Post-transcriptional and epigenetic regulation of estrogen signaling

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Abstract
Post-translational and epigenetic regulation are important mechanisms controlling functions of genes and proteins. Although the "classic" estrogen receptors (ERs) have been acknowledged to function in mediating estrogen effects via transcriptional mechanisms, estrogogenic agents modulate the turnover of several proteins via post-transcriptional and post-translational pathways including epigenetics. For instance, the metabolic and angiogenic action of G-protein coupled estrogen receptor (GPER) in vascular endothelial cells has been recently elucidated. By interacting with GPER, 17β-estradiol (E2) and the GPER agonist G1 enhance endothelial stability of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and capillary tube formation by increasing ubiquitin specific peptidase 19 (USP19) levels, thereby reducing PFKFB3 ubiquitination and proteasomal degradation. In addition to ligands, the functional expression and trafficking of ERs can be modulated by post-translational modification, including palmitoylation. MicroRNAs (miRNAs), the most abundant form of endogenous small RNAs in humans, regulate multiple target genes and are at the center of the multi-target regulatory network. This review discusses also the emerging evidence how miRNAs affect glycolytic metabolism in cancer, as well as their regulation by estrogens. Restoring dysregulated miRNA expression represents a promising strategy to counteract the progression of cancer and other disease conditions. Accordingly, estrogen post-transcriptional regulatory and epigenetic mechanisms represent novel targets for pharmacological and non-pharmacological intervention for the treatment and prevention of hormone-sensentive non-communicable diseases, including estrogen-sensitive cancers of the reproductive system in women.

Keywords: estrogen, microRNA, glycolysis, endothelium, cancer, angiogenesis, epigenetics

Significance Statement: The effects of estrogen are mediated by several mechanisms that are not limited to the transcriptional regulation of target genes. Slowing down the turnover of master regulators of metabolism by estrogens allows cells to rapidly adapt to environmental cues. Identification of estrogen-targeted miRNAs may lead to the development of novel RNA therapeutics that disrupt pathological angiogenesis in estrogen-dependent cancers.
Introduction
Steroid hormones such as estrogen interact with specific receptors in target tissues that mediate biological responses affecting different cellular functions such as metabolism, proliferation, ion transport, excitability and contraction (Deroo and Korach, 2006). In general, estrogen responses that are dependent upon latent (hours-days) nuclear and transcriptional events are genomic responses and involve, among others, ‘classical’ nuclear estrogen receptors (Hewitt and Korach, 2018). Non-genomic, rapid responses (seconds-minutes) do not require nuclear transcriptional events and involve cytosolic signaling (i.e. protein kinases, cAMP, pH, Ca²⁺) downstream interactions of hormone with estrogen receptors expressed at the plasma membrane (Levin and Hammes, 2016).

Many of the genomic effects of estrogen are largely mediated through its binding to estrogen receptors (ER) ERα and ERβ isoforms, members of the nuclear receptor superfamily (Alexander et al., 2021). ERα and ERβ display distinct patterns of expression and function in various tissues (Matthews and Gustafsson, 2003). They both act as ligand-dependent transcription factors either by binding to the estrogen-responsive element with the consensus palindromic repeat, or by interacting with other transcription factors, and by recruitment of cell-specific co-regulators (Hall and McDonnell, 2005). Transmembrane estrogen receptors such as G protein-coupled estrogen receptor (GPER) have been shown to mediate non-genomic actions of estrogen in several tissues (Barton et al., 2018) as well as chronic (genomic) effects (Barton et al., 2018; Meyer et al., 2016). In extra-nuclear-initiated pathways, ERα, ERβ and GPER activate rapid kinase signaling upon ligand binding (Bologa et al., 2006). Regulation of gene transcription by GPER involves MAPK pathways and regulation of transcription factors (Prossnitz and Barton, 2023). The clinical and translational aspects of rapid non-genomic responses to estrogen (and the resulting downstream chronic effects) mediate estrogen signaling in a variety of diseases such as breast cancer, cardiovascular and neurodegenerative diseases (Mauvais-Jarvis et al., 2022; Prossnitz and Barton, 2023). Of note, rapid responses have been recently reported to determine sexual
dimorphism of estrogen actions in hormone-producing, non-reproductive tissues such as the adrenal gland (Caroccia et al., 2016).

In addition to ligands, the activities of ERs can be modulated by post-translational modifications. ERα, for example, is subject to phosphorylation, ubiquitylation, sumoylation and acetylation (Anbalagan et al., 2012). This will be further described in a separate section below. mRNA decay and translation, together with transcription, determine the abundance of cellular RNA and protein (Hu and Coller, 2012). Additional post-transcriptional mechanisms found in eukaryotes include the regulation of splicing and polyadenylation (Schaefke et al., 2018). The post-translational changes of components involved in the signal transduction cascades, typically initiated by the binding of a ligand to its receptor, are strong indicators of the cell response in which it occurs (Benayoun and Veitia, 2009). Acetylation and deacetylation of a lysine residue on proteins are among the most common post-translational modifications that act as molecular switches in regulating important cellular events (Drazic et al., 2016). The lysine residue is reversibly modified through acetylation and deacetylation by lysine acetyltransferases and lysine deacetylases, respectively (Haberland et al., 2009).

This review focusses on mechanisms involved in non-transcriptional action of estrogens, in particular the metabolic and angiogenic responses mediated by estrogen-activated GPER signaling. The ability of estrogens in regulating glycolytic metabolism through microRNAs in cancer will also be covered.

**Protein regulation by post-transcriptional and epigenetic mechanisms**

Post-transcriptional regulation occurs in a variety of biological processes (Corbett, 2018). However, a given process is often regulated through multiple mechanisms that afford fine-tuning and lead to cell responses within different time frames (Corbett, 2018). By way of example, one such process is the regulation of proteins involved in cellular energy production through glucose metabolism (Kim and Lee, 2012). Fast proliferating cells, e.g. tumor cells, must be able to tightly control and coordinate glucose metabolism and the cell cycle to guarantee sufficient ATP and anabolic
substrates at distinct phases of the cell cycle (Huber et al., 2021). Protein production can be regulated at post-transcriptional levels by several mechanisms affecting mRNA and protein turnover (Mata et al., 2005). Regulation of mRNA stability of glycolytic proteins has been investigated in different cell types, mainly in cancer cells (Wang et al., 2014; Qi and Pekala, 1999). The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) enzyme is a key player in glycolysis/oxidative stress, and regulates metabolism and chemosensitivity in various cancer models (Mondal et al., 2019). The mRNA of all PFKFB3 isoforms is highly unstable. The PFKFB3 gene generates many mRNA transcripts through alternative splicing (Kessler and Eschrich, 2001). A common feature of splicing variants is the presence of multiple copies of the AUUUA sequence in the 3'-UTR region (Bando et al., 2005). The AUUUA motif typifies the 3'-UTR region structures of several proto-oncogenes and proinflammatory cytokines and confers instability and enhanced translational activity to mRNAs (Shaw and Kamen, 1986). PFKFB3 is the first metabolic enzyme to be identified whose mRNA contains the AUUUA instability element (Chesney et al., 1999). Regulation of mRNA stability also plays a role in regulating glucose transporter 1 (GLUT1)/solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1) gene expression. In terms of structure, GLUT1 mRNA contains a 3'-UTR of 884 nucleotides and is considered to be A+U rich (Mueckler et al., 1985). Several laboratories have reported alterations in the stability of GLUT1 transcript in various cell lines and tissues under conditions including both glucose deprivation and hyperglycemia, hemangioblastoma as well as cytokine, hormone, and metabolite stimulation (Qi and Pekala, 1999).

In addition to the above-mentioned mechanisms that affect mRNA stability, cancer cells can adjust PFKFB3 activity according to their own metabolic demand both by varying PFKFB3 gene expression and through post-translational protein modifications. For instance, S-glutathionylation results in decreased PFKFB3 activity (Seo and Lee, 2014), whereas phosphorylation enhances its activity and it is associated with increased glycolysis and cancer progression (Bando et al., 2005). Effects of phosphorylation on PFKFB3 activity have also been investigated in non-cancer cells. For example, monocytes exposed to hypoxia rapidly stimulate glycolysis by activating PFKFB3 through
protein phosphorylation on serine 461, located at the protein C-terminal domain (Marsin et al. 2002). Similar to PFKFB3, GLUT1 undergoes post-translational modifications such as phosphorylation, resulting in increased protein activity; in particular, phosphorylation of GLUT1 by protein kinase C on serine 226 enhances cell surface localization and glucose uptake in endothelial cells (Lee et al., 2015). Specific post-translational modifications including methylation and phosphorylation of specific residues promotes proteasomal protein degradation. For example, in the case of reduced methylation, PFKFB3 activity is reduced as the enzyme undergoes polyubiquitination and is degraded by the proteasome (Yamamoto et al., 2014). Overall, the amount of glycolytic proteins such as PFKFB3 and GLUT1 can be modulated via post-transcriptional mechanisms including protein translation and mRNA or protein stability. It is conceivable that the mechanisms involved in the regulation of key glycolytic promoters are not the same in healthy and tumor cells and/or vary depending on the stimulus.

**Post-transcriptional regulation of glycolytic proteins and angiogenesis by estrogen**

Endothelial cells are considered the body's largest endocrine organ, with a weight that is similar to the liver, covering a surface area of about 400 m² and counting about 1.2 billion cells in a healthy human (Anggård, 1990). Indeed, endothelial cells are metabolically highly active and rely primarily on glycolysis to produce energy in a short timeframe, thus allowing rapid adaptation to micro-environmental changes (Eelen et al., 2015). In particular, GLUT1 and the glycolytic enzyme activator PFKFB3 play a relevant role in the control of endothelial function, including physiopathological angiogenesis. In fact, angiogenic signaling pathways converge into metabolism (Schoors et al., 2014; Draoui et al., 2017), and pathologic angiogenesis can be inhibited by blocking PFKFB3. Fructose-2,6 bisphosphate, the product of PFKFB3, is the allosteric activator of the key glycolytic enzyme phosphofructokinase 1. In endothelial cells, which largely rely on glycolysis for ATP generation (Culic et al., 1997), PFKFB3 represents the most abundant PFKFB isoenzyme (De Bock et al., 2013). Because PFKFB3 plays a role in transcription and its product increases the expression and activity of cyclin-dependent–kinase-1 (Yalcin et al., 2009), PFKFB3 represents a novel pharmacological for targeting angiogenesis and tumor migration and cell...
The glucose transporters GLUT1 and GLUT4, members of the solute carrier family 2, are responsible for taking up extracellular glucose into endothelial cells (Leung and Shi, 2022). It has also been shown that GLUT1 protein deficiency arrests angiogenesis, resulting in overt manifestations of brain dysfunction (Tang et al., 2017).

Estrogens are involved in metabolic regulation (Cignarella et al., 2010; Meyer et al., 2011). Specifically, whether estrogen modulate glycolysis by targeting the stability of glycolytic proteins, and in particular that of PFKFB3 and GLUT1, has been actively investigated. Both GLUT1 and PFKFB3 are finely regulated at both transcriptional and post-transcriptional level by a variety of stimuli including estrogens (Obach et al., 2004; Veschini et al., 2007). In particular, the biologically most relevant estrogen, 17β-estradiol (E2), upregulates GLUT1 mRNA and protein levels, respectively, in different cell types including microvascular endothelial and cancer cells (Shi and Simpkins, 1997). In breast cancer cells, E2-induced alterations in glucose metabolism involve increased transcription of glycolytic proteins including PFKFB3 (Neeman and Degani, 1989; Imbert-Fernandez et al., 2014). Conversely, we reported that E2 rapidly increases PFKFB3 protein levels in a concentration-dependent manner without affecting PFKFB3 mRNA levels (Trenti et al., 2017; Boscaro et al., 2020a), suggesting that non-genomic mechanisms drive estrogen-boosted glycolysis in the vascular endothelium. Thus, E2 promotes glycolytic metabolism in human endothelial cells, where PFKFB3 is a downstream effector of angiogenesis, highlighting that estrogens contribute to adaptable changes of endothelial glycolytic metabolism (Trenti et al., 2017).

The G protein-coupled estrogen receptor (GPER) can cross-talk and interact with classical membrane-associated and nuclear ERs (Barton, 2012; Prossnitz and Barton, 2023). Although the classic ERs have been acknowledged to function in mediating estrogen effects on glucose metabolism, metabolic and angiogenic actions of GPER have been elucidated in the vascular endothelium and in other cell types (Sharma and Prossnitz, 2021). As noted above, estrogens contribute to rapid adaptation of metabolic demand in endothelial tissue by regulating glycolytic protein levels through post-translational mechanisms. GPER promotes glycolysis in endothelial
cells, and mediates the interface between estrogenic agents and PFKFB3 (Trenti et al., 2017; Boscaro et al., 2020a). In fact, treatment with E2 and the selective GPER agonist G1 increases PFKFB3 protein stability by enhancing ubiquitin specific peptidase 19 (USP19) protein amount (Boscaro et al., 2020a), thus decreasing PFKFB3 ubiquitination and proteasomal degradation, resulting in enhanced angiogenesis (Figure 1). This effect is already detectable within 60 minutes of exposure, as indicated by the rapid increase in PFKFB3 protein levels and the involvement of a membrane receptor. GPER silencing abolished the E2-mediated increase in USP19 levels, further supporting the role of GPER-dependent signaling pathways in the rapid post-transcriptional regulation of endothelial PFKFB3. It is tempting to speculate that estrogens could induce the phosphorylation of PFKFB3 in endothelial cells. Overall, these findings are consistent with the notion that estrogen rapidly promotes endothelial glycolysis through a variety of nongenomic mechanisms that may also account for at least some of the proangiogenic effect of estrogenic agents (reviewed in Cignarella et al., 2022).

Other mechanisms of non-genomic protein regulation by estrogens

Some evidence shows that E2 affects the rate of protein translation, thereby modulating protein levels (Sudhagar et al, 2011). For instance, nanomolar concentrations of E2 increase protein S-nitrosylation in endothelial cells via ERα and nitric oxide synthase (NOS) 3, also known as endothelial NOS (eNOS), which attenuates the pro-inflammatory actions of angiotensin II (Chakrabarti et al., 2010). The selective ER modulator (SERM) raloxifene up-regulates telomerase activity in HUVECs via increased phosphorylation through the PI3K/Akt cascade (Doshida et al., 2006). Through activation of the same pathway, E2 treatment increases programmed cell death 1 ligand 1 (PD-L1) protein abundance in ERα-positive cancer cell lines as a result of increased mRNA stability (Yang et al., 2017). Finally, exposure to E2 or G1 rapidly stabilizes hypoxia-inducible factor 1-alpha (HIF-1α) protein with a peak effect at 15 minutes in endometrial tissue without affecting its mRNA levels (Zhang et al, 2017). This suggests that estrogen effects are mediated by a variety of mechanisms that do not involve the transcription of target genes.
Estrogen signaling and intracellular protein degradation via the proteasomal pathway

Regulation of protein levels due to changes in the degradation rate affords rapid adaptation to environmental changes; thus, key players in essential cellular pathways often undergo rapid ubiquitin-proteasome degradation (Stangl and Stangl, 2010). The ubiquitin/proteasome pathway is a possible mechanism for post-transcriptional regulation. The 8-kDa peptide ubiquitin is found in eukaryotes. Ubiquitin or a polyubiquitin chain is conjugated with the targeted protein to form polyubiquitinated protein complexes, which are delivered to and degraded by the 26S-proteasome (Miller and Gordon, 2005). The covalent bonding of ubiquitin occurs via the sequential action of four enzyme families: E1, E2, E3 and E4 (Micel et al., 2013). This process is finely tuned, and intracellular levels of ubiquitinated proteins at any given time depend on the balance between ubiquitination and deubiquitylation reactions in the ubiquitin-proteasome system (Ciechanover et al., 2000).

In non-cancer cells, estrogens appear to modulate protein levels by targeting protein translation or proteasomal degradation rate, possibly via specific E3 ligases (Tschlugguel et al., 2003). For instance, so-called conjugated estrogens (CE), a mixture of hormones derived from pregnant horses' urine also containing equine estrogens, progestins and androgens (Barton et al., 2007) increase endoplasmic reticulum-associated degradation (ERAD)-associated E3 ubiquitin-protein ligase (HRD1) abundance without increasing HRD1 mRNA levels in pancreatic beta-cells (Xu et al, 2018). Whether this also applies to human sex hormones, and which receptor pathways are activated by equine sex hormones is not clear. Through post-translational mechanisms involving stabilization of the E3 ubiquitin ligase HRD1, equine sex hormones also promote ubiquitination and proteasomal degradation of misfolded proteins via the ERAD system, thus preventing endoplasmic reticulum stress and beta-cell dysfunction in vitro and in vivo (Xu et al, 2018; Sachs et al., 2020). In skeletal muscle, E2 increases USP19 protein levels leading to reduced degradation of proteins that are involved in the repression of myogenesis (Ogawa et al., 2015). USP19 is a sex-specific potent down-regulator of muscle mass in young female mice (Ogawa et al., 2015). Systemic genetic deletion of ERα increases soleus muscle mass by repressing USP19 expression in female, but not
in male mice. By binding to ERα, E2 upregulates Usp19 expression. USP19 removes ubiquitin from ubiquitinated target proteins, leading to skeletal muscle atrophy in young female mice (Ogawa et al., 2015). In humans, the impact of estrogen-containing menopausal hormonal therapy on muscle mass or strength is controversial, probably due to the use of a large number of different formulations (Ikeda et al., 2019). Of note, USP19 has also been identified as a potent inhibitor for TNF-α- and IL-1β-induced activation of nuclear factor (NF)-κB (Lei et al., 2019), suggesting a potential novel regulatory mechanism underlying the anti-inflammatory actions of estrogenic agents.

In cancer cells, estrogen promotes K-Ras stabilization by inhibiting its polyubiquitylation, thus contributing to endometrial cellular transformation and tumor growth (Koo et al., 2015). Estrogenic agents target both specific E3 ubiquitin ligases and USPs with different effects according to cell types. As an example, in endometrial cancer cells, estrogens promote the proteasomal degradation of the tumor suppressor p27, which is prevented by inhibitors of the SCF-Skp2/Cks E3 ligase (Pavlides et al., 2013). Taken together, the complex interplay between estrogen signaling and proteasome regulation has been increasingly elucidated, and estrogenic agents appear to play a significant role in this process with consequences in health and disease (Xu et al., 2018).

**Post-transcriptional modification of estrogen receptor expression and function**

Site-specific covalent post-translational modifications, including acetylation at lysine's 266 and 268, increase both the DNA-binding and transcriptional activities of human ERα (Kim et al., 2006). Post-translational modifications impact on ER expression and stability, subcellular localization and sensitivity to pharmacological agents. Discrepancy between ER gene expression and protein accumulation has been reported. For instance, short-term transdermal E2 treatment for one week, an increase in adipose tissue ESR1 gene expression was observed in both early and late postmenopausal women, although ERα protein levels did not change (Park et al., 2017). This may be due to post-translational modification such as protein degradation or ubiquitination. In target tissues such as the uterine endothelium, E2 regulates expression of ERα by rapid degradation via a proteasome-mediated pathway (Tschugguel et al., 2003). ER palmitoylation, a protein
modification determining its stability, trafficking to the plasma membrane and membrane signaling, is essential in mediating neuronal membrane-initiated E2 signaling (Meitzen et al., 2013). E2-bound ERα has been reported to be more susceptible to its degradation in the non-palmitoylated state (La Rosa et al., 2012). Mice expressing an ERα mutated for the palmitoylation site (C451A-ERα) are a model for membrane-induced steroid signaling loss-of-function (Adlanmerini et al., 2014). Of note, simvastatin treatment reduces ERα palmitoylation and increases ubiquitin-mediated ERα degradation in immortalized human leiomyoma cells (Afrin et al., 2021).

ERα signaling is regulated by other proteins and transcription factors. For example, resolvin D2, a member of the specialized pro-resolving lipid mediator family, indirectly modulates ERα signaling in ER-positive breast cancer cells transcription via activation of the PI3K/Akt signaling pathway (Al-Zaubai et al., 2014). Because of the interplay between growth factor and E2 signaling that regulates transcriptional effects (Marquez et al., 2001), resolvin D2 may activate signaling cascades leading to post-translational modifications of ERα or its co-activators or co-repressors (Al-Zaubai et al., 2014). FOXA1 is a transcription factor that enables ER binding to chromatin. In breast cancer cells, FOXA1 is responsible for most ER binding events in the genome, and its upregulation is associated with enhancer reprogramming in endocrine resistance (Fu et al., 2016). Of note, FOXA1 is post-translationally modified in response to proinflammatory cytokines at two evolutionarily conserved amino acids (Franco et al., 2015). The post-translational modification of FOXA1 in response to external stimuli suggests a mechanism underlying FOXA1 regulation and function (Toska et al., 2017). In particular, estrogen-directed post-translational modification of FOXA1 may dictate binding site selection and drive ERα to non-canonical enhancers across the genome, thereby activating expression programs that underlie the tumorigenesis of breast cancer (Fu et al., 2019).

Pharmacological modification of ER stability may represent an interesting therapeutic approach. In particular, SERMs and selective ER antagonists and degraders (SERDs), such as fulvestrant, are approved for the treatment of ER-positive breast cancer. However, the emergence of mutations in the *ESR1* gene encoding ERα (*ESR1m*; Scott et al., 2020) has been described as a major mechanism of resistance to endocrine-based therapy (O’Leary et al., 2018). Novel...
compounds are likely to provide superior clinical benefit to existing endocrine therapies through improved target engagement and modulation in patients with hormone receptor-positive breast cancer. AZD9833 is an orally active, potent, next-generation SERD that has demonstrated antitumor activities in several ER-positive cell lines and patient-derived breast cancer xenograft models (Scott et al., 2020). It also holds promise to counteract or delay the emergence of endocrine resistance, which develops in patients treated with ERα-targeting drugs. A Phase I study of AZD9833 (SERENA-1) showed good oral bioavailability (Gangl et al., 2020) to support once daily oral administration. In addition, promising antitumor activities and an encouraging safety profile (Hamilton et al., 2020) were seen with AZD9833 monotherapy. In this context, although SERMs and SERDs inhibit cell survival and proliferation through the inhibition or degradation of ERα, respectively, at the same time they show agonistic effects towards GPER (Filardo et al., 2000; Thomas et al., 2005; Petrie et al., 2013). Sustained stimulation of GPER in women taking SERM/SERD therapies possibly represents one of the mechanisms contributing to resistance or even stimulation of certain recurring breast tumors, as well as the increased risk of endometrial hyperplasia and cancer (Pepermans and Prossnitz, 2019). This suggests that ERα inhibition in the absence of GPER activation might reduce anti-hormone therapy resistance with the future possibility of improved outcomes for women with breast cancer.

Accumulating evidence suggests that ERβ shifts from a predominantly ligand-activated transcription factor (its major role during reproductive years) to a ligand-independent transcription factor after menopause with constitutive activity (Kim et al., 2018). The main molecular factors that facilitate the ligand-independent function of ERβ in the aged brain are post-translational phosphorylation of the receptor (Pinceti et al., 2015), alternative RNA splicing, and co-regulatory protein interactions. Therefore, understanding the basic molecular signaling pathways of E2 in the aging brain will help drive therapeutic advances and inform treatment strategies for postmenopausal women. In addition, GPER also drives constitutive, ligand-independent expression and activity on NADPH oxidase 1 (NOX1; Meyer et al, 2016), an NADPH oxidase isoform that is key to many chronic diseases and is involved in the physiological aging process (Zhu et al., 2015; Meyer et al, 2016; Tsai et al., 2016; Yoon et al., 2023).
Regulation of protein translation by microRNAs and the role of estrogen

The discovery of microRNAs (miRNA), a class of small non-coding RNAs molecules comprising 22-25 nucleotides, has revealed the existence of a new level of gene expression regulation (Cech and Steitz, 2014). Post-transcriptional protein regulation includes several mechanisms affecting mRNA and protein turnover, which are not mutually exclusive (Maier et al., 2009). Among the post-transcriptional mechanisms involved in the regulation of protein levels, miRNAs act as negative post-transcriptional regulators of gene expression by targeting multiple mRNAs and inducing repression of translation and/or RNA degradation (Chamorro-Jorganes et al., 2013; Iorio et al., 2007; Oliveto et al., 2017). In particular, miRNAs mediate translational repression through direct binding to the 3'-UTR of target mRNA (Kong et al., 2008). In addition, miRNAs are emerging rapid post-transcriptional regulators of protein abundance and function. The intracellular pool of functional miRNAs is controlled at multiple levels, including pre-miR maturation and miRNA degradation (Kir et al., 2018). Moreover, miRNAs are critical regulators of cell proliferation, differentiation and cellular responses to stress, but their biogenesis is poorly understood. For example, transcription rates of miRNAs often do not correlate with the level of the corresponding mature miRNA, suggesting that post-transcriptional events determine the fate of miRNAs during these cellular processes (Ding et al., 2009). The miRNA pathway can be regulated through post-transcriptional modification. Specifically, the BCDIN3D RNA methyltransferase methylates specific miRNA precursors to inhibit their processing into mature miRNAs by Dicer (Xhemalce et al., 2012). Given that miRNAs are de-regulated in human diseases, obtaining a comprehensive mechanistic view of miRNA biogenesis could greatly expand potential therapeutic opportunities.

MiRNAs are recognized as rapid post-transcriptional regulators of protein abundance, and a single miRNA has the potential to simultaneously regulate multiple proteins (Fabian et al., 2010). Emerging evidence establishes miRNAs as novel players in the regulation of glycolytic metabolism in cancer cells (Subramaniam et al., 2019). Remarkably, the amount of PFKFB3 and GLUT1 is regulated by the action of specific miRNAs in several cancer cell types, and deregulation of miRNA expression has been attributed to estrogen-related cancers (Ge et al., 2015; He et al., 2019). There is strong evidence, at least in estrogen-sensitive cancer cells, for a role of miR-206 and miR-
26b in the down-regulation of PFKFB3 protein levels, which impacts on cell growth and migration (Du et al., 2015; Ge et al., 2015). Several miRNAs are up- or down-regulated in ovarian cancer, suggesting that they play a role as a novel class of oncogenes or tumor-suppressor genes depending on the targets they regulate (Iorio et al., 2007). A further layer of complexity is due to the possible combination of the competing endogenous RNA phenomenon, whereby coding and non-coding RNA molecules targeted by the same miRNAs compete for binding, and epigenetic silencing of miRNA by target proteins, as demonstrated using mathematical models (Huang et al., 2022). In non-cancer cells, emerging evidence in post-ischemic tissues suggests a pro- or anti-angiogenic role of specific miRNAs in the regulation of metabolism and neovascularization depending on their targets (Kir et al., 2018; Azzouzi et al, 2015). Remarkably, mice lacking DICER, a key enzyme in miRNA processing maturation, display defective vessel formation and die (Singh et al., 2011). In addition, miRNAs affect angiogenesis at distant sites, being packaged into vesicles (exosomes) and secreted (Kir et al, 2018). Hence, plasma miRNAs could also represent disease biomarkers (Backes et al., 2016).

To date, over 200 estrogen-regulated miRNAs have been identified, and estrogen is known to suppress or stimulate miRNA expression and/or activity acting at multiple levels, including pre-miR maturation and miRNA degradation (Klinge, 2015). For example, E2 reduces endogenous miR-26b and miR-206 expression in the MCF-7 breast cancer cell line (Ge et al., 2015; Tan et al., 2014), which has been correlated with increased cell proliferation or migration. miR-206 negatively regulates estrogen-responsive genes in a complex interplay (Tan et al., 2014). Consistently, overexpression of these miRNAs reduces E2-dependent cell growth (Ge et al., 2015; Tan et al., 2014). In addition, E2 can regulate miRNA function by acting at the post-transcriptional level. There is evidence that disrupted ERα signaling in breast cancer is mediated by expression of argonaute 2 (AGO2), a protein that regulates translation and microRNA function (Conger et al., 2016). E2 has been shown to regulate AGO2 expression or phosphorylation by interacting with the EGF pathway (Adams et al., 2009). Notably, AGO2 can also be regulated by E3 ubiquitin ligases, which promote its degradation, thereby inhibiting miRNA activity (Klinge, 2015). An involvement of the "classical",
i.e. nuclear estrogen receptors (e.g. ERα) in miRNA regulation by estrogens has also been established (Klinge, 2015). Expression of the ER-α36 variant in breast cancer cells is inversely correlated with let-7 miRNAs, which also increase sensitivity to tamoxifen treatment in resistant cells (Zhao et al., 2011). Recently, GPER has been shown to mediate the inhibition of miR-338-3p expression induced by E2 in an ER-negative breast cancer cell line. Consistent with these observations, miR-338-3p mimics decrease E2-induced cancer cell proliferation, providing evidence for a novel role of GPER signaling in miRNA regulation (Vivacqua et al., 2018).

In ovarian cancer cells, E2 treatment increases PFKFB3 protein levels already at early time points (3-6 h) and for up to 24 h. PFKFB3 upregulation occurs without changes in mRNA levels, in line with what is observed in human endothelial cells (Trenti et al., 2017; Boscaro et al., 2020a). Unexpectedly, the E2-dependent increase in PFKFB3 protein levels did not correlate with the growth response (Boscaro et al., 2022), suggesting additional levels of regulation of cell proliferation. Similarly, E2 treatment increases c-myc and c-fos mRNA levels in the absence of any proliferative response in SKOV3 cells (Hua et al., 1995). However, estrogen-induced growth has been associated with increased c-fos expression in several cancer cell lines (Liu et al., 2014), suggesting that the functional activation of ERs depends on the specific environment and cell type.

Based on the observation that E2 treatment increases PFKFB3 protein levels via non-genomic mechanisms (Trenti et al., 2017; Boscaro et al., 2020a), miRNAs may be involved in this regulation by targeting PFKFB3 3′-UTR. In fact, both miR-26b and miR-206 negatively regulate PFKFB3 expression in a luciferase assay (Boscaro et al., 2022). Transfection of SKOV3 cells with miR-26b and miR-206 significantly reduces PFKFB3 protein abundance, which is not reverted by E2 pre-treatment (Boscaro et al., 2022). This is consistent with previous findings in breast cancer cells transfected with a miR-338-3p mimic, where treatment with E2 or G1 failed to upregulate c-fos mRNA and protein levels (Vivacqua et al., 2018). Conversely, miR-206 directly interacts with the 3′-UTR of PFKFB3 mRNA in breast cancer cells lines, and E2 treatment was partially able to revert this miRNA’s effect on PFKFB3 levels (Ge et al., 2015). Overall, it appears that estrogenic agents can inhibit endogenous miRNAs’ functions but cannot counteract the effect of long-term exposure to exogenous miRNAs. Notably, both miR-206 and miR-26b are significantly
downregulated in ovarian cancer with respect to normal tissue (Dai et al., 2018; Lin et al., 2015), consistent with the hypothesis that tissues permanently exposed to high estrogen concentrations express lower levels of specific miRNAs. miR-206 overexpression impairs proliferation and migration of estrogen-dependent cancer cells by modulating PFKFB3 amounts (Boscaro et al., 2022), which is functionally linked to protein tyrosine kinase 2 (FAK), a master regulator of cell migration (Boscaro et al., 2020b; Figure 2). miR-206 is downregulated in ERα-positive tissues and in MCF7 cells, and ERα agonists may further modulate this effect (Adams et al., 2007; Kondo et al., 2008; Li et al., 2014). This is consistent with a direct effect of tumor-suppressor miR-206 on the negative regulation of estrogen-responsive genes, including ERα (Kondo et al., 2008; Chen et al., 2012).

While estrogens can stimulate or suppress miRNA activity in different types of cancer cells, it is unclear whether this occurs in healthy tissues as well (Klinge, 2015; McCall et al., 2011). miR-7a is expressed in granulosa cells in the ovary, is involved in estrogen synthesis and regulates ovarian function (Li et al., 2022). Because miRNAs are rapid post-transcriptional regulators of protein abundance as well as emerging players in endothelial cell metabolism and function including angiogenesis, miRNAs may participate in the functional regulation of endothelial glycolytic programs, with a possible role for GPER in this process (Boscaro et al., 2020a; Wade et al., 2019; Vidal-Gomez et al., 2018). miR-mediated PFKFB3 downregulation may also be relevant in vascular ischemic disorders, where rapid metabolic and functional adaptation to environmental changes occurs (Li et al., 2019). Along the same lines, although the underlying basis for sexual dimorphism in autoimmune diseases is yet to be determined (Whitacre 2001), the X chromosome is highly enriched for miRNAs, whose expression can be regulated by estrogens: dysregulated miRNA expression has been documented in some autoimmune disease conditions, including rheumatoid arthritis (Dai and Ahmed, 2011).

**Epigenetic regulation of adipose tissue by estrogens**

Reduced endogenous production of estrogens, for example following menopause, predisposes to or facilitates visceral adiposity (Papadakis et al., 2018). Adipose tissue morphology and biology in
males and females in turn are differentially programmed by early life exposures, as has been suggested in some preclinical studies (Rodgers and Sferruzzi-Perri, 2021). Estrogen-mediated epigenetic regulation of adipogenic genes via interaction with enzymes involved in DNA methylation and histone tail post-translational modifications are key mechanisms believed to mediate the interaction between the in-utero environment and clinical outcomes (Jorgensen et al., 2016). These events may mediate the transgenerational inheritance of adipose tissue regulation and obesity (Sun et al., 2019). Moreover, ERα and/or ERβ are thought to be involved in the epigenetic regulation of adipogenesis (Bitirim et al., 2021). Histone modifications by ERα are described: for instance, ERα interacts with and promotes the activity of lysine methyltransferase 2B (MLL2), which is also required for ERα-activated gene transcription (Shi et al., 2011).

**Conclusion**

In addition to their genomic effects, multiple estrogen-dependent effects and mechanisms that do not involve transcription of target genes. The main pathways discussed in this article are summarized in Figure 3. For instance, slowing down glycolytic protein turnover via estrogen receptors such as GPER allows estrogens to rapidly and finely tune functions in several cell types including cancer, endothelial and immune cells that rely on glycolysis to rapidly adapt to environmental cues (Li et al., 2019). These observations might have implications in estrogen’s protective and prophylactic effects in chronic ischemic vascular or myocardial diseases as well as in heart failure, where rapid metabolic and functional adaptation to environmental changes is required (Mauvais-Jarvis et al., 2020; Barton and Meyer, 2020; DeFilippis et al., 2022; Shuaishuai et al., 2022). Identification of E2-targeted miRNAs, which in turn regulate glycolytic protein levels as well as cell growth and invasiveness, may pave the way to the development of miRNA-based treatments for blocking adverse hormone functions such as pathological angiogenesis in E2-dependent cancers. In addition, drugs affecting ER stability and turnover are possible strategies to overcome resistance to ER-dependent cancer endocrine therapy. Thus, posttranslational and epigenetic regulation of estrogen signaling offers exciting new possibilities for the development of new therapeutics, particularly in the fields of oncology and cardiovascular diseases.
Data Availability Statement

This article contains no datasets generated or analyzed during the current study.

Authorship contribution

Drafting of the manuscript: Cignarella

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Footnotes

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Competing interests
M.B. is an inventor on U.S. patent Nos. 10,251,870, 10,682,341 and 10,980,785 for the therapeutic use of compounds targeting GPER.
Legend to Figures

**Figure 1.** Mechanisms of estrogen-dependent effects on phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) target gene stability: Estrogenic agents rapidly increase PFKFB3 levels by promoting protein stability, namely by decreasing PFKFB3 ubiquitination and proteasomal degradation. Treatment with 17β-estradiol (E2) and the GPER agonist G1 enhances PFKFB3 protein stability via upregulation of ubiquitin specific peptidase 19 (USP19).

**Figure 2.** Graphical depiction summarizing the tumor suppressor role of miR-206 in ovarian cancer cells. In cells expressing low levels of miR-206, exogenous administration of miR-206 induces anti-migratory and anti-proliferative effects through downregulation of the PFKFB3/FAK axis. EOC: epithelial ovarian cancer.

**Figure 3.** Estrogen-regulated post-transcriptional mechanisms involved in different human diseases. Estrogens are involved in several regulatory mechanisms that do not require gene expression regulation. These include modulation of protein ubiquitination and degradation, phosphorylation, palmitoylation, nitrosylation and miRNA expression, which have been associated with cancer, aging and cardiovascular diseases. SERMs: selective ER modulators. PD-L1: programmed cell death 1 ligand 1.
Figure 1
Figure 2

This figure illustrates the role of miR-206 in EOC cells. MiR-206 targets PFKFB3 mRNA, leading to decreased PFKFB3 protein levels and subsequently decreased FAK protein levels. This results in cell growth and migration inhibition.
**Breast cancer**
- Proteins phosphorylation (PFKFB3 Ser^481^)
- Reduced miR-206/miR-26b expression → enhanced PFKFB3 expression
- Reduced miR-338-3p expression (via GPER) → increased cell proliferation
- ERα S^118^ phosphorylation → increased SERM responsiveness
- ERα palmitoylation → enhanced ERα stability

**Visceral adipose tissue**
- Epigenetic regulation of adipogenesis → reduced visceral adiposity

**Gynecological cancer**
- Increased PD-L1 mRNA stability
- Reduced K-Ras ubiquitination → cellular transformation and tumor growth
- Increased p27 proteasomal degradation → reduced tumor suppression activity

**Endothelium**
- PFKFB3 decreased proteasomal degradation → increased angiogenesis via GPER
- Increased protein S-nitrosylation → vascular protection

**Pancreatic β-cells**
- Ubiquitination and protein degradation regulation → β cells protection

**Skeletal muscle**
- USP19 upregulation and protein degradation regulation → reduced age-associated muscle loss