Trends in Endocrinology & Metabolism

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Review

Natural Estrogen Receptor Modulators and Their Heterologous Biosynthesis

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Estrogen receptors (ERs) are transcription factors highly involved in physiological development and metabolism in the human body. They also play important roles in the treatment of cancer and metabolic diseases. Chemicals that interact with ERs can be used to treat diseases and maintain health. Phytoestrogens are natural chemicals that have been documented to possess significant ER modulatory activities. However, since phytoestrogens usually exist at low quantities in nature, heterologous biosynthesis techniques have quickly developed in recent years in order meet the demands for needed therapeutic amounts. In this review, the performance of phytoestrogens as ER modulators is described along with recent advances in biosynthesis techniques.

Highlights

ERs are highly involved in physiological development and metabolism. They play important roles in actions of immunomodulation and neuroprotection.

Phytoestrogens are effective for maintaining health and treating diseases through regulation of ER signaling pathway.

Heterologous biosynthesis is a promising technique for phytoestrogens production.

Phytoestrogens: Natural ER Modulators

ERs (ERα and ERβ) belong to the nuclear receptor superfamily of ligand-regulated transcription factors. They function as signal transducer and transcription factors to regulate the expression of target genes [1]. Their structural characteristics and regulatory mechanisms have been extensively investigated (Box 1). Various physiological functions and bioactivities (Box 2) of ERα and ERβ have also been reported. ERα is essential for the maturation and function of the reproductive system, bone development, cognitive system development, and metabolism [2]. While the ERβ isoform contributes less to these aspects than ERα contributes, it does play an important role in treatment of disease. Furthermore, ERβ can interact with ERα to exert novel physiological function. In clinical cancer studies, the absence of ERβ expression is associated with larger tumors, higher histological grade, and increased metastasis to the lymph nodes [3]. Due to the important functions of ERs in human development and health, searching for potent ERs modulators remains a hot area of investigation. Since many of the undesirable effects of estrogens are involved in the activation of ERα signaling, selective ERα/ERβ agonists or antagonists would be useful. Tissue-selective or non-nuclear selective ERα agonists could be good candidates for activating ERα in bone and adipose tissue, but not in tissues containing ER-related cancers.

Phytoestrogens are natural ER modulators with structural and functional similarities to endogenously produced mammalian estrogens [4]. Flavonoids, stilbenoids, coumarins, and lignans are phytoestrogens with impressive affinity and selectivity for ERs. These phytoestrogens are usually safe and can be good candidates to target the ER signaling pathway in health and disease (Box 3).

Flavonoids

Flavonoids are a family of phytoestrogens that contains several subclasses, including flavones, flavanols, flavonols, flavans, anthocyanins, chalcones, isoflavones, isoflavonols, and isoflavans [5]. Due to their structural similarity to E2, many flavonoids act as significant ER modulators. Epidemiological studies suggest that the phytoestrogen-enriched diets, like those containing

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Box 1. Structure and Regulatory Mechanisms of ERs

ERs contain eight transcribed exons that give rise to six polypeptide domains: domains A/B at the N terminus containing AF-1, domain C (DNA-binding domain), hinge region D (epicenter of recruitment, binding and function of receptor comodulators and post-translational modifications), domain E containing ligand-binding domain and AF-2, and domain F. The ligand-binding domain is folded into three-layered antiparallel α-helical sandwich comprising a central core layer of three helices (H5/H6, H9, and H10) sandwiched between two additional layers of helices (one formed by H1–H4 and another by H7, H8, and H11). The classic regulatory mechanism is that ligand binds to ER, launching a conformation change. An activated state is formed for homodimerization, which orients the AF-2 region to recruit coregulators, such as coactivators and cosuppressors. The DNA-binding domain contacts the hormone response elements. The ER–coactivator complex recruits proteins like histone acetyltransferase to upregulate the expression of target gene, while the ER–corepressor complex recruits histone deacetylase or other proteins to inhibit the expression of target genes. The most notable coactivators are p160 family members, and they interact with AF-2 by LxxLL sequences. Common corepressors include nuclear receptor corepressor and silencing mediator of retinoic acid and thyroid hormone receptor, which interact with AF-2 through the LxxI/HIxxxI/L sequence (CoRNR-box). Several alternative signaling mechanisms diverge from the classical model. ER tethers the heterologous DNA-binding transcription factor to regulate genes that lack hormone response element sequences within promoters. Ligand-independent actions are also observed when ERs are activated via intracellular second messenger and related signaling pathways [54]. cAMP is a second messenger of G protein-coupled receptors and an activator of the protein kinase A pathway. It can stimulate the ER-mediated transcription of target genes in the absence of estrogens. The AF-2 domain is postulated to be the interaction position. Polypeptide growth factor can mimic the effect of E2 to activate ERα by interacting with AF-1 domain [55]. This mechanism relies mainly on cellular kinase pathway to modify the phosphorylation state of ERs and associated proteins, like coactivators.

Glossary

Modular pathway engineering: design several multigene pathways to improve the flux of desired pathway and prevent the flux of branch pathway.

Vector development: design the vector to support the assembly of multiple molecular components, including regulatory signal elements (promoter, operator, ribosome binding site and terminator) and multigene pathway.

soybean, are positively associated with decreased risk of hormone-induced cancers. Genistein (Table 1) is a characteristic isoflavone found in soybean and is the most abundant natural ERβ agonist. It has a ninefold preferential affinity to ERβ than ERα. Genistein exerts anticancer activity through its regulation of ERβ expression. It can reduce promoter methylation of the ERβ gene, which increases overall expression. The phosphorylation, nuclear translocation, and transcriptional activity of ERβ can be increased by genistein administration [6]. Moreover, genistein can enhance or sensitize the preventive and inhibitory effects of tamoxifen on ERα-positive breast cancer. For ERα-negative breast tumor, genistein can also prevent cancer development and reduce the growth through epigenetic regulation. It does this by remodeling the chromatin structure in the ERα promoter through histone acetylation, which reactivates ERα expression as well as resensitizes ERα-dependent cellular responses to activator E2 and antagonist tamoxifen [7]. These findings are in addition to the previously reported anticancer mechanisms of genistein, including induction of apoptosis, and inhibition of angiogenesis and

Box 2. Physiological Functions and Bioactivities of ERs

ERα is essential for physiological development and metabolic normalization. Knockout of ERα leads to infertility, reduced prolactin production, immature mammary glands, reduced bone size, insulin resistance, and obesity. Whereas, knockout of ERβ can result in reduced fertility, decreased oocytes in the reproductive system, and improved body weight, with increased serum cholesterol, leptin, and glucose [56]. No apparently macroscopic phenotype differences are observed for bone and metabolism [57]. ERβ can act as a dominant-negative regulator of the estrogen signaling pathway and shows a repressive effect on ERα-mediated transcriptional activity. Unliganded ERβ can also regulate gene expression, which is normally regulated by estrogen through the ERα signaling pathway. A high level of ERβ expression serves as a good prognostic marker in clinical cancer treatment. ERs play important roles in immune modulation, which can regulate cells and pathways in the innate and adaptive immune systems [58]. At a low physiological level of E2, ERs, especially ERα, generally promote the production of type I interferon and proinflammatory cytokines. At a high E2 level, anti-inflammatory responses are produced, and ERβ signaling may be responsible for this behavior as it is more effective in attenuating proinflammatory cytokines than ERα is [59]. Neuroprotection is another important function that ERs perform. ERβ is the principal ER in the human hippocampus [60]. The endogenously synthesized E2 in the hippocampus is enough to bind and activate ERα during menopause. When a high level of E2 is presented, ERβ shows a positively regulatory activity to improve memory. ERα/ERβ ratio is important for the regulatory behavior and is sensitive to age. ERβ is expressed in serotonergic neurons of the dorsal raphe and mediates the of tryptophan hydroxylase, which is involved in serotonin synthesis. Selective ERβ agonists can restore the tryptophan hydroxylase loss due to menopause, and alleviate depression as well as mood disorder [61].

Box 3. Detrimental Effects of Phytoestrogens

The safety of phytoestrogens has been extensively debated for decades. Most of the literature shows that phytoestrogens can give beneficial effects without side effects. However, there are also some reports on the detrimental effects. The Sister study has pointed out that phytoestrogen-enriched soy formula consumption links to a greater risk of developing uterine fibroids [62]. In a meta-analysis of climacteric syndrome treatment, phytoestrogens are found to moderately elevate the rates of gastrointestinal side effects [63]. Very high doses of phytoestrogen may stunt mammary gland development, while lower doses produce the opposite effect [64]. Age, health status, gut microenvironment, and dose are important parameters influencing the positive or negative effects of phytoestrogens.

proliferation. Aside from its anticancer effects, genistein has other bioactivities through the ERβ signaling pathway, such as having beneficial effects on skeletal muscle regeneration. During muscle regeneration, high levels of isoflavone (100 μM) substantially reduces DNA and protein synthesis, whereas a low level (10 μM) improves DNA synthesis and repair. A daily dose of 1 mg/kg genistein administered to rats stimulates the expression of the muscle satellite cell marker MyoD; an important factor in muscle repair [8].

Another flavonoid subclass, chalcones show multitarget biological activities through microtubule formation, inhibition of receptor tyrosine kinase, aldose reductase, and cyclooxygenase, as well as modulation of ER activity [9]. In a ER binding assay, 4-hydroxychalcone shows considerable binding activity with an IC_{50} value of 2.4×10^{-5} M [10]. Flavon-3-ols have been documented to protect the vascular system by activation of ERα *in vivo* [11]. When ERα is knocked out, the beneficial effects of flavan-3-ols on plasma triglycerides and visceral adiposity as well as the antioxidant effect in liver are prevented or reduced [11].

Stilbenoids

As a commercial ER modulator, diarylpropionitrile shares a stilbenoid skeleton and is a selective agonist with a 70-fold higher affinity to ERβ than ERα. Resveratrol is the most investigated stilbenoid, which is abundantly present in grapes and peanuts. It acts as a partial agonist to ERα; however, it does not stimulate breast cancer cell proliferation as other ERα agonists do. Resveratrol binds to ERα to associate fully with steroid receptor coactivator (SRC)2, partially with coactivator SRC1 and SRC3, leading to pathway-selective activation. Resveratrol shows an anti-inflammatory effect through a direct, ER-mediated transrepression mechanism [12]. Tumor necrosis factor (TNF)α triggers rapid translocation of nuclear factor (NF)-κB and causes activation of inflammatory genes, such as interleukin (IL)-6. Resveratrol inhibits the TNFα-induced secretion of IL-6 protein through ER signaling pathway, while ER antagonist reverses this inhibition. TNFα-induced genes, like IL-6, prostaglandin E receptor 4 and TNF receptor superfamily member 11b, are equally suppressed by resveratrol or E2. Studies using an AMPK inhibitor or knockdown of AMPK subunits indicate that the PDE/cAMP and AMPK pathways are not involved in resveratrol-mediated inhibition of IL-6. Resveratrol shows a rare binding way to ERα with two different orientations. The phenol moiety or the resorcinol moiety can form the conserved hydrogen bond with helix 3 of ERα, causing the receptor to adopt a mixture of active and inactive conformations, generating partial agonistic activity. In ovariectomized mice subjected to transient middle cerebral artery occlusion, resveratrol shows protective effects by reducing the infarct volume and neurologic deficits and by increasing the basal level of tight junction protein expression. This effect is reversed by a nonspecific ER antagonist (ICI182,780), but not by specific ERα or ERβ antagonist, suggesting that resveratrol exerts neuroprotective effects through nonspecific ER signaling pathways. Since resveratrol is a potent ER modulator, structural modification might lead to finding better anticancer therapeutics. 4-(E)-((p-tolylimino)-methylbenzene-1,2-diol) has an increased anticancer activity in a ERβ-dependent fashion compared to resveratrol. It can induce ERβ expression and inhibit ERα expression. This inhibits

Table 1. Natural and Synthesized ER Modulators

Compounds	Classes	Sources	Refs
Natural ER modulators			
Coumestrol	Coumarin	*Cicer arietinum*	[15]
Daidzein	Flavonoid	*Glycine max*	[35]
Dehydrodiconiferyl alcohol	Lignan	*Cucurbita moschata*	[23]
8,8′-Dihydroxypinoresinol	Lignan	*Schisandra sphenathera*	[20]
3,4′-Dihydroxystilbene	Stilbenoid	*Hydrangea macrophylla*	[14]
4-Dihydroxystilbene	Stilbenoid	*Pinus griffithii*	[14]
Diosmetin	Flavonoid	*Citrus limon*	[28]
Ebenosin I–III	Flavonoid	*Onobrychis ebenoides*	[32]
Genistein	Flavonoid	*Glycine max*	[6]
Glyceollidin II	Stilbenoid	*Glycine max*	[36]
Glyceollin II	Stilbenoid	*Glycine max*	[36]
Hydroxypinoresinol	Lignan	*Schisandra sphenathera*	[20]
4-Hydroxychalcone	Flavonoid	*Glycyrrhiza glabra*	[10]
8-Prenylnaringenin	Flavonoid	*Humulus lupulus*	[29]
8-Prenylgenistein	Flavonoid	*Glycyrrhiza uralensis*	[30]
Psoralidin	Coumarin	*Psoralea corylifolia*	[33]
Resveratrol	Stilbenoid	*Vitis vinifera*	[12]
Schizandrin	Lignan	*Schisandra sphenathera*	[20]
Sesamin	Lignan	*Sesamum indicum*	[21]
Wedelolactone	Coumarin	*Wedelia calendulacea*	[17]
Xanthohumol	Flavonoid	*Humulus lupulus*	[31]
Synthesized ER modulators			
Diarylpropionitrile		Resveratrol derivative	[65]
4-(E)-((p-tolylimino)-methylbenzene-1,2-diol)		Resveratrol derivative	[13]
ICI 182,780		E2 derivative	[12]
Tamoxifen			[7]

cell proliferation by downregulating the expression of c-Myc and cyclin D1 [13]. Two natural stilbenoids (3,4′-dihydroxystilbene and 4-dihydroxystilbene) and a synthesized one (4,4′-dihydroxystilbene) show better agonistic activities to ERα than resveratrol [14]. Therefore, further exploration of stilbenoid-type phytoestrogens with strong estrogenic activities will likely be of great benefit for researchers in the field.

Coumarins

Coumarins are fragrant chemicals in the benzopyrone class. Coumestrol, found in chick peas and alfalfa, is a representative of a subclass called coumestans, which have been well documented for their estrogenic activity. Coumestrol can upregulate ERβ expression, activating the ERβ signaling pathway in the gut to induce apoptosis, which has been found to reduce small intestinal mucosal tumor number and colon tumor number in $Apc^{Min/+}$ mice [15]. Moreover, coumestrol can mediate the activity of the ERβ variant, ERβ2 [16]. Wedelolactone

shares a similar structure to coumestrol with one more hydroxyl and methoxyl. It acts as an agonist to both ERα and ERβ, stimulating the growth of ER-positive cells and expression of estrogen-responsive genes through ER genomic signaling pathway. Moreover, a rapid non-genomic estrogen signaling pathway is also activated [17]. However, these effects can be inhibited by pure ER antagonist ICI 182,780, which helps support the positive effect of wedelolactone on the ER signaling pathway.

Lignans

Lignans have two phenol moieties mimicking A and D rings of E2 for ER binding, giving an agonistic or antagonistic activity. There are various pathways through which lignans exert their activities, including transcription regulation of ERs, immune response, regulation of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) pathways, apoptosis, and autophagy [18]. Schizandrin, hydroxypinoresinol, and 8,8′-dihydroxypinoresinol are lignans present in *Schisandra sphenathera* stem. The fruit of *S. sphenathera* can be used to make tea, and it has been used in traditional medicine for treatments of insomnia and gastrointestinal diseases [19]. Schizandrin can activate ERα activity and the effect was only tenfold less than E2 at 0.1 μM, while hydroxypinoresinol and 8,8′-dihydroxypinoresinol are 50-fold less than it. Moreover, these chemicals have good safety as no cytotoxicity to multiple cells has been detected [20]. Sesamin and its enterolignans can activate two pathways, extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), through the nongenomic estrogen signaling pathway. The enterolignans also show positive effects on cell cycle progression and chemokine monocyte chemoattractant protein-1 secretion through direct binding to the ligand binding domain of ERα to activate transcription activation function (AF)-1 and/or AF-2 [21]. Dehydrodiconiferyl alcohol is an agonist to both ERα and ERβ. It shows a great osteoblastogenetic effect by inducing the expression of alkaline phosphatase, osteocalcin, and osteoprotegerin via the ER signaling pathway [22]. Furthermore, the differentiation of osteoclasts is inhibited as six osteoclastogenic genes are downregulated [23]. These findings suggest that dehydrodiconiferyl alcohol has beneficial effects in preventing bone loss and osteoporosis. High intake of lignans and high blood concentration of its main biomarker, enterolactone, are associated with good breast cancer prognosis [24]. A higher enterolactone level is also associated with a lower risk of all-cause and breast cancer-specific mortality [24]. Due to the impressive bioactivities and high levels in whole grains and fiber-containing plant foods, lignans have been proposed as a marker of healthy dietary patterns [25].

Derivatization to Alter ER Affinity

Methylation and prenylation are two derivatization techniques to effectively improve the affinity of a phytoestrogen for ERs, while glycosylation usually reduces the affinity due to the steric hindrance [26]. Isoflavone Rx-phytoestrogen contains abundant glycosidic phytoestrogens, which make the extract show a lower estrogenic activity than those mainly containing aglcones [27]. Methylation of hydroxyl groups on phytoestrogens can directly remove the necessary hydrogen bond formed in the ligand-binding domain, changing the position in the binding pocket and determining the regulatory effect on ER. Schizandrin is a lignan containing multiple methoxyl groups on its aromatic ring. When these groups are removed, the estrogenic activities of schizandrin are sharply decreased [20]. Diosmetin (3′-*O*-methylated luteolin) shows a preferential affinity (>3-fold) to ERβ than to ERα. It can increase the expression level of TNFα via the ERβ signaling pathway and can activate caspase 8 in acute myeloid leukemia cells, leading to apoptosis and tumor growth inhibition *in vivo* [28].

Prenylation may turn a flavonoid from an agonist to an antagonist and improve the selectivity to both ERs depending on the position of the substitution. *C*-8 prenylation appears to promote antagonism to a larger extent than *C*-6 in ERα due to steric hindrance against generation of agonistic conformation. Prenylation affords naringenin a good ER modulatory activity that is supposed to be negligible. 8-Prenylnaringenin has a stronger agonistic activity to ERβ (EC_{50} = 50 nM) than to ERα (EC_{50} = 124 nM) [29]. 8-Prenylgenistein has enhanced binding affinity to ERβ and improves trabecular bone properties by inhibiting the expression of RANKL/osteoprotegerin, alkaline phosphatase, type-1 collagen, osteocalcin, cathepsin K, and ERα in bone tissue of ovariectomized mice. However, unprenylated genistein gives a different phenotype by increasing the ERα expression [30]. Xanthohumol is a prenylated chalcone present as a bioactive compound of hops used in beer production. Xanthohumol prevents the interaction between brefeldin-A-inhibited guanine nucleotide exchange protein 3 (BIG3) and prohibitin 2 (PHB2), which releases the latter allowing it to bind to ERα, resulting in complete suppression of ERα signaling pathway and inhibition of breast cancer progress [31]. Triggering the phosphorylation of ERK1/2 and p38 MAPK is another anticancer mechanism of xanthohumol. Several novel prenylated isoflavonoids (Ebenosin I–III) have been found in *Onobrychis ebenoides* and they show estrogenic activities in a cell-dependent manner [32]. Psoralidin is a monoprenylated coumestrol and has better ER modulatory activity than coumestrol. It can work as an agonist to both ERα and ERβ. At 10 μM, psoralidin activates the reporter gene expression to the maximum [33]. It shows an osteoprotective effect through ER signaling pathway [34]. The osteoblast proliferation and differentiation are promoted, along with increased formation of alkaline phosphatase colony and calcified nodules, enhanced secretion of collagen-I, bone morphogenetic protein-2, osteocalcin, and osteopontin. The expression of insulin-like growth factor 1, β-catenin, runt-related transcription factor 2 (RUNX2), osterix, and osteoprotegerin is induced, leading to inhibition of osteoclast formation and osteoclastic bone resorption [34]. It is worth mentioning that furan and pyran prenylation may lead to antagonistic activity for phytoestrogens. Pyran prenylation transforms daidzein from an ER agonist to an antagonist [35]. Glyceollin II acts as an ER antagonist, which is the prenyl cyclization product of glyceollidin II, an ER agonist [36].

Heterologous Synthesis of Phytoestrogens

Natural phytoestrogens are usually present at limited quantities in nature, and organic synthesis of these chemicals is costly and raises environment concerns. Heterologous synthesis has several advantages, like being cost-effective, environment friendly, and easy to operate, and it is a desired technique for chemical production in the future. Reconstruction of heterologous biosynthetic pathways in microbial hosts offers promise for a scalable technique to provide the required quantity of desired phytoestrogens from inexpensive precursors or simple carbon sources, such as glucose and glycerol.

Strategies of Heterologous Synthesis

For specific phytoestrogen biosynthesis, finding a relevant enzyme with high efficiency and good specificity is of priority. In our previous work, a flavonoid prenyltransferase FoPT1 can only catalyze the prenylation of isoflavones, flavonols, but not chalcones, flavanols, and stilbenes [37]. Therefore, in the bioconversion of prenylated isoflavones and flavonols, this enzyme could be used. To obtain a desired enzyme, the first step is to find a suitable gene. Then, strategies, including codon optimization, promoter engineering, translation regulation sequence mutation, post-translation modification, and protein engineering, are applied to improve the expression level and enzyme activity. By mutation P212A of dihydrodaidzein reductase, it increases the dihydrodaidzein enantioselectivity and improves the titer of equol by 15% [38].

When the desired genes encoding multiple enzymes in a pathway have been selected, strategies to balance the pathway and improve the product titer are applied, including protein fusion, DNA assembly, and **modular pathway engineering** (Figure 1; see Glossary). Protein fusion puts active sites of enzymes into a closer proximity that facilitates channeling and relieves the side pathway competition. Flavonoid 3′-hydroxylase is functionally expressed as a fusion protein with cytochrome P450 reductase in *Escherichia coli* to successfully produce eriodictyol from tyrosine by simultaneously coexpressing fusion protein with tyrosine ammonia lyase (TAL), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) [39]. DNA assembly allows the design and rapid construction of biochemical pathways in a one-step fashion via *in vivo* homologous recombination in yeast [40]. **Vector development**, like ePathBrick [41], supports the modular assembly of multigene pathways and several molecular components, including promoter, operator, ribosome binding site, and terminator. To produce high titers of a target compound, modular pathway engineering is usually carried out, which can be classified into three categories: increasing the supply of substrate, elevating the whole flux of the desired biosynthetic pathway, and downregulating or eliminating the flux of branch pathway. Combination of three categories is called a push–pull–block strategy [42]. Through modular pathway design of the resveratrol biosynthetic pathway, optimization of electron transfer to cytochrome P450 monooxygenase, increase of precursors supply and decrease of pathway intermediates degradation in *Saccharomyces cerevisiae*, a titer of 800 mg/l resveratrol is obtained. Further integration of methyltransferase leads to production of pinostilbene and pterostilbene [43].

De novo Biosynthesis of Phytoestrogens

De novo synthesis will construct a complete pathway in the engineered microbial host to produce a target chemical, which only uses simple carbon and nitrogen sources in the medium. Any intermediates for the biosynthesis of a target chemical are not required. *De novo* biosynthesis of a specific phytoestrogen by metabolic engineering is always the target pursued by researchers in this field. Glycerol is more relevant for aromatic chemical biosynthesis than

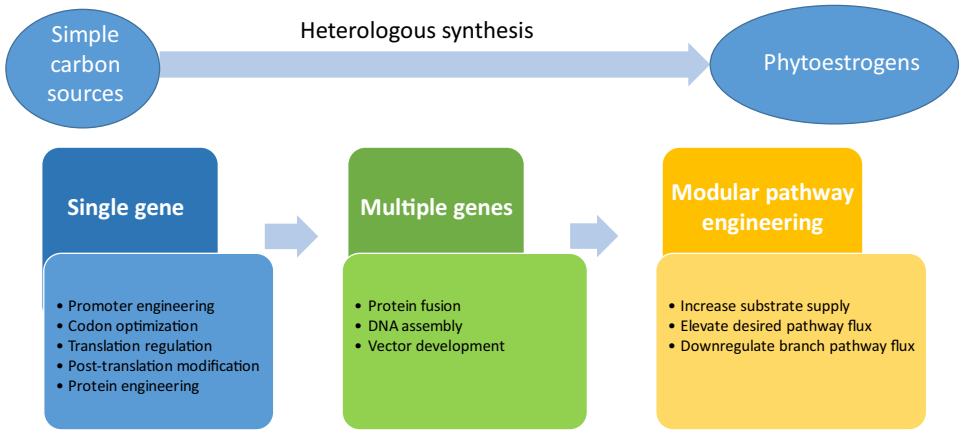

Figure 1. Strategies for Heterologous Synthesis of Phytoestrogens. In the first step, a single gene encoding a specific enzyme needs to be selected, and strategies including promoter engineering, codon optimization, translation regulation, post-translation modification, and protein engineering are utilized to optimize protein expression and activity. After multiple genes in a pathway are selected, strategies like protein fusion, DNA assembly, and vector development are applied to balance the pathway and improve the product titer. The last step is modular pathway engineering, which further improves the product titer by increasing the substrate supply, elevating desired pathway flux and downregulating branch pathway flux.

glucose due to a higher efficiency and a lower cost. The biosynthetic pathways from glycerol or glucose to phytoestrogens are shown in Figure 2. *De novo* biosynthesis of scopoletin is constructed in *E. coli*. The genes encoding 4-hydroxyphenylacetate 3-hydroxylase, TAL, 4CL, caffeoyl-CoA *O*-methyltransferase, and flavonoid 3′-hydroxylase are incorporated as a module to construct the pathway from native tyrosine to scopoletin [44]. As phenylalanine or tyrosine are the common precursors of above phytoestrogens, improvement of their levels in *E. coli* remains a hot topic. Combinatorial overexpression of fructose-1,6-bisphosphatase and transketolase increases 80% of phenylalanine level, when using glycerol as the only carbon source [45]. Furthermore, construction the biosynthetic pathway from phenylalanine to target phytoestrogens has been extensively investigated. Four metabolically engineered yeast strains have been designed, which harbor plasmids with plant-derived genes for enzymes involved in resveratrol, naringenin, genistein, kaempferol and quercetin biosynthesis, with phenylalanine as the initial substrate [46]. Three genes, *4CL*, *CHI*, and *CHS*, which encode flavanone biosynthesis, are cloned into an expression plasmid and transformed into *E. coli* to yield naringenin

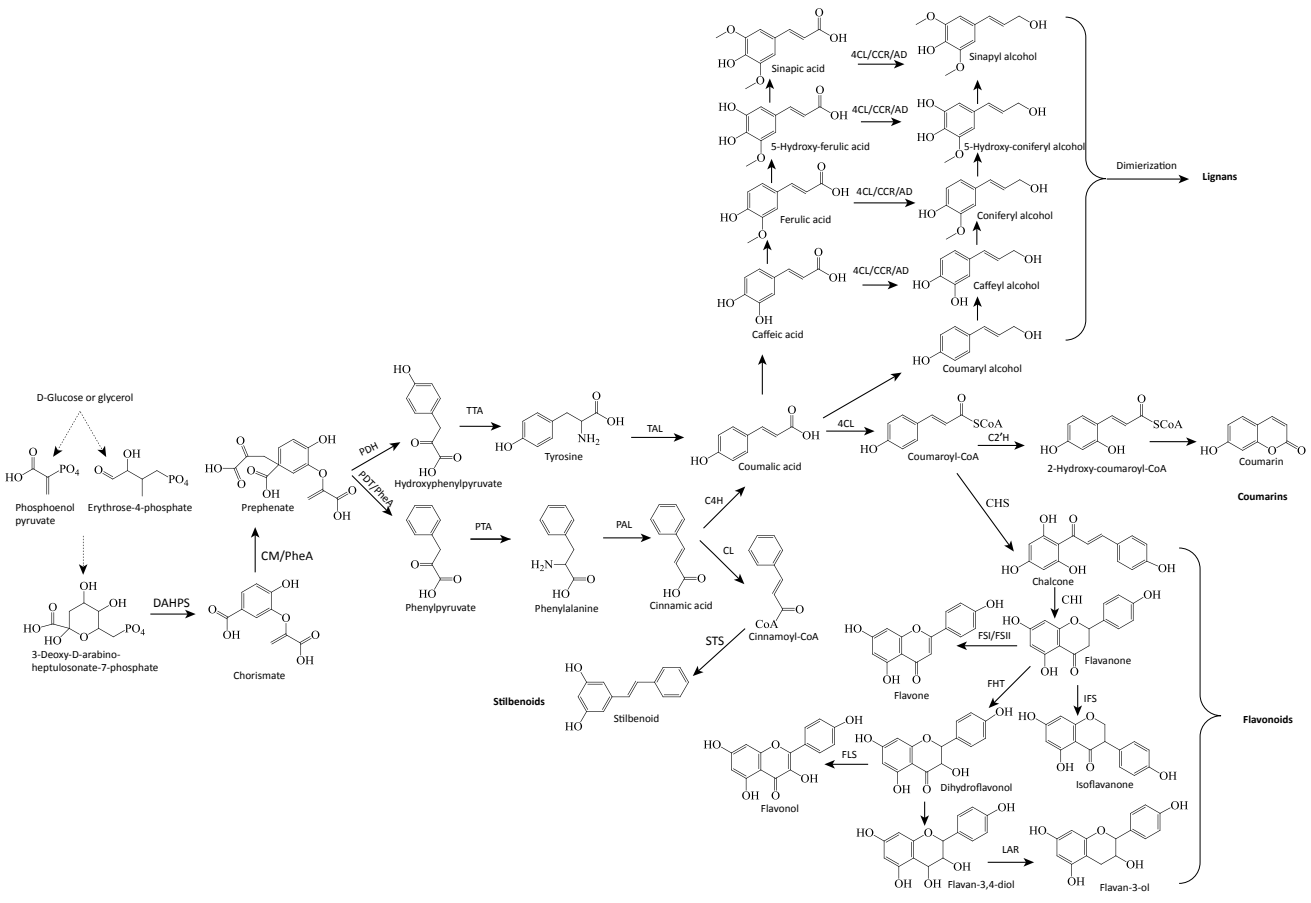

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Figure 2. Biosynthetic Pathways of Phytoestrogens. Abbreviations: C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; 4CL/CCR/AD, 4-coumarate:CoA ligase/cinnamoyl-CoA reductase/alcohol dehydrogenase; C2′H, coumaroyl-CoA 2′-hydroxylase; C3H, coumarate 3-hydroxylase; CM, chorismate mutase; COMT, caffeic acid *O*-methyltransferase; DAHPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DFR, dihydroflavonol reductase; F5H, ferulate 5-hydroxylase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; FSI/FSII, flavone synthase I/II; HFOMT, 5-hydroxy-ferulic acid *O*-methyltransferase; IFS, isoflavanone synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia lyase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PTA, phenylalanine transaminase; STS, stilbene synthase; TAL, tyrosine ammonia lyase; TTA, tyrosine transaminase.

from tyrosine [47]. Equol is the *in vivo* metabolite of genistein and it contributes to the observed ER modulatory effect of genistein. In the engineered *E. coli* that can produce genistein, further overexpressing daidzein reductase, dihydrodaidzein reductase, tetrahydrodaidzein reductase and dihydrodaidzein racemase originating from gut bacterium *Slackia isoflavoniconvertens* leads to the production of 5-hydroxy equol with a yield of 230 mg/L [48].

p-Coumaric acid is another important intermediate of phytoestrogen biosynthesis. Increase the titer of *p*-coumaric acid will be beneficial for the accumulation of phytoestrogens. Through metabolic engineering strategies, the titer of *p*-coumaric acid in *E. coli* reaches as high as 2.51 g/l when glycerol serves as the carbon source [49]. Furthermore, a *p*-coumaric acid overproduction platform in *S. cerevisiae* has been developed [50]. The byproduct (aromatic alcohols) formation is reduced by knocking out phenylpyruvate decarboxylase and pyruvate decarboxylase. The feedback-resistant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase/chorismate mutase, and the shikimate kinase II, which is a flux-controlling enzyme and a TAL from *Flavobacterium johnsoniaeu*, are overexpressed in *S. cerevisiae*. The highest titer of *p*-coumaric acid can reach 1.93 g/l when glucose is used as the carbon source. This engineered host supplies a platform to produce phytoestrogens by further incorporation of the downstream biosynthetic pathway. Coumarins can be produced by incorporating 4CL and coumaroyl-CoA 2′-hydroxylase into this platform. By incorporating 4CL, CHS, CHI, and flavone synthase, flavones are generated. Lignans can be produced by further expressing hydroxylase and 4-coumarate:CoA ligase/cinnamoyl-CoA reductase/alcohol dehydrogenase. Downregulation of the fatty acid biosynthesis pathway to decrease the malonyl-CoA consumption can help improve flavonoid accumulation. As mentioned by Yang *et al*. [47], when *fabD* gene (a gene involved in fatty acid biosynthesis) is knocked down, the naringenin production can be elevated 1.53-fold. The Koffas group has successfully constructed *de novo* biosynthesis of flavanoids and stilbenoids. By incorporation of 4CL, CHS, CHI, and FSI into *E. coli*, flavanones and flavones are produced in minimum medium. Increase of malonyl-CoA supply and inhibition of fatty acid generation are two efficient strategies to increase flavonoid accumulation [51]. In the recombinant *E. coli* expressing *At4CL* and *VvSTS* genes, overexpression of pyruvate dehydrogenase multienzyme complex, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase, and deletion of fumarase can lead to apparent accumulation of malonyl-CoA. It improves the final yield of resveratrol to 1.6 g/l [52].

S. cerevisiae and *E. coli* are the two most-used heterologous hosts to produce secondary metabolites, even though there are many other microbial hosts that have the same functions. As the production in *S. cerevisiae* is usually lower than in *E. coli*, more attention is placed on engineered *E. coli* to design the pathway for phytoestrogen production. However, *E. coli* and *S. cerevisiae* have their own specialized environments or compartment for optimal functions. It is practicable to coculture both engineered strains with each harboring partial pathway that is most suitable to the host. Such strategy can give an opportunity to produce phytoestrogens that cannot be synthesized in a single strain, or to give a higher titer [53].

Concluding Remarks

ERs are positively involved in physiological behavior of humans. Regulation of ER signaling pathways can help to maintain health status and treat diseases. Phytoestrogens are good candidates as ER modulators due to high safety and good performance, even though weaker than synthesized commercial ER modulators. By using modern techniques, like computer simulation, to search for selective ERα/ERβ agonists/antagonists from a natural product database, more potent phytoestrogens with specific functions will be found. Through *de novo* heterologous synthesis of these chemicals, it is possible to produce any desired

Outstanding Questions

How can we precisely regulate the positive effects of ERα and ERβ by using phytoestrogens to improve memory?

How do we find a phytoestrogen that is specifically effective for an ER-related disease?

How do we find the most suitable gene from thousands of candidates for *de novo* biosynthesis of a specific phytoestrogen.

phytoestrogens in an efficient way. This technique solves the quantity limits of phytoestrogens in nature and makes them available for industrial applications. The quick development of natural product research and metabolic engineering technique will bring more benefits to human health. There are some questions that need to be solved in the future (see Outstanding Questions). Answering these questions will be good to utilize and prepare phytoestrogens.

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References

1. Flamment, M. *et al.* (2012) New insights into ER stress-induced insulin resistance. *Trends Endocrinol. Metab.* 23, 381–390
2. Coyoy, A. *et al.* (2016) Metabolism regulation by estrogens and their receptors in the central nervous system before and after menopause. *Horm. Metab. Res.* 48, 489–496
3. Marotti, J.D. *et al.* (2010) Estrogen receptor-beta expression in invasive breast cancer in relation to molecular phenotype: results from the Nurses' Health Study. *Mod. Pathol.* 23, 197–204
4. Gaya, P. *et al.* (2018) Incomplete metabolism of phytoestrogens by gut microbiota from children under the age of three. *Int. J. Food Sci. Nutr.* 69, 334–343
5. Yang, B. *et al.* (2016) The plant resources, structure characteristics, biological activities and synthesis of pyranoflavonoids. *Curr. Med. Chem.* 23, 3078–3115
6. Mahmoud, A.M. *et al.* (2015) Genistein increases estrogen receptor beta expression in prostate cancer via reducing its promoter methylation. *J. Steroid Biochem.* 152, 62–75
7. Li, Y.Y. *et al.* (2013) Epigenetic reactivation of estrogen receptor-alpha (ER alpha) by genistein enhances hormonal therapy sensitivity in ER alpha-negative breast cancer. *Mol. Cancer* 12, 9
8. Velders, M. *et al.* (2012) Selective estrogen receptor-beta activation stimulates skeletal muscle growth and regeneration. *FASEB J.* 26, 1909–1920
9. Zhuang, C.L. *et al.* (2017) Chalcone: a privileged structure in medicinal chemistry. *Chem. Rev.* 117, 7762–7810
10. Branham, W.S. *et al.* (2002) Phytoestrogens and mycoestrogens bind to the rat uterine estrogen receptor. *J. Nutr.* 132, 658–664
11. Leonetti, D. *et al.* (2018) Extract enriched in flavan-3-ols and mainly procyanidin dimers improves metabolic alterations in a mouse model of obesity-related disorders partially via estrogen receptor alpha. *Front. Pharmacol.* 9, 406
12. Nwachukwu, J.C. *et al.* (2014) Resveratrol modulates the inflammatory response via an estrogen receptor-signal integration network. *eLife* 3, e02057
13. Ronghe, A. *et al.* (2016) 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol}, 1 a novel resveratrol analog, differentially regulates estrogen receptors alpha and beta in breast cancer cells. *Toxicol. Appl. Pharmacol.* 301, 1–13
14. Lappano, R. *et al.* (2009) Structure–activity relationships of resveratrol and derivatives in breast cancer cells. *Mol. Nutr. Food Res.* 53, 845–858
15. Williams, C. *et al.* (2016) Estrogen receptor beta as target for colorectal cancer prevention. *Cancer Lett.* 372, 48–56
16. Donoghue, L.J. *et al.* (2017) Differential activation of a mouse estrogen receptor beta isoform (mER beta 2) with endocrine-disrupting chemicals (EDCs). *Environ. Health Perspect.* 125, 634–642
17. Nehybova, T. *et al.* (2015) Wedelolactone induces growth of breast cancer cells by stimulation of estrogen receptor signalling. *J. Steroid Biochem.* 152, 76–83
18. Zheng, N. *et al.* (2015) ER alpha down-regulation plays a key role in silibinin-induced autophagy and apoptosis in human breast cancer MCF-7 cells. *J. Pharmacol. Sci.* 128, 97–107
19. Szopa, A. *et al.* (2017) Current knowledge of *Schisandra chinensis* (Turcz.) Baill (*Chinese magnolia* vine) as a medicinal plant species: a review on the bioactive components, pharmacological properties, analytical and biotechnological studies. *Phytochem. Rev.* 16, 195–218
20. Liu, H.W. *et al.* (2013) Lignans from *Schisandra sphenathera* Rehd. et Wils. and semisynthetic schisantherin A analogues: absolute configuration, and their estrogenic and anti-proliferative activity. *Eur. J. Med. Chem.* 59, 265–273
21. Zhu, Y. *et al.* (2017) Differential and directional estrogenic signaling pathways induced by enterolignans and their precursors. *PLoS One* 12, e0171390
22. Lee, W. *et al.* (2018) Dehydrodiconiferyl alcohol promotes BMP-2-induced osteoblastogenesis through its agonistic effects on estrogen receptor. *Biochem. Biophys. Res. Commun.* 495, 2242–2248
23. Lee, W. *et al.* (2018) Dehydrodiconiferyl alcohol inhibits osteoclast differentiation and ovariectomy-induced bone loss through acting as an estrogen receptor agonist. *J. Nat. Prod.* 81, 1343–1356
24. Kyro, C. *et al.* (2017) Pre-diagnostic plasma enterolactone concentrations and breast cancer prognosis among postmenopausal women. The Danish diet, cancer and health cohort. *Clin. Nutr.* 37, 2217–2225
25. Reger, M.K. *et al.* (2016) Urinary phytoestrogens and cancer, cardiovascular, and all-cause mortality in the continuous National Health and Nutrition Examination Survey. *Eur. J. Nutr.* 55, 1029–1040
26. Powers, C.N. and Setzer, W.N. (2015) A molecular docking study of phytochemical estrogen mimics from dietary herbal supplements. *In Silico Pharmacol.* 3, 4
27. Basu, P. *et al.* (2016) Estrogenic and antiestrogenic activities of commercial dietary supplements containing herbal ingredients and isoflavones. *FASEB J.* 30, 1176.18
28. Roma, A. *et al.* (2018) Diosmetin induces apoptosis of acute myeloid leukemia cells. *Mol. Pharm.* 15, 1353–1360
29. van de Schans, M.G.M. *et al.* (2015) Involvement of a hydrophobic pocket and helix 11 in determining the modes of action of prenylated flavonoids and isoflavonoids in the human estrogen receptor. *ChemBioChem* 16, 2668–2677
30. Zhang, Y. *et al.* (2018) 8-Prenylgenistein, a prenylated genistein derivative, exerted tissue selective osteoprotective effects in ovariectomized mice. *Oncotarget* 9, 24221–24236
31. Yoshimaru, T. *et al.* (2014) Xanthohumol suppresses oestrogen-signalling in breast cancer through the inhibition of BIG3–PHB2 interactions. *Sci. Rep.* 4, 7355
32. Sakavitsi, M.E. *et al.* (2016) Isolation and structure elucidation of twelve new prenylated flavonoids from *Onobrychis* spp. (*Leguminosae*) employing LC-HRMS, HSCCC and NMR techniques. *Planta Med.* 82, S1–S381

33. Liu, X. *et al.* (2014) Psoralidin, a coumestan analogue, as a novel potent estrogen receptor signaling molecule isolated from *Psoralea corylifolia*. *Bioorg. Med. Chem. Lett.* 24, 1403–1406
34. Zhai, Y.K. *et al.* (2017) Psoralidin, a prenylated coumestan, as a novel anti-osteoporosis candidate to enhance bone formation of osteoblasts and decrease bone resorption of osteoclasts. *Eur. J. Pharmacol.* 801, 62–71
35. Jiang, Q. *et al.* (2010) Effects of 7-O substitutions on estrogenic and antiestrogenic activities of daidzein analogues in MCF-7 breast cancer cells. *J. Med. Chem.* 53, 6153–6163
36. van de Schans, M.G.M. *et al.* (2016) Glyceollins and dehydroglyceollins isolated from soybean act as SERMs and ER subtype-selective phytoestrogens. *J. Steroid Biochem.* 156, 53–63
37. Yang, X.M. *et al.* (2016) Regiospecific synthesis of prenylated flavonoids by a prenyltransferase cloned from *Fusarium oxysporum*. *Sci. Rep.* 6, 24819
38. Lee, P.G. *et al.* (2016) P212A mutant of dihydrodaidzein reductase enhances (S)-equol production and enantioselectivity in a recombinant *Escherichia coli* whole-cell reaction system. *Appl. Environ. Microbiol.* 82, 1992–2002
39. Zhu, S.J. *et al.* (2014) Efficient synthesis of eriodictyol from L-tyrosine in *Escherichia coli*. *Appl. Environ. Microbiol.* 80, 3072–3080
40. Shao, Z.Y. and Zhao, H.M. (2012) DNA assembler: a synthetic biology tool for characterizing and engineering natural product gene clusters. *Methods Enzymol.* 517, 203–224
41. Xu, P. *et al.* (2012) ePathBrick: a synthetic biology platform for engineering metabolic pathways in *E-coli*. *ACS Synth. Biol.* 1, 256–266
42. Chen, Y. *et al.* (2013) Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. *Metab. Eng.* 15, 48–54
43. Li, M.J. *et al.* (2016) Engineering yeast for high-level production of stilbenoid antioxidants. *Sci. Rep.* 6, 36827
44. Lin, Y.H. *et al.* (2013) Combinatorial biosynthesis of plant-specific coumarins in bacteria. *Metab. Eng.* 18, 69–77
45. Gottlieb, K. *et al.* (2014) Improvement of L-phenylalanine production from glycerol by recombinant *Escherichia coli* strains: the role of extra copies of *glpK*, *glpX*, and *tktA* genes. *Microb. Cell Fact.* 13, 96
46. Trantas, E. *et al.* (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. *Metab. Eng.* 11, 355–366
47. Yang, Y.P. *et al.* (2015) Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. *Metab. Eng.* 29, 217–226
48. Lee, P.G. *et al.* (2017) Biosynthesis of (-)-5-hydroxy-equol and 5-hydroxy-dehydroequol from soy isoflavone, genistein using microbial whole cell bioconversion. *ACS Chem. Biol.* 12, 2883–2890
49. Jones, J.A. *et al.* (2017) Complete biosynthesis of anthocyanins using *E. coli* polycultures. *mBio* 8, e00621-17
50. Rodriguez, A. *et al.* (2015) Establishment of a yeast platform strain for production of *p*-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. *Metab. Eng.* 31, 181–188
51. Leonard, E. *et al.* (2008) Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. *Mol. Pharm.* 5, 257–265
52. Bhan, N. *et al.* (2013) Redirecting carbon flux into malonyl-CoA to improve resveratrol titers: proof of concept for genetic interventions predicted by OptForce computational framework. *Chem. Eng. Sci.* 103, 109–114
53. Zhou, K. *et al.* (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat. Biotechnol.* 33, 377–383
54. Bennesch, M.A. *et al.* (2016) LSD1 engages a corepressor complex for the activation of the estrogen receptor alpha by estrogen and cAMP. *Nucleic Acids Res.* 44, 8655–8670
55. Berno, V. *et al.* (2008) Activation of estrogen receptor-alpha by E2 or EGF induces temporally distinct patterns of large-scale chromatin modification and mRNA transcription. *PLoS One* 3, e2286
56. Yepuru, M. *et al.* (2010) Estrogen receptor-beta-selective ligands alleviate high-fat diet- and ovariectomy-induced obesity in mice. *J. Biol. Chem.* 285, 31292–31303
57. Lee, H.R. *et al.* (2012) Functions and physiological roles of two types of estrogen receptors, ERalpha and ERbeta, identified by estrogen receptor knockout mouse. *Lab. Anim. Res.* 28, 71–76
58. Kovats, S. (2015) Estrogen receptors regulate innate immune cells and signaling pathways. *Cell. Immunol.* 294, 63–69
59. Lewis, D.K. *et al.* (2008) Effects of estrogen receptor agonists on regulation of the inflammatory response in astrocytes from young adult and middle-aged female rats. *J. Neuroimmunol.* 195, 47–59
60. Foster, T.C. (2012) Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging. *Hippocampus* 22, 656–669
61. Warner, M. and Gustafsson, J.A. (2015) Estrogen receptor beta and Liver X receptor beta: biology and therapeutic potential in CNS diseases. *Mol. Psychiatry* 20, 18–22
62. D'Aloisio, A.A. *et al.* (2010) Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the Sister Study. *Environ. Health Perspect.* 118, 375–381
63. Wisniewska, I. *et al.* (2016) The pharmacological and hormonal therapy of hot flushes in breast cancer survivors. *Breast Cancer* 23, 178–182
64. Padilla-Banks, E. *et al.* (2006) Neonatal exposure to the phytoestrogen genistein alters mammary gland growth and developmental programming of hormone receptor levels. *Endocrinology* 147, 4871–4882
65. Moradi, F. *et al.* (2017) Estradiol and estrogen receptor-beta agonist diarylpropionitrile promote mitochondrial network fusion. *Free Radic. Biol. Med.* 112, 172–173