]. These actions are attributed to estradiol inhibiting appetite indirectly through cannabinoid receptors [119]. Further,

blocking estrogen receptors with ICI182,270 ablates any action of estradiol on appetite [119]. What is more interesting is that appetite is influenced by the microbiome present in the GIT. Bacterial peptides signal hunger or satiation [117, 120]; in essence, the bacteria control our desire to eat. Locally synthesized estrogen produced in response to microbiome composition in turn may influence immune responses, bringing us back to control of microbiome composition.

Immune function. Estrogenic compounds in the gut lumen suppress immune function through targeted apoptosis and inhibition of cell proliferation in the germinal centers of ileal Pp [121]. The Pp are important in generating protective immune responses to pathogens through both innate and cell medicated responses [121] and are also key in tolerizing the host to food antigens. The mucosal surfaces of the gut must maintain homeostasis, allowing sufficient function of Pp to prevent immune responses to food antigens yet not responding prolifically to commensal bacteria in the gut. Abnormal Pp function through estrogenic compounds is responsible for initializing autoimmune responses and impaired innate responses. Again, we see the constituents of the gut signaling control of the microbiome composition. This leads into the next topic of estrogens and cancer.

Cancer. The small intestine is the main absorptive area of the gastrointestinal tract. To maximize absorption, the epithelial layer is covered with invaginations or crypts of Lieberkühn and exists as a sheet of single cells. These cells are prone to injury and are therefore replaced every 3-5 days [122]. To facilitate this replacement, the base of the crypts is populated with stem cells that differentiate into the mature epithelium as they migrate towards the crest of the crypt. ERα and ERβ are both expressed in the crypt cells. However, they are distributed such that ERα is predominantly expressed in the cells at the base of crypts and ERβ is expressed in the cells towards the crest. ERα signaling stimulates proliferation [123] and ERβ signaling opposes this action [124, 125], and the net signaling from the two receptors controls proliferation. To further support the role of estrogen receptors in tumor development, ERβ–deficient mice demonstrate a hyper-proliferation of the colonic epithelium with progression to colon carcinoma [91, 126]. More than 30 years ago, it was established that there is an associative risk between reduced estrogen levels and colorectal cancer in menopausal women [127] and that hormone (estrogen) replacement therapy reduces the incidence of colorectal cancer [128]. Recent literature on estrogen and colorectal cancer confirms an anti-tumorigenic role for estrogen signaling in the gut due to preferential ERβ signaling [129].

However, estrogen in the gut is not always good. A recent review by Kwa *et al* [107] associated the “estrobolome” [130], bacteria with the capacity to metabolize estrogens, with level of risk for breast cancer. A phylogenetic diverse microbiome favors metabolism of conjugated estrogens. Once metabolized, the free estrogens are more easily reabsorbed increasing systemic estrogen levels. Increased circulating estrogens levels increases relative risk for hormone dependent malignancies such as breast cancer. As described above, our recent unpublished work has demonstrated that not only are Pp able to respond to estrogens, but they are also a significant site of estradiol synthesis. Thus, Pp are able to monitor the

bacterial diversity of the gut lumen and secrete estradiol. This estradiol then regulates immune responses locally and ultimately alters the diversity of the microbiome.

In conclusion, although estradiol is best recognized as sex hormone that regulates the development and function of reproductive hormone across the entire mammalian species, ever-growing evidence demonstrates its multi-faceted nature in exerting its role in non-reproductive organs and systems under normal as well as pathological conditions. It will be exciting to see what other functions estradiol may play in local tissues and from where the hormone is supplied to those sites.

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