REVIEW

Oestrogen receptor-independent actions of oestrogen in cancer

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Abstract

Oestrogen, the primary female sex hormone, plays a significant role in tumourigenesis. The major pathway for oestrogen is via binding to its receptor [oestrogen receptor (ERα or β)], followed by nuclear translocation and transcriptional regulation of target genes. Almost 70% of breast tumours are ER +, and endocrine therapies with selective ER modulators (tamoxifen) have been successfully applied. As many as 25% of tamoxifen-treated patients experience disease relapse within 5 years upon completion of chemotherapy. In such cases, the ER-independent oestrogen actions provide a plausible explanation for the resistance, as well as expands the existing horizon of available drug targets. ER-independent oestrogen signalling occurs via one of the following pathways: signalling through membrane receptors, oxidative catabolism giving rise to genotoxic metabolites, effects on mitochondria and redox balance, and induction of inflammatory cytokines. The current review focuses on the non-classical oestrogen signalling, its role in cancer, and its clinical significance.

Keywords Estrogen receptor-independent signalling · GPER1 · Estrogen metabolites · Breast cancer · Tamoxifen resistance

Introduction

Oestrogens are the primary female sex hormones, mainly synthesised in the ovaries of premenopausal women, and to a lesser extent in the liver, heart, muscle, bone, adipose tissue, and brain. Three physiological oestrogens, namely, oestrone (E1), oestradiol (E2), and oestriol (E3), are formed during cholesterol metabolism. E2 is the predominant and most potent oestrogen found in premenopausal women [1]. The majority of the effects of oestrogen, also known as the genomic effects, are attributed to their binding to the oestrogen receptors (ERα and ERβ) and subsequent translocation to the nucleus. The ligand-bound ER acts as a transcription factor and binds to oestrogen response elements on DNA [2]. ER-dependent oestrogen signalling can also be non-genomic, non-nuclear. Protective effects of E2 on vascular tissues is an example of such signalling, where E2-bound ER interacts with PI3K (phos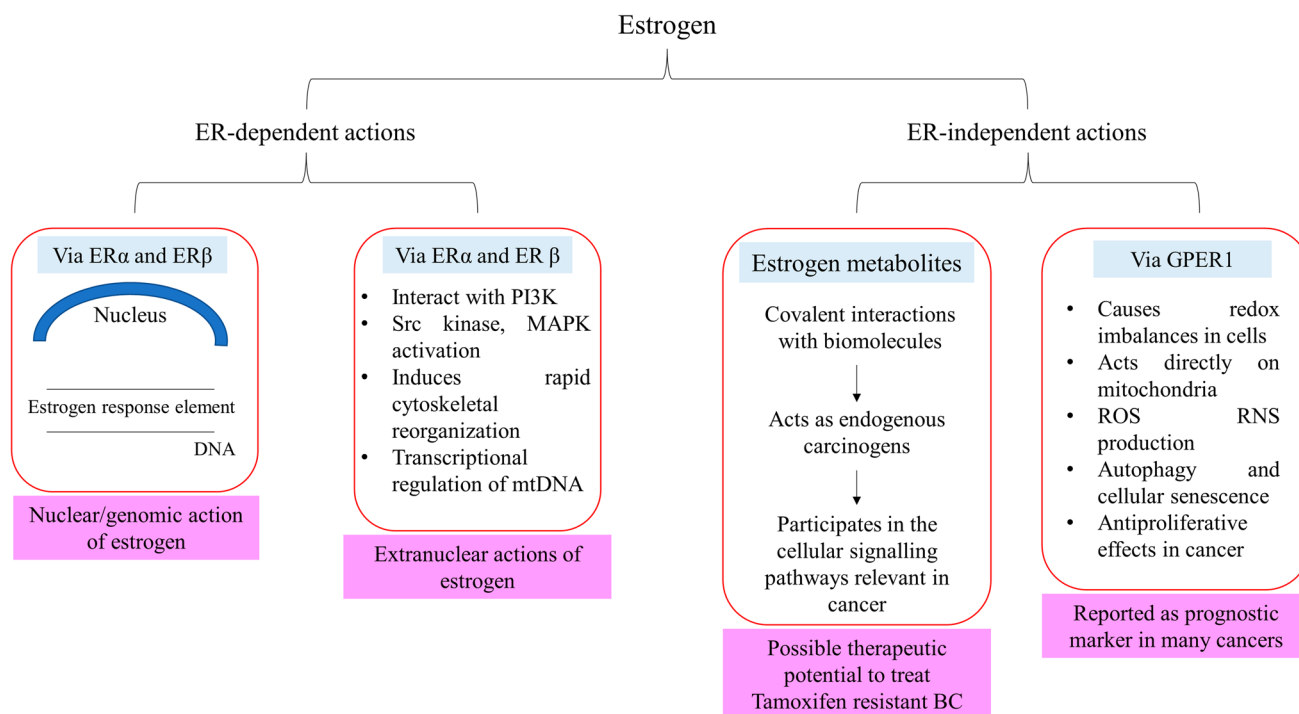phoinositide 3-kinase) activating the PI3K/Akt axis resulting in the activation of endothelial nitric oxide synthase [3, 4]. Non-genomic effects of hormones in the extranuclear compartments is rapid and includes important physiological changes in reproductive and nonreproductive cells [5]. ER-independent signalling, which mostly (not exclusively) comprises of non-genomic effects of oestrogen include signalling cascades through membrane receptors and those that directly involve covalent interactions between oestrogens and their metabolites with other biomolecules [6] (Fig. 1). Oestrogens act as endogenous chemical carcinogens through the generation of reactive oxygen species (ROS) and reactive nitrogen species via (a) their direct effect on mitochondria, (b) their oxidative metabolism forming genotoxic metabolites, and (c) their induction of proinflammatory cytokines IL-1β and TNFα [7]. While oestrogens and its receptors also acts as a protective agent in several disease conditions like cardiovascular [8, 9], exercise-induced muscle damage in women [10] and also regulate erythropoiesis under hypoxic condition, thereby can be a target for erythrocytosis [11].

ER-independent oestrogen signalling converges with major cellular signalling pathways. In brain cells derived from mice lacking ERα expression, oestrogen activates Mitogen-activated protein kinases (MAPK) signalling. In a similar model in Breast cancer (BC), exogenous oestrogen treatment increased the levels of phosphorylated

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Estrogen

ER-dependent actions

ER-independent actions

Via ERα and ERβ

Nucleus

_____

Estrogen response element

_____

DNA

Nuclear/genomic action of estrogen

Via ERα and ER β

- Interact with PI3K
- Src kinase, MAPK activation
- Induces rapid cytoskeletal reorganization
- Transcriptional regulation of mtDNA

Extranuclear actions of estrogen

Estrogen metabolites

Covalent interactions with biomolecules

↓

Acts as endogenous carcinogens

↓

Participates in the cellular signalling pathways relevant in cancer

Possible therapeutic potential to treat Tamoxifen resistant BC

Via GPER1

- Causes redox imbalances in cells
- Acts directly on mitochondria
- ROS RNS production
- Autophagy and cellular senescence
- Antiproliferative effects in cancer

Reported as prognostic marker in many cancers

Fig. 1 The role of oestrogen in the ER-dependent and ER-independent signalling and the possible therapeutic potential of ER-independent signalling especially in tamoxifen-resistant BC. (ER: oestrogen receptor, PI3K: phosphoinositide 3-kinase, MAPK: Mitogen-activated protein kinases, ROS: reactive oxygen species, RNS: reactive nitrogen species)

protein kinase B (or AKT). Treatment of these cells with ER antagonists did not significantly alter AKT phosphorylation. Moreover, the transient activation of AKT disproved the ER-based nuclear events [12]. Similarly, the interaction of E2 with G-protein-coupled ER 1 (GPER1) culminates in epidermal growth factor receptors (EGFR) [13, 14], PI3/AKT, and MAPK/ERK signalling [15] and NF-κB activation [16]. The current review elaborates on the history and recent reports on ER-independent signalling, the role of oestrogen and its metabolites in cellular physiology, and their clinical significance.

Oestrogen metabolism

Oestrogen synthesis in premenopausal women occurs in the ovaries during cholesterol catabolism. The formation of pregnenolone is the first step in oestrogen synthesis via the action of the cytochrome P450 side-chain cleavage enzyme on cholesterol. Pregnenolone is converted into progesterone by 3β-hydroxysteroid dehydrogenase. Testosterone is formed by the sequential action of cytochrome P450 17α-hydroxylase and 17β-hydroxysteroid dehydrogenase (17β-HSD). Androgens are transported from thecal cells to granulosa cells. In the final step, the conversion of testosterone to E2 is catalysed by aromatase, which belongs to the

cytochrome P450 superfamily. This enzyme is expressed in the brain, gonads, blood vessels, liver, bones, skin, adipose tissue, and endometrium. The tissue-specific expression of aromatase depends on three factors: alternate splicing, tissue-specific promoters, and different transcription factors. Distinct promoters are differentially regulated by hormones and cytokines. In the brain, aromatase is regulated by post-translational phosphorylation. In non-reproductive women, the main source of E2 is extragonadal organs, where it functions as a paracrine and/or intracrine factor, whereas ovarian E2 is released into the blood [6]. Under physiological conditions, oestrogen metabolism involves conjugation to inactive sulphates and glucuronides [1]. Oestrone sulphate (E1S) is a sulphated oestrogen that serves as the precursor for E2 synthesis. Desulphonation of E1S, a biologically inactive but highly stable derivative, by steroid sulfatase forms E1, which is reduced to E2 by 17β-HSD [17]. E1 and E2 are metabolised via two routes: formation of catechol oestrogen and, to a lesser extent, 16α-hydroxylation [18]. 2-hydroxy oestradiol (2-OHE) and 4-hydroxy oestradiol (4-OHE) are catechol derivatives of E2 that possess tumourigenic potential and are detoxified by catechol-O-methyltransferase (COMT), which has greater catalytic activity toward 2-OHE, thus making it less carcinogenic than 4-OHE [19]. Furthermore, O-methylation of 2-OHE results in the formation of 2-methoxy oestradiol (2-MeOHE) [19]. The inactivation of

oestrogen occurs via a conjugation reaction and conversion of E2 (most potent) to E1 and E3 (less active) forms, which help regulate oestrogen activity [1, 20].

In recent decades, advances in research have revealed the involvement of gut microbial flora in the metabolism of oestrogen, especially dietary phytoestrogens. This group of organisms was called astrobleme, broadly classified as Bacteroidetes, Firmicutes, Verrucomicrobia, and Proteobacteria that mainly involve β-glucuronidases and β-glucosidases. The enterohepatic circulation of oestrogen leads to alterations in circulating and excretory oestrogen levels [21]. The use of antibiotics four or more times per year is associated with a moderate increase in BC risk [22, 23]. Antibiotics modulate the gut flora, leading to reduced phytochemical oestrogen metabolism and hence increased circulating oestrogen levels, thereby increasing the cancer risk.

In-vitro and *in-vivo* studies provide early evidence of ER-independent signalling in cancer

Available data on ER-independent mechanisms of oestrogen are mostly from BC studies, as they are the most implicated in the disease. Direct evidence of oestrogen metabolite genotoxicity was obtained from two independent studies: the big blue rat cell culture mutation assay [20] and Chinese hamster VP-79 cell mutation assay [24]. *In-vitro* evidence has arisen from studies in which oestrogen metabolites were shown to cause malignant transformation in MCF-10F cells, which are benign breast epithelial cells lacking ER [25, 26]. Furthermore, exposure to physiological concentrations of E2 or 4-OHE in MCF-10F cells resulted in a loss of heterozygosity at common human mammary tumour hot spots. Comparative genomic hybridisation techniques showed that 1 μM 4-OHE induced several damages, including DNA gain (8q24, 9q34) and loss at 13q21 [27]. ER-independent effects of oestrogen were confirmed based on two observations: First, the short time between the stimulus and downstream signalling activation and consistent levels of effector expression, and second, the inability of ER inhibitors to block these actions [14].

In-vivo studies in SCID mice showed that oestradiol-exposed, non-malignant MCF-10 cells formed serially transplantable tumours. Direct and concrete evidence in support of this hypothesis was obtained from an experiment with the ERKO/Wnt-1 model, a transgenic *Wnt-1* mouse model with ER knockout. The mice were subjected to oophorectomy, and oestradiol was administered at physiological concentrations, which revealed a dose-dependent increase in breast tumour formation. That study was designed such that the confounding effects of progesterone and other ovarian factors were minimised. A limitation of that study was the presence of minimally functional (confirmed by PCR)

ERβ. To overcome any leaky expression of ERα, the ER antagonist fulvestrant was used, which did not have any effect on tumour incidence. However, the use of letrozole in combination with a minimally active form of E2 delayed tumour formation, even in animals with intact ovaries [27, 28]. These studies laid the foundation for the possibility of oestrogen-induced tumourigenesis beyond the pretext of ER.

Clinical evidence for the ER-independent effects of oestrogen came from the fact that in patients with *BRCA1* mutation, almost 75% of whom were ER − , bilateral oophorectomy reduced the BC risk by 53% [29, 30]. Additionally, post-menopausal hormone therapy increased the risk of both ER + and ER − disease [27, 31]. More recently, growing experimental data suggests ER-independent, immunomodulatory, DNA damaging effect of selective oestrogen receptor modulators (SERMs) like tamoxifen and raloxifene in triple negative BC (TNBC).

Oestrogen metabolites play a vital role in tumourigenesis

17α-Oestradiol (17α-E2) is a weak oestrogen produced in the ovarian follicles that is converted to E1 and further to E2. 17α-E2 was shown to induce *VEGFA* mRNA expression in a dose- and time-dependent manner via the PI3K/AKT signalling pathway, independent of ER [32]. 4-OHE was found to be tumourigenic in hamster kidney [33]. Repeated exposure to 4-OHE induced ROS production, malignant transformation of MCF10A cells, and growth in nude mice via PI3K/AKT signalling, leading to the overexpression of cell cycle genes *cdc2*, *PRC1*, and *PCNA* and the transcription factor NRF-1 [34]. In addition, 4-OHE induced the invasion of human breast epithelial cells (MCF10F) [35]. The enzyme involved in the formation of 4-OHE, cytochrome P4501B1 (CYP1B1), showed significant activity in the extrahepatic targets of oestrogen, including the breast, where it was upregulated in tumours compared to normal breast. Polymorphism in *CYP1B1* at position 432 was observed in BC and endometrial cancer populations [21, 36].

In-vitro studies revealed that both 2-OHE and 2-MeOHE had a negligible affinity to the ER. In MCF-7 cells, 2-OHE significantly enhanced cell growth and protein synthesis in the G2/M phase of the cell cycle compared with E2. However, 2-MeOHE showed cytostatic activity by inhibiting DNA synthesis and mitosis [37]. In cultured endothelial cells, fulvestrant did not alter the dose-dependent inhibition of endothelin-1 synthesis and MAPK activity by 2-MeOHE, indicating an ER-independent mechanism [38]. *In-vitro* studies using the osteosarcoma (OS) model yielded similar results, and 2-MeOHE was shown to induce time- and dose-dependent apoptosis without considerable changes in normal osteoblasts or decreased resorption in the bone

tumour microenvironment. Here, ER-independent action was confirmed in three experiments: (a) only a negligible effect on cell survival was observed with E2, 2-OHE, and oestrone; (b) 2-MeOHE exerted similar effects irrespective of the ER status of the cell; and (c) addition of ICI182780 did not significantly alter the anti-proliferative effect [39–41]. This was accompanied by an increase in interferon (IFN) expression in OS cells. 2-MeOHE also increased the phosphorylation of eIF-2α, a downstream effector of IFN-mediated anti-proliferative effects. The analysis of clinical samples also indicated decreased phosphorylation in the tumour compared with that in normal OS tissues, without significant differences in eIF-2α levels [42]. The specificity of 2-MeOHE for OS cells was later demonstrated *in-vivo*, providing insights into its therapeutic potential [2, 43]. More recently, the mechanism of 2-MeOHE action was reported to be via a decrease in Bcl-2 and VEGF expression and increase in caspase-3 expression, leading to G2/M cell cycle arrest and premature apoptosis [44]. In the bone marrow microenvironment, 2-MeOHE also decreased the expression of cytokines that mediate tumour growth, survival, and angiogenesis [45, 46]. Mechanistically, *in-vitro* and *in-vivo* studies have demonstrated macrophage stimulatory protein-1 receptor, a receptor tyrosine kinase, as a target for 2-MeOHE [47]. In summary, unlike other E2 metabolites, 2-MeOHE plays a pronounced tumour-suppressive role by inhibiting cell proliferation and promoting apoptosis (Table 1).

Oestrogens and their genotoxic metabolites impair the cellular redox state

Evidence for the production of genotoxic metabolites via the oxidative metabolism of endogenous and synthetic oestrogens originated in the late 90 s. Oestrogen can act as an endogenous chemical carcinogen, with its oxidative metabolites forming adducts with DNA, which may result in tumour initiation [20]. An imbalance in oestrogen metabolism that leads to oxidative damage can be attributed to various factors. Overexpression of aromatase leads to excessive synthesis of E2, increased production of 4-OHE, loss or decreased function of COMT, low levels of inactivation of the oestrogen quinone pathway and GSH, and/or low levels of quinone oxidoreductase and/or CYP reductase [56]. For instance, BRCA1/BARD1 transcriptionally controls the P450 subsets of genes, namely, *CYP1A1* and *CYP3A4*. Thus, alterations in the expression of either of these proteins may contribute to tumourigenesis [57]. Oestrogen-induced DNA damage can be of three types: generation of free radicals, formation of oestrogen (or its metabolites)—DNA adducts, and endogenous DNA modifications [58].

The oxidation of catechol oestrogens to semiquinones and o-quinones concomitantly produces superoxide anions and hydroxyl radicals, and redox cascades lead to DNA, protein, and lipid damage (Fig. 2). One-electron oxidation of E2 generates reactive phenoxyl radical intermediate, further giving rise to glutathione thiyl, NAD$^\bullet$ radical, and H_2O_2. Accumulation of H_2O_2 leads to DNA base lesions and affects redox-sensitive transcription factors such as NRF2 and NF-κB, which are key players of inflammatory responses [59]. Oestrogen-induced proliferation and activation of

Table 1 Tumor suppressive functions of 2-MeOHE in different cancers

Sl. No	Cancer	Model	Mechanism	References
1	T lymphoblastic leukemia	CEM cells	p21 (↑) p53 and BCl-2 (↓)	[48]
2	Ovarian cancer	BG1, HeyC2, TOV112D	Catalytic activation of protein kinase Cδ	[49]
3		SKOV-3 cells	Not studied	[50]
4	Endometrial cancer	Ishikawa cells	F-spondin signaling	[51]
5	Prostate cancer	PC-3 cells	BCl2 (↓) Bax (↑)	[52]
6		PC-3 cells	ROS production (↑) E-Cadherin (↑) N-Cadherin and vimentin (↓) Cyclin D1 and β-catenin (↓)	[53]
7	Chronic myeloid leukemia	K562 and KCL22 cells, BALB/C nude mice	ROS production (↑) Mitochondrial membrane potential (↓) miR223 (↑) PI3K/AKT (↓)	[54]
8	Non-small cell lung cancer (NSCLC)	2D and 3D culture of A549 cells	Inhibition of acyl-protein thioesterases 1 and thus enhanced cellular protein S-palmitoylation	[55]

(↑) upregulation and (↓) downregulation.
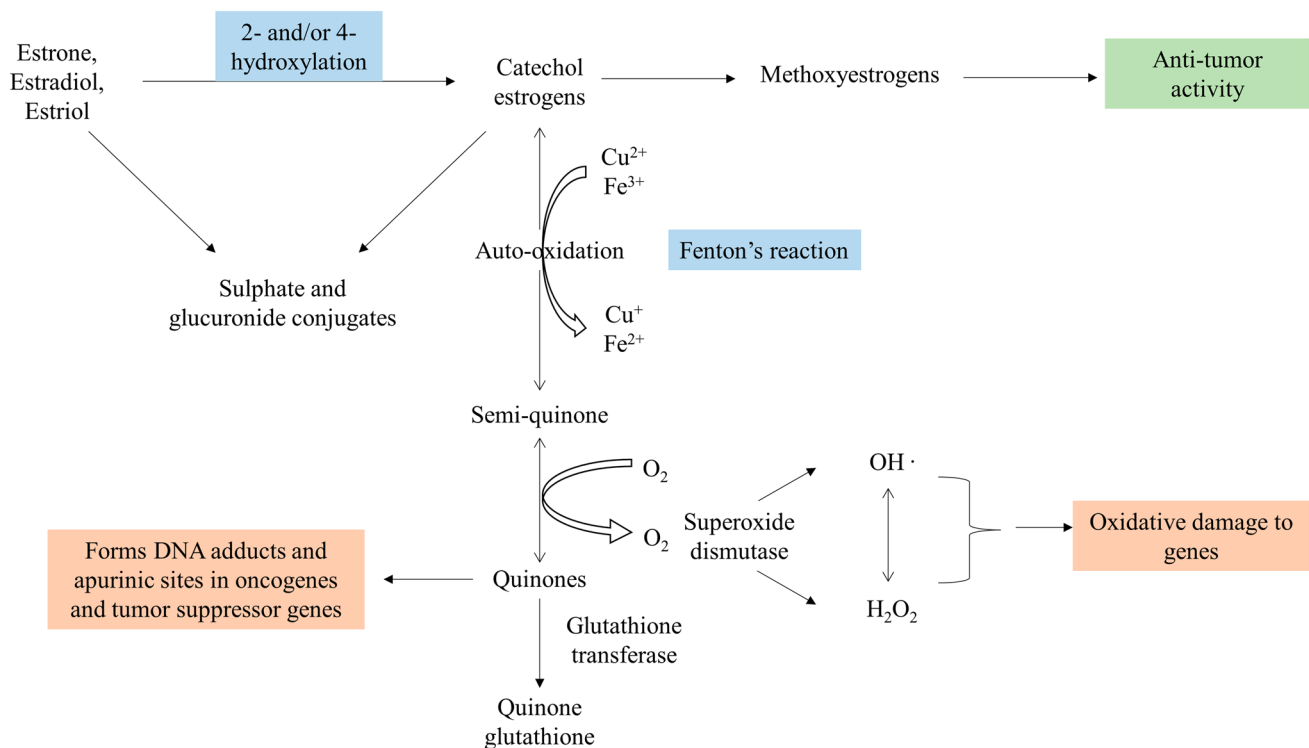
Fig. 2 Oestrogen metabolites and its association with cancer

macrophages also produce ROS and modify the function of polymorphonuclear leukocytes, resulting in the formation of hypochlorites (OCl⁻) [7].

Redox cycling of 2-OHE simultaneously accumulates ROS in MCF-10A cells, activating the IκB kinase signalling and thus, anchorage-independent growth [60]. Similarly, this process was found to produce H_2O_2 and OH· in MCF-7 and MDA-MB-231 cells, leading to oxidative DNA modification expressed by 8–oxo–7,8–dihydroxy–2′–deoxyguanosine [61]. O-Methylation of 2-OHE by COMT to form 2-MeOHE protected BC cells from catechol oestrogen-induced oxidative DNA damage (formation of 8-hydroxy-2′-deoxyguanosine) [19]. Catechol oestrogens and quinones, in the presence of lactoperoxidase, tyrosinase, or prostaglandin H synthase, react with DNA to form stable depurinated adducts, which act as endogenous initiators of breast tumorigenesis [61]. Oestrogen-3,4-quinones react with adenine and guanine in DNA to form depurinated 4-OHE-1-N3 adenine and 4-OHE-1-N7 guanine adducts, respectively [18, 62]. When hamsters were consistently treated with oestrogen, DNA adducts containing endogenous electrophiles were observed in the renal cortex. Furthermore, in that study, when the animals were treated simultaneously with tamoxifen, DNA damage blocking was unsuccessful [58]. Although the authors were unable to conclude that tamoxifen fails to counteract oestrogen-induced damage, this can now be postulated as an ER-independent mechanism.

An oestrogen metabolite-induced redox imbalance has also been implicated in tumourigenesis in oestrogen-independent organs. The role of oestrogen in the development of thyroid cancer was evaluated as the prevalence of thyroid cancer was 3–4 times higher in women than in men [20]. Follicular thyroid cells produce thyroid hormones via iodination of thyroglobulin [63]. *In-vivo* studies have revealed higher exposure to oxidative stress in the thyroid glands of female rats owing to increased ROS production and decreased ROS degradation [64]. Further experimental evidence suggests an imbalance in E2 metabolism, as detected by a higher ratio of depurinated oestrogen-DNA adducts in the urine of women with thyroid cancer [65]. Thus, it can be concluded that an imbalance in oestrogen metabolism impairs the cellular redox balance, contributing to tumourigenesis.

Effects of oestrogen on mitochondria

Mitochondria are the powerhouse of the cell that derive energy for the cells from metabolic fuels. This is achieved through oxidative phosphorylation, in which electrons travel through a series of carrier complexes to the final acceptor, O_2, which is reduced to water. Electron transfer is accompanied by the expulsion of protons across the inner mitochondrial membrane, which creates a potential difference

across the membrane. The change in free energy upon re-entry of protons into the mitochondrial matrix is coupled with ATP synthesis [66]. Lipid-rich mitochondria have been described to act as a "sink" for the highly lipophilic oestrogen. Moreover, the two main enzymes of oestrogen anabolism, 3-βHSD and aromatase, were found to be localised in the mitochondria in ovarian tumour epithelial cells. [67]. E2, through anchorage and integrin-dependent signalling, was found to induce ROS, especially H_2O_2 in the perinuclear mitochondria. This ROS induction was accompanied by enhanced cell motility; phosphorylation of c-Jun and CREB; and binding of the oxidant-sensitive transcription factors AP-1, CREB, and NRF1 [67]. Functionally, it also modified G1 to S transition and some of the early G1 genes through an ER-independent, non-genomic signalling pathway [68]. These findings provide a novel perspective on the role of E2-induced changes in mitochondria and their impact on cell cycle regulation.

The treatment of human spermatozoa with endogenous E2 and xenoestrogens genistein and bisphenol A alters the mitochondrial membrane potential and increases $O_2^\bullet$ levels [69]. In isolated rat liver mitochondria, E2 reduces ROS generation, consequently protecting mitochondrial integrity and preventing the release of cytochrome c, thereby inhibiting apoptosis [70]. In ovariectomised mice, E2 directly modulated and protected the mitochondrial functions in the absence of ERα [71]. *In-vitro* BC study showed that E2 induced the expression of ATG3 and Beclin1 and reduced the expression of p53, leading to mitochondrial damage by autophagy and cellular senescence [72]. Another *in-vivo* study on cardiovascular disease showed that E2 administration induced E2-mediated autophagy which is related to the Rab9-dependent autophagy pathway [73]. *In-vivo* study using porcine oocytes found E2 to enhance autophagy, reducing ROS levels and apoptosis activity promoting efficacy of the development of porcine oocytes [74]. Exogenous E2 protected mitochondrial functions in cardiomyocytes of ovariectomized rats having insulin resistance [75]. Menopausal women with reduced E2 levels lead to adiposity and reduced insulin sensitivity and diabetes.

In summary, these studies demonstrate that E2 plays an important role in the regulation of mitochondrial membrane potential, ROS production, autophagy, and apoptosis. The mechanism of E2-mediated regulation of mitochondrial function is yet to be fully elucidated.

Membrane-bound receptor of E2 in cancer

Among the ER family, ERα is expressed abundantly in tissues due to its critical role in cellular processes. ERα dysregulation has been reported in many cancers, including breast [76], ovary [77], and uterus [78]. Apart from ERα and ERβ, the G-protein coupled receptor superfamilies are also known to exert important effects of oestrogen. While ERs are intracellularly localised to the mitochondria, GPER1 or GPR30 are plasma membrane-bound receptors that activate signalling cascades via G-proteins. GPER1 is a 375-amino acid transmembrane receptor that binds to E2 and mediates non-genomic oestrogen responses [79]. E2-bound GPER1 phosphorylates ERK1/2 via EGFR, cAMP, Ca^{2+}, and PKC activation. GPER1 is expressed in normal breast mammary cells (MCF10A) and is considerably downregulated in dedifferentiated BC cells. Additionally, GPER1 was found to reduce cell viability but favoured the migration of metastatic BC cells [80]. This could be due to the different downstream signalling pathways in the two phenotypes and requires further characterisation. Two independent studies confirmed this pattern of expression in clinical tumour samples, with all normal breast epithelia showing positive immunoreactivity for GPER1, whereas in tumours, its expression varied. Crosstalk between GPER1 and CXCR1 was found to be involved in the migration and invasion of BC cells [81]. In tumours, GPER1 expression correlated with histological grade, ER negativity, HER2 positivity, tumour size, and metastasis [82, 83]. Direct *in-vivo* evidence for the proliferative role of GPER1 was obtained using a mouse model. GPER1 knockout mice were crossed with the transgenic mammary tumourigenesis model, MMTV-PyMT. Interestingly, the initial tumour development process was similar in wild-type and GPER1 deficient mice. After a few weeks, the GPER1 null mice showed smaller tumours, lower proliferation, lower grade, and lesser metastasis [84] GPER1 was reported to have the potential to function as a prognostic marker in various cancers [85]. Plasma membrane-bound GPER1 expression is a negative prognostic factor in ER +, tamoxifen-treated, and high-risk BC [86, 87].

Anti-oestrogens tamoxifen and fulvestrant, natural compounds hydroxytyrosol and oleuropein, phytoestrogen coumestrol, and endocrine disruptor bisphenol A bind to and act as non-selective agonists of GPER1 [88]. The selectivity of receptors and cross-reactivity of ligands depend on the effective ligand concentration [89]. E2 and genistein were reported to protect fibroblasts and keratinocytes from ROS-induced damage by activating GPER1 [90]. Genistein promoted the proliferation of thyroid squamous cell carcinoma SW579 cells via GPER1 activation [91]. Chlorobisphenol A, an oestrogenic compound, was found to induce cell proliferation in neuronal cells *in-vitro* via both ERα and GPER1 pathways [92]. C–C motif chemokine ligand 18 (CCL18), a cytokine overexpressed in tumours, was also shown to bind to GPER1 [93]. GPER1 antagonists such as G-15 [94], G-36 [95], and C4PY (*meso*-(*p*-acetamidophenyl)-calix[4]pyrrole) and GPER1 agonists such as 131I-fulvestrant [96] and 5408–0877 [97] are among the GPER1 modulating compounds identified previously (Table 2).

Table 2 Different modulators of GPER1 from literature

Compound	Agonist/Antagonist	Model	Cellular effects	References
Lupeol	Antagonist	*In-silico*	–	[98]
Quercetin	Antagonist	*In-vitro*, BM cells	↓ AKT phosphorylation ↓ NF-κB activation	[99]
Bisphenol S	Agonist	Murine hypothalamic cell lines	↑ *Agrp* expression	[100]
17β-aminoestrogens	Agonist	MCF7 and SIHA cells	↓ c-fos phosphorylation	[101]
Synthetic compounds SK0 and SK0P	Agonist	*In-silico* and *in-vitro*	↓ Cell proliferation	[102]

(↑) increase, (↓) decrease, and BM Bone marrow.

In osteoblasts, E2 inhibits autophagy and apoptosis via the GPER1/AKT axis [103]. Evidence shows lower mortality rates in women with colorectal cancer (CRC), which may be due to the role of oestrogen. While ERβ is downregulated in CRC, public datasets on CRC suggest that the overexpression of GPER1 can selectively rescue tumourigenic effects via the Wnt/β-catenin pathway [104]. Increased expression of GPER1 was observed in gastric cancer and reported to be associated with EMT and poor prognosis [105, 106]. In NSCLC A549 cells, GPER1 agonist decreased the proliferation and enhanced the apoptotic activity by modulating the redox enzymes of the cell [107]. Histological analysis of NSCLC tissues revealed that higher nuclear localisation of GPER1 correlated with poor recurrence-free and overall survival [108]. In papillary thyroid cancer, GPER1 has been implicated in tumourigenesis and is a potential therapeutic target [109]. GPER1 is overexpressed in the cell membrane and cytoplasm of uterine cervical adenocarcinoma in situ and adenocarcinoma, unlike the strong nuclear expression of GPER1 in normal tissue. GPER1-positive patients have poor overall survival [110]. In leiomyosarcoma cells, insulin was found to increase the levels of GPER1 mRNA and protein via the PRKCD/MAPK1/c-Fos/AP axis [87]. GPER1 was reported to be overexpressed in prostate cancer-associated fibroblasts and to regulate its interaction with tumour-associated macrophages, which have the potential to regulate the tumour microenvironment [111]. In glioblastoma, premenopausal women with high GPER1 levels showed better prognosis [112]; however, there is a need to understand the complex signalling pathways that occur parallelly inducing the genomic and non-genomic effects of GPER1 signalling.

Oestrogen signaling via GPER1 has been studied in several non-cancer models [113, 114]. Mechanistically, these studies showed the involvement of necroptosis, ERK signalling, which are crucial in tumorigenesis as well. *In-vitro* studies also showed the protective role of Genistein/E2 on mitochondria via GPER1 pathway in non-alcoholic fatty liver disease in postmenopausal women and mice muscle cells [115]. GPER1 also partially mediates genistein stimulation and improves glucose tolerance *in-vivo* [116]. *In-vivo* studies showed that E2 protects the mitochondrial functions of muscle cells. Estrogen-supplemented postmenopausal women and ovariectomized mice restored complex I function in muscle and liver cells [117]. In both cancer and other diseases, estrogen signaling via GPER1 confirmed to have protective effects over mitochondria functions. However, more research on the mechanism of GPER1 pathway on mitochondria will help to identify novel therapeutic approaches. Further studies are required to shed light on the relationship and balance between ER and GPER1 activities. The role of GPER1 in TNBC might be of clinical significance, that requires experimental evidence.

Therapeutic significance of ER-independent oestrogen signalling

ER-independent oestrogen signalling provides a plausible explanation for resistance to ER antagonist therapies, such as RET signalling mediated Raloxifene resistance demonstrated *in-vitro* [118]. A recent study demonstrated novel analogues of Tamoxifen, showing potential toxicity against TNBC cell lines MDA-MB-231 and MDA-MB-468 [119]. With the emerging success of drug repurposing, off-target effects of these ER modulators are being explored for their roles in other diseases [120]. 2-MeOHE sensitises BC cells to taxane treatment by centrosome de-clustering [121]. Apart from cell growth arrest and apoptosis, 2-MeOHE also demonstrated the potential to overcome drug resistance in multiple myeloma (MM). Similarly, in OS cells, it inhibited mitochondrial biogenesis via PPARγ, coactivator 1α, and cytochrome c oxidase I. It was also proved to be a potent inhibitor of succinate dehydrogenase complex subunit A and sirtuin 3 [122]. *In-vitro*, MM models of melphalan (LR-5), doxorubicin (Dox-40 and Dox-6), and dexamethasone (MM.1R) resistance were shown to undergo apoptosis upon treatment with 2-MeOHE. Its therapeutic potential was evaluated in clinical trials involving patients with hormone-refractory prostate cancer and reported to be a well-tolerated drug, with non-linear pharmacokinetics and suboptimal

plasma levels. Despite its poor availability for oral administration, it showed some anti-cancer activity at 1200 mg/day [123]. In prostate cancer, the administration of 2-MeOHE in combination with eugenol showed better efficacy, thus reducing the therapeutic concentration and adverse effects (1). 2-MeOHE was effective against melanoma *in-vitro* and *in-vivo* and acted synergistically with PD-1 blockade immunotherapy [124]. Synergistic treatment with 2-MeOHE and erlotinib induced apoptosis in hepatocellular cancer (HCC) cells and inhibited the stemness of erlotinib-resistant hypoxic cells [125]. To improve the efficacy of 2-MeOHE, polymeric micelles were used for delivery, which increased the cellular uptake and enhanced the anti-tumour activity *in-vitro* [126]. Similarly, the micellar administration of 2-MeOHE showed a beneficial effect in lung cancer cells by modulating hypoxia, cell cycle, and apoptosis [126, 127]. Liposomal delivery of 2-MeOHE nanoparticles was effective in the treatment of uterine leiomyoma *in-vivo* using patient-derived xenografts. There was an almost two-fold difference in the tumour size, proliferation, and apoptosis [128]. There is a need to conduct rigorous clinical trials to establish 2-MeOHE as a therapeutic agent in conjunction with existing therapies.

An *in-vitro* study showed that overexpression of ERα36 and GPER1 can affect responsiveness to tamoxifen and promote tumour progression, thereby not supporting this treatment in ER + BC [129]. E2-mediated GPR30 activation was reported to induce the mTOR signalling pathway, promoting p62 phosphorylation which, in turn, increased ESR1 expression. Targeting this signalling pathway to decrease ESR1 expression holds therapeutic potential in oestrogen-sensitive cells [130]. The antidepressant drug fluoxetine was found to exert oestrogen-like actions through GPER1 activation, leading to the activation of PI3/AKT and ERK signalling cascades [131]. The n-butanol extract of Huaier was found to inhibit gastric cancer proliferation, invasion, and metastasis *in-vitro* by inhibiting GPER1-mediated PI3/AKT signalling [105]. siRNA-mediated knockdown of GPER1 suppressed the proliferation, invasion, and migration of gastric cancer cells by attenuating PI3/AKT-mediated EMT [106]. The GPER agonist G1 altered the mitochondrial membrane potential, DNA damage, and apoptosis in mantle cell lymphoma (MCL), thereby confirming the potential of G1 in combination with ibrutinib as a candidate therapy for MCL [132]. Targeting GPER1 is a promising approach for modulating cisplatin resistance in gastric cancer. *In-vitro* GPER1 knockdown increased sensitivity to cisplatin and induced changes in EMT [133]. Cepharanthine hydrochloride was reported to induce mitophagy by binding to GPER1 in HCC primary cells, cell lines, and mouse xenograft models [134]. The GPER1 agonist G1 showed a potential anti-tumour effect in glioblastoma *in-vivo* [112]. However, there are few

data from mechanistic studies on targeting GPER1 and sufficient clinical evidence to prove its specificity to GPER1.

In summary, the expression of ER and GPER1 was reported to be independent in BC, and both receptors showed distinct binding affinities to various oestrogen metabolites [135]. In addition, most ER antagonists serve as GPER1 agonists; hence, treatment modalities require a deep understanding of these pathways, especially in BC [135]. GPER1/EGFR signalling plays an important role in increasing tamoxifen resistance in ER-positive BC [136]. Increased expression of GPER1 and GPER1-EGFR mediated signalling play a major role in TNBC progression [137, 138] modulating the GPER1 signalling pathway could be an effective approach in the treatment of both hormone-positive cancer and TNBC. Recent advances in research have provided other options such as proteolysis-targeting chimeras (PROTACs) to target specific proteins of interest. Hence, GPER1 holds potential as a therapeutic target in cancer, and the modulation of its expression can complement existing anti-oestrogen therapies and help manage drug resistance in oestrogen-driven cancers.

Conclusion

Almost 70% of breast tumours are ER + , and endocrine therapies with selective ER modulators (tamoxifen) or downregulators (fulvestrant) are a common treatment modality for such subtypes. Approximately 25% of tamoxifen-treated patients present with disease recurrence 5 years post-therapy. Several mechanisms, including MAPK- and AKT-induced downregulation of ER and epigenetic modifications, have been implicated in tamoxifen resistance. Furthermore, 20–30% of ER + patients develop either *de-novo* or acquired resistance to tamoxifen. As described previously, ER-independent actions may represent a mode of resistance to anti-ER therapy. Furthermore, modulating the oestrogen metabolism imbalance leading to free radical-mediated DNA damage, addressing the therapeutic potential of oestrogen-mitochondrial interactions, and targeting GPER1 offer novel treatment strategies, especially for TNBC.

Declarations

Conflict of interest Authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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