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NON-GENOMIC MECHANISMS IN THE REGULATION OF GLYCOLYTIC PROTEINS BY ESTROGEN RECEPTOR LIGANDS IN CELLS WITH HIGH GLYCOLYTIC RELIANCE

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SUMMARY

17β-estradiol (E2) affects multiple aspects of tissue and cell metabolism with implications in physiological and pathological conditions. Few studies have been conducted to explore the mechanisms coupling hormonal signals to metabolic demand in cells with high glycolytic reliance such as endothelial cells. We recently showed that E2 triggers angiogenesis via the membrane estrogen receptor GPER and the key glycolytic protein PFKFB3 as a downstream effector. In particular, we reported that E2 rapidly increases PFKFB3 protein levels in a concentration-dependent manner without affecting PFKFB3 mRNA levels, suggesting that non-genomic mechanisms drive estrogen-boosted glycolysis in the vascular endothelium.

We investigated whether estrogenic agents contribute to rapid adaptation of metabolic demand in human umbilical cord vascular endothelial cells (HUVECs) by regulating glycolytic protein levels through non-genomic mechanisms involving protein stability and/or degradation. Similarly to E2, the GPER selective agonist G1 increased PFKFB3 protein amounts peaking at 3 h, without affecting mRNA levels. When protein synthesis was inhibited by cycloheximide, E2 treatment prevented PFKFB3 degradation over time. Similar results were obtained with G1. In addition, the selective E3 ubiquitin ligase (SMER-3) as well as the proteasome inhibitor MG132 rapidly increased PFKFB3 protein levels, thus mimicking the effect of estrogenic agents. Accordingly, the levels of ubiquitin-bound PFKFB3 decreased in HUVECs treated with either E2 or G1. Notably, both ER ligands increased the deubiquitinase USP19 levels whereas GPER siRNA counteracted this effect. Moreover, E2 and G1 treatment enhanced GLUT1 expression in a short time frame (1 h) through a mechanism unrelated to transcriptional activation. Differently from what observed for PFKFB3, we found that treatment with E2 did not affect GLUT1 stability, at least over 24 hours. Accordingly, HUVECs treatment with the proteasome inhibitor MG132 for 3-6 h did not change GLUT1 levels, suggesting that alternative post-transcriptional mechanisms are likely involved.

MiRNAs may contribute to post-transcriptional regulation of protein abundance by binding to the 3'-UTR of target mRNAs, leading to translational repression. Based on the evidence that E2 downregulates the expression of miRNA-206 and miRNA-26, thereby increasing PFKFB3 levels and breast cancer cell proliferation and invasion, we hypothesized that estrogenic agents would regulate glycolytic proteins post-transcriptionally via shared miRNAs. To test the involvement of specific miRNA in PFKFB3 turnover, we set up a luciferase gene assay. For this purpose we first generated a vector containing the 3'-UTR region of human PFKFB3. Since we were unable to efficiently deliver the vector to HUVECs, we decided to use the ovarian cancer cell line SKOV3. We showed that treatment with E2 or G1 did not induce a proliferative response in SKOV3, and that the proliferation was not inhibited by the selective ERα and GPER antagonists MPP and G15, respectively. However, treatment with E2 as well as G1 induced SKOV3 migration in a concentration-dependent manner, suggesting that SKOV3 are responsive to estrogenic agents. E2 treatment increased PFKFB3 levels in SKOV3 at early time points and for up to 24 h, without affecting PFKFB3 mRNA levels, in line with what observed in HUVECs. Next, to explore the role of specific miRNAs in PFKFB3 turnover and the interplay with estrogenic agents, we transfected SKOV3 with the vector containing human PFKFB3-3'UTR with high transfection

efficiency. Using the luciferase assay, we found that both miRNA-26b and miRNA-206 reduced luciferase activity with respect to miRNA-negative control. Consistently, miRNA-26b and miRNA-206 mimics significantly decreased PFKFB3 protein levels, and pretreatment with E2 did not revert the effect of the above miRNA mimics. Further studies are required to establish whether estrogenic agents up-regulate PFKFB3 by inhibiting endogenous miRNA expression/activity.

In conclusion, we showed that the membrane receptor GPER mediated the post-transcriptional regulation of endothelial GLUT1 and PFKFB3 abundance. We add knowledge to the mechanisms coupling hormonal signals with metabolic demand in cells that rely on glycolysis to exert their functions, showing that estrogenic agents in endothelial cells enhanced the amount of a) GLUT1 protein but not mRNA, and b) PFKFB3 protein by increasing deubiquitinase USP19 levels, thereby reducing its ubiquitination and further degradation; in addition, c) E2 controlled PFKFB3 protein levels in SKOV3 cells via post-transcriptional mechanisms, likely involving miRNA regulation.Overall, these results might have implications in estrogen's protective and prophylactic effects in vascular ischemic disorders where rapid metabolic and functional adaptation to environmental changes is required. In addition, the identification of E2-targeted miRNAs in cancer cells, which in turn regulate glycolytic protein levels and thus likely cell growth and invasiveness, will pave the way to the development of miRNA-based treatments for blocking adverse hormone functions such as pathological angiogenesis in E2-dependent cancers.

1. INTRODUCTION

1.1 ESTROGENS

1.1.1 Biosynthesis and regulation of endogenous estrogen levels

Estrogens are the primary female sex hormones, recognized predominantly for their function in female reproductive system and for the development of secondary sex characteristics. Estrogens are also critical mediators of multiple and diverse physiologic effects throughout the body in both woman and men, including the reproductive, cardiovascular, endocrine, nervous, and immune systems (Barkhem et al., 2004).

In healthy premenopausal women, the most active natural physiologic form and the main circulating estrogen is 17β-estradiol (E2), formed from the aromatization of testosterone in multiple tissues but predominantly in the ovaries. Additional forms of estrogen include estrone (E1), the least abundant estrogen, derived from aromatization of androstenedione, and estriol (E3), produced primarily during pregnancy (Gruber et al., 2002).

In women with normal menstrual cycles, E2 functions as a circulating hormone that acts mainly on distant target tissues. In postmenopausal women, when the ovaries fail to produce E2 and in men, who have naturally low circulating levels, E2 is synthesized in extragonadal sites where it acts locally as a paracrine or intracrine factor (Mauvais-Jarvis et al., 2013; Fig. 1.1). These sites include the mesenchymal cells of adipose tissue including that of the breast, osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain (Simpson et al., 2003). Therefore, among both postmenopausal women and men, the determinant of E2 action is not circulating estrogens; rather, E2 function depends on estrogen biosynthesis from a circulating source of androgens. Consequently, in these individuals, a major driver of E2 action is the aromatization of androgens to estrogens (Simpson et al., 2005).

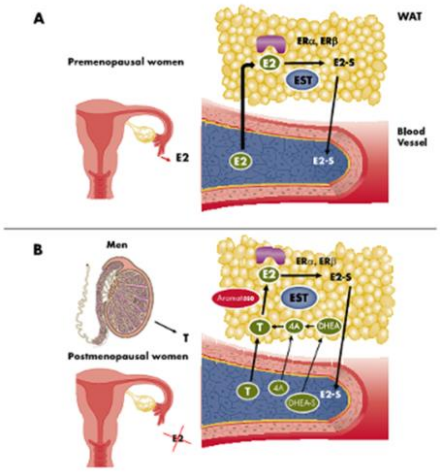

Figure 1.1 Origin of circulating and tissue estrogens. A, In healthy premenopausal women, 17β-estradiol (E2) is produced by the ovaries and functions mainly as a circulating hormone that acts on distant target tissues. B, In postmenopausal women and in men, E2 is synthesized in extragonadal sites from circulating androgenic precursors such as testosterone, androstenedione (4A), or dehydroepiandrosterone (DHEA) (Mauvais-Jarvis et al., 2013).

Estrogen biosynthesis derives by the synthesis of the 19-carbon steroid hormone pregnenolone from cholesterol (Fig. 1.2). This compound is converted to testosterone and then to the estrogens, estrone and 17β-

estradiol. Estrogen biosynthesis is catalyzed by aromatase, a microsomal member of the cytochrome P450 superfamily (Gruber et al., 2002). Aromatase activity has also been detected in muscle, fat, nervous tissue (Gruber et al., 2002) and in the endothelium (Arnal et al., 2010).

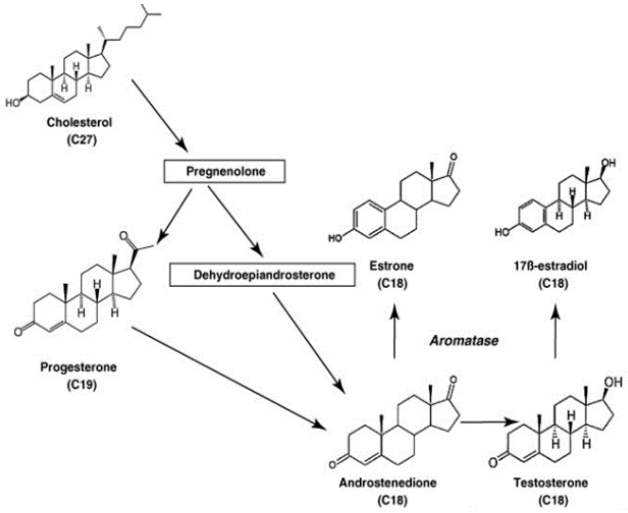

Figure 1.2 The metabolic pathway for estrogen. Estrogens are derived from cholesterol, with pregnenolone formed from cholesterol through the activity of cytochrome P-450. The ovarian granulosa cells express the highest levels of aromatase in premenopausal women, but the adipose tissue becomes the major aromatase-expressing site after menopause (Cignarella et al., 2010).

The primary sources of E2 in women are the theca and granulosa cells of the ovaries. The theca cells secrete androgens that diffuse to the granulosa cells to be aromatized to E2. Estrone and estriol are primarily formed in the liver from E2 metabolism. Estrogens are further metabolized by sulfation or glucuronidation, and the conjugates are excreted into the bile or urine. Estrogens are also metabolized by hydroxylation and subsequent methylation to form catechol and methoxylated estrogens (Hughes et al., 2002).

After synthesis, E2 is secreted into the bloodstream, where it binds to sex-hormone–binding globulin (SHBG) and albumin. Free estrogens (about 2 to 3 percent) diffuse into target tissues to exert their specific genomic or non-genomic effects, by interacting with estrogen receptors. It is generally believed that only unbound (free) hormones are able to act on their respective receptors, and that SHBG could influence hormone bioavailability (Gruber et al., 2002).

During menstrual cycles, E2 production varies cyclically, with the highest rates and serum concentrations in the preovulatory phase (Gruber et al., 2002). Total serum estrogen levels in premenopausal women generally range from 0.1 to 1.3 nM, whereas in perimenopausal period depletion of ovarian follicles leads to a steady decline in ovarian E2 production, although serum estradiol concentrations vary considerably. Significantly lower levels of E2 are found in postmenopausal women (0.02–0.2 nM) and men (0.07–0.25 nM) (Kushnir et al., 2008; Blair, 2010); however, local tissue concentrations of E2 may be considerably higher, up to 8-fold (Huhtinen et al., 2012).

1.1.2 Estrogen receptor subtypes and signaling

Estrogens mediate their effects through multiple receptor subtypes, including the traditional intracellular estrogen receptors ERα and ERβ and the recently described membrane G protein–coupled estrogen receptor, GPER, belonging to the G protein–coupled receptor (GPCR) family. The endogenous estrogen receptor agonist E2 has a binding affinity for ERα and ERβ of 0.1–0.4 nM, whereas the affinity of E2 for GPER is 10-fold (3–6 nM) (Revankar et al., 2005). To differentiate between the functions of different receptor subtypes, selective ligands have been syntesized (Fig. 1.3) (Harrington et al., 2003; Bologa et al., 2006).

In particular, GPER agonists and antagonists are available, which are valid tools for studying the function of this receptor. In particular, the selective and potent agonist for this receptor was developed, G1 1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone, which shows activity for neither ERα nor ERβ. Selective antagonists such as G15 ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline) and G36 ((±)-(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-8-(1-methylethyl)-3*H*-cyclopenta[*c*]quinolone) were also produced (Bologa et al., 2006; Dennis et al., 2011). An overview of GPER ligands according to the IUPHAR nomenclature has been provided by Prossnitz and Arterburn *(*2015). Compounds belonging to different structural classes including phytoestrogens (e.g. genistein), xenoestrogens (e.g. bisphenol A, BPA) and anti-estrogens (e.g. tamoxifen, ICI182,780 (Faslodex/fulvestrant), raloxifene) appear to act as GPER agonists.

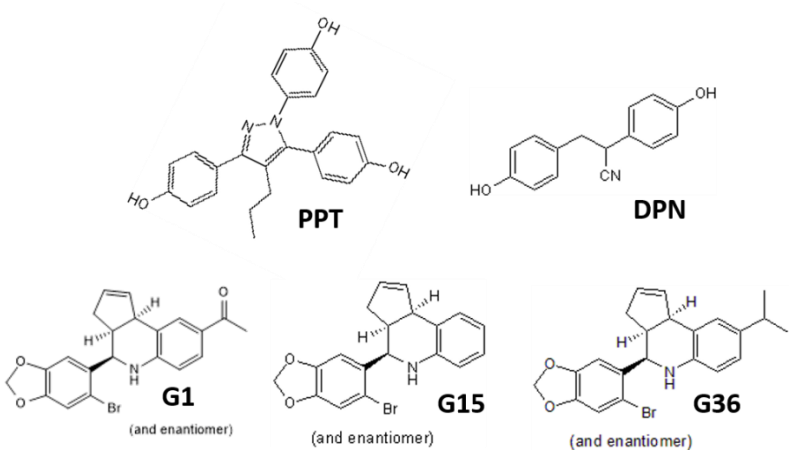

Figure 1.3 Selective synthetic agonist and antagonist for estrogen receptor subtypes. The selective ERα agonist PPT (1,3,5-(4-hydroxyphenyl)-4-propyl-1H-pyrazole) is 410 times more selective for ERα than ERβ. The selective ERβ agonist DPN (2,3-bis-(4-hydroxyphenyl) propionitril) has binding affinity 70 times greater for ERβ than ERα (Harrington et al., 2003). The selective GPER agonist, G1 (1-(-4(-6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetraidro-3H-ciclopenta[c]chinolin-8-il)-etanone), has no activity on ERα or ERβ. G15 (1(-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone) and G36 ((±)-(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-8-(1-methylethyl)-3*H*-cyclopenta[*c*]quinolone) are selective GPER antagonists (Bologa et al., 2006; Dennis et al., 2011).

The effects induced by E2 in different tissues are the result of the activation of genomic (long-term effects) and non-genomic (rapid effects) signaling pathways (Fig. 1.4, Iorga et al., 2017). Although both receptor families can initiate rapid cell signaling and transcriptional regulation (Hadjimarkou and Vasudevan, 2018),

the nuclear receptors act as ligand-activated transcription factors and are traditionally associated with genomic effects, whereas GPER is recognized as mediating non-genomic rapid cellular signaling (Prossnitz and Arterburn, 2015). In the next sections nuclear receptor and GPER structural features and effector mechanisms will be described in more detail.

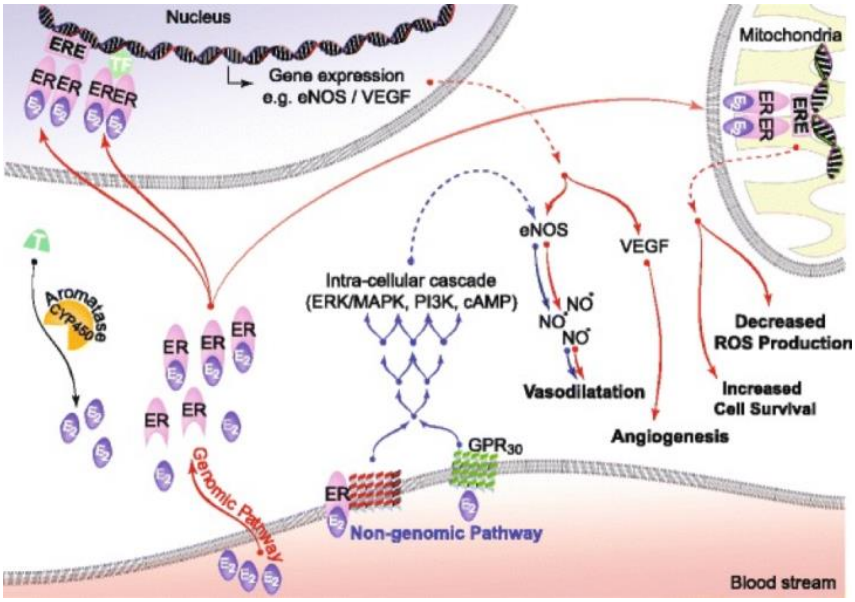

Figure 1.4 Genomic and non-genomic actions of E2. In genomic regulation, binding of E2 to the ER promotes the formation of homo/hetero dimers, translocation to the nucleus and direct binding to estrogen response elements (ERE), or to transcription factors which regulate transcription of its target genes. In non-genomic regulation, binding of E2 to ERs and GPER (also called GPR30) at the plasma membrane leads to activation of MAPK/ERK/PI3K/cAMP, which induce eNOS, a potent vasodilator. Genomic pathways are shown in red arrows, whereas non-genomic pathways are shown in blue arrows. Abbreviations: E2, estrogen; ERs, traditional estrogen receptor; ERE, estrogen response element; T, testosterone; GPCR, G-protein-coupled receptor; PI3K, phosphoinositide 3-kinase; MAPK, mitogen activated protein kinase; AKT, protein kinase B; VEGF, vascular endothelial growth factor (Iorga et al., 2017).

ERα and ERβ

The estrogen receptors (ERs) ERα and ERβ are encoded by two distinct genes. The human ERα gene (*ESR1*) is located at chromosome 6 and encodes the full-length 66 kDa protein composed of 595 amino-acids (Kos et al., 2001). The ERβ gene (*ESR2*), mapping to chromosome 14, consists of 530 amino acids, with a molecular mass of 60 kDa (Ogawa et al., 1998).

ERs share structural characteristics that are responsible for similar functional features. Distinct aminoacid compositions at various structural regions are responsible for subtype-specific properties in conveying E2 signaling. ERs are subdivided into functionally distinct domains (Fig. 1.5). Starting from NH_2- to COO-terminus, the principal domains are: (1) the transcriptional regulation domain (AF-1); (2) DNA-binding domain (DBD) responsible for binding to specific DNA sequences (the estrogen response elements, EREs); (3) the ligand binding domain (LBD), which contains the activator factor-2 (AF-2), necessary for ligand binding. Two activation function (AF) domains, AF1 and AF2, located within the N-terminal domain (NTD) and LBD, respectively, are responsible for regulating the transcriptional activity of ER (Kumar et al., 2011).

Full transcription activity of the ER is thought to be achieved by synergism between the two AFs, and their activities are promoter and cell specific. AF1 functions as hormone independent, whereas AF2 function requires the presence of hormone/steroid (Kumar et al., 2011).

ERα and ERβ exhibit high homology in their DNA binding domain (97%), low homology (17%) in their AF-1 domain and partial homology (55%) in their ligand binding domain. Differences in AF-1 and AF-2 could allow drugs to be designed in order to recruit different cofactors to ERα and ERβ. Depending on the ligand and the tissue, ERs regulate both positively and negatively the expression of thousands of genes (Katzenellenbogen et al., 2000; Charn et al., 2010) recruiting coactivators to stimulate and/or corepressors to inhibit gene expression. For example, tamoxifen is defined as a selective estrogen receptor modulator, being an antagonist in the breast, but an agonist in the endometrium where coactivator expression is higher (Burris et al., 2013).

Several ERα and ERβ isoforms and splicing variants (hERβ1 long, hERβ1 short, hERβ2, hERβ4, hERβ5, hERα-46) have also been described (Kassi and Moutsatsou, 2010).

Figure 1.5 Comparison of the structures and homology between ERα and ERβ. Human ERα contains 595 amino acids whereas ERβ contains 530 aminoacids. The DNA binding domains are nearly identical whereas the transcriptional regulation domain and LBD, which contains AF-1 and AF-2, respectively, have the least homology (Leitman et al., 2010).

ERα and ERβ mediate their effects via different molecular pathways, as illustrated in Fig. 1.6. In the classical transcription pathway, following ligand binding, ER undergoes conformational changes and biochemical modifications that induce release of inhibitory proteins (heat shock proteins), receptor dimerization, and interaction with DNA. In fact, the nuclear ER acts as a transcription factor that modulates gene expression by directly binding to DNA at specific EREs (Fig. 1.6, pathway 1). Genes that regulate endothelial functions including angiogenesis-related genes and genes affecting cell metabolism have been described to have an ERE. Examples of such genes are those encoding the angiogenic factor VEGF (Hyder et al., 2000), the hypoxia-inducible factor HIF-1α (Yang et al., 2015) as well as the glycolytic protein PFKFB3 (Imbert-Fernandez et al., 2014).

In the non-classical transcription pathway, the estrogen/ER-complex starts transcription by binding to alternative transcription factors such as AP-1, SP-1, FoxA1 and NF-κB, which bind non-ERE sites (Fig. 1.6, pathway 2). As mentioned above, a rapid non-genomic pathway has recently been described that is mediated

by membrane-associated ERs. Some studies have indicated that ERα may also be membrane associated in some cells. Binding of these transmembrane receptors mediates several rapid cellular effects of estrogens, including activation of other transcription factors (TF) such as the mitogen-activated protein kinase (MAPK) signaling cascade and intracellular calcium mobilization, leading either to non-genomic signaling or altered transcriptional activity (Fig. 1.6, pathways 3 and 4). The rapid effects of E2 that are believed to originate at least in part from membrane-bound populations of ERs have been explored using estrogen-dendrimer conjugates as well as various forms of transgenic mice expressing mutant forms of ERα (Chambliss et al., 2010; Adlanmerini et al., 2014).

Different tissue distribution suggests that ERs mediate at least in part distinct biological functions. For example, ERα is the most abundant isoform expressed in ovary, prostate, bladder, and lung in the cardiovascular system. Moreover, within the same tissue, the expression of a receptor subtype can be limited to a specific cell type. For example, although the ovary expresses both nuclear ERs, ERα is expressed by the theca cells while ERβ is localized on granulosa (Barkhem et al., 2004). In addition to nuclear localization, it has been shown that ERα is also associated with the plasma membrane in endothelial cells from pig and rat aorta (Cignarella et al., 2009; Wu et al., 2011).

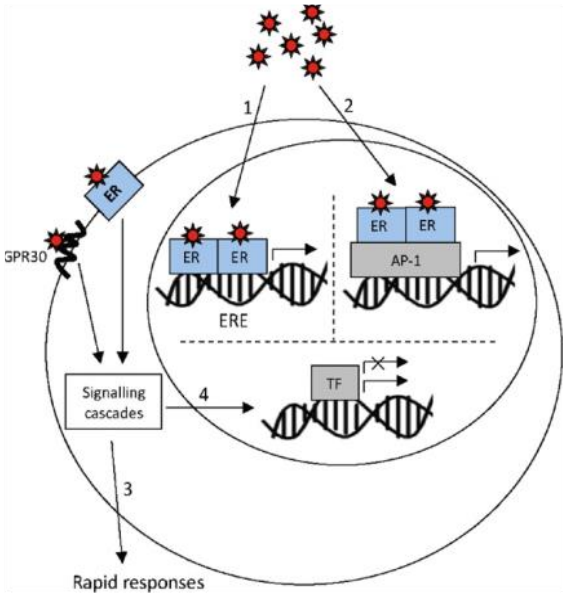

Figure 1.6 Estrogen genomic and non-genomic transcription pathways (Islander et al., 2011).

G Protein–Coupled Estrogen Receptor

The G-protein-coupled estrogen receptor-1 (GPER) is a newly discovered G-protein-coupled receptor. GPER has recently been suggested to be tightly coupled to estrogen receptor membrane signaling and may thereby contribute to normal physiological as well as pathophysiological estrogenic effects. The human GPER is located on chromosome 7 and comprises 375 amino acids with a molecular mass of approximately 41 kDa (Mizukami, 2010). It is a member of the G protein-coupled receptor superfamily, characterized by the presence

of 7 transmembrane helices; its structure comprises the amino terminal portion localized to the cell exterior, where it is often glycosylated, and the carboxy terminus localized to the cytoplasm. Cytoplasmic loops are involved in the selective binding and activation of heterotrimeric G proteins (Prossnitz and Arterburn, 2015).

GPER binds E_2 with high affinity and may be involved in estrogen signaling (Islander et al., 2011). Several GPER activated pathways regulate diverse cellular functions with profound implications for the role of GPER in normal physiology and disease.

Activation of GPER by estrogenic agents results in the activation of several transduction cascades including MAPKs and PI3K/Akt, which in turn affect the production of phosphatidylinositol 3,4,5-trisphosphate and the phosphorylation of proteins such as eNOS (Prossnitz and Barton, 2011). Additional downstream pathways reported to be activated by GPER include the PKC and p-38 MAPK pathways (Sathya et al., 2015; Prossnitz and Barton, 2014; Prossnitz and Arterburn, 2015). GPER agonists elicit the mobilization of intracellular calcium and cAMP synthesis; nevertheless, the exact signaling pathways has not been described (Prossnitz and Maggiolini, 2009; Prossnitz and Barton, 2014). In addition to rapid nongenomic signaling, GPER may also indirectly regulate gene expression (Prossnitz and Maggiolini, 2009; Romano and Gorelick, 2017). Among the genes whose expression is regulated by GPER are fatty acid synthase (Santolla et al., 2012) and VEGF (De Francesco et al., 2014) in breast cancer cells.

Antibodies raised against GPER have demonstrated its expression and distribution in several tissues and cell types. Beyond reproductive tissues, adipose tissue, liver, skeletal muscle and inflammatory cells, GPER is expressed in the heart and blood vessels as well as in human endothelial and smooth muscle cells (Barton et al., 2018). GPER has been found also in cancer cells, in particular in breast cancer, but its functional role has not been completely characterized (Prossnitz and Barton, 2011).

GPER has been first localized to the endoplasmic reticulum and Golgi apparatus of COS7 cells (Revankar et al., 2005). Subsequent experiments through confocal microscopy demonstrated that in many cell types including human umbilical vein endothelial cells (HUVECs) the majority of receptors was localized to intracellular membranes (Fredette et al., 2018). In HUVECs GPER has been observed also at the nuclear level (Chakrabarti and Davidge, 2012), and we recently detected it in the plasma membrane (unpublished data).

Interestingly, GPER is co-expressed with ERα and ERβ in several tissues and cell types, suggesting that interactions between these receptors and their signaling pathways may occur (Prossnitz and Barton, 2011). Evidence shows that GPER interacts with ER signaling. When combined with the actions of classic ERs at the cellular level, ERs and GPER may act either in concert (synergistically or with a requirement for the other receptor) or to antagonize aspects of the other's activity, with the ultimate cellular output being dependent on the integration of all the stimulated and inhibited pathways (Romano and Gorelick, 2017). Two independent studies provide evidence for a physical association between GPER and ERα proteins in human primary monocytes and in a human endometrial adenocarcinoma cell line (Ishikawa cells) (Romano and Gorelick, 2017). Moreover, in the hippocampus of male mice, GPER activation has been shown to phosphorylate the classical intracellular estrogen receptor ERα, suggesting that crosstalk with ERα is important in the display of social behaviors, many of which are absent in ERα-null mice (Hadjimarkou and Vasudevan, 2018). A

functional cross-talk has been reported, where GPER expression is required along with ERα for estrogen-mediated activity in cancer cells (Albanito et al., 2007) or for inhibiting ERα-mediated functions in uterine epithelial cells (Gao et al., 2011), or is also evident from functional vascular studies in porcine coronaries, where acute NO-dependent vasodilation is observed only with ERα-selective agonists such as PPT, but is completely abrogated when ERα, ERβ, and GPER are activated simultaneously by estrogen (Traupe et al., 2007).

1.1.3 Effects of estrogens on the vessel wall and the angiogenic process

Experimental and observational studies suggest that estrogen induces a number of protective effects on the cardiovascular system. The rate of coronary heart disease among women in the childbearing years is lower than in age-matched men and in postmenopausal women when circulating estrogen levels decrease dramatically (Bush and Barrett-Connor, 1985; Wenger et al., 1993).

The protective effects of estrogen on the vascular wall are attributed principally to the hormone's effects on serum lipid concentrations, the coagulation cascade and the modulation of vasoactive proteins. A more detailed description of cardiovascular systemic effects of estrogen has been given by Knowlton and Lee (2012) and Mendelshon and Karas (1999).

The cardiovascular protective effects of estrogen are largely mediated by their direct action on multiple cell types involved in the atherogenic process including immune cells, smooth muscle cells and endothelial cells, which are involved in the maintenance of vascular integrity (Cignarella et al., 2001; Mendelsohn and Karas, 1999). The maintenance of endothelial integrity is known to be closely linked to the artery-protecting effect of estrogen including vasodilation, anti-adhesion, and anti-inflammatory effects by controlling the release of vasoactive molecules.

In particular, *in vitro* and *in vivo* studies indicate that estradiol can promote the generation of nitric oxide (NO) and prostacyclin in the endothelium (Cignarella et al., 2009; Evans et al., 2014). In addition, E_2 prevents monocyte adhesion to endothelial cells, the production of pro-inflammatory cytokines, neutrophil chemotaxis in injured arteries, and contributes to vascular wall remodeling by inhibiting vascular smooth muscle cell proliferation (Mendelsohn and Karas, 1999; Geraldes et al., 2002; Miller et al., 2004) (Fig. 1.7).

With regard to endothelial effects, E_2 has been observed to improve vascular function and endothelial regrowth after arterial injury also through circulating endothelial progenitor cells and new vessel formation (Arnal et al., 2010; Fadini et al., 2008). Indeed, several studies show that estrogens inhibit apoptosis and promote the migration and proliferation of endothelial cells that represent important steps in the angiogenic process (Spyridopoulos et al., 1997; Geraldes et al., 2002; Morales et al., 1995) (Fig. 1.7).

During the menstrual cycle and pregnancy, increased estrogen production and angiogenesis are temporally related (Rubanyi et al., 2002). Pathological estrogen-dependent capillary growth is associated with conditions such as breast cancer (Folkman, 1995). Conversely, angiogenesis stimulation by the ovarian steroid hormone accelerates functional endothelial recovery after arterial injury, which could be beneficial in coronary artery

disease, peripheral vascular disease, cerebral ischemia (stroke) and congestive heart failure (Losordo and Isner, 2001; Evans et al., 2014; Rubanyi et al., 2002; Billinger et al., 2002).

Among the mechanisms of estrogen's proangiogenic effects, E_2 up-regulates well-recognized proangiogenic factors such as vascular endothelial growth factor (VEGF) and regulates expression of adhesion molecules (Arnal et al., 2010). Yet it is increasingly evident that growth factors and transcriptional pro-angiogenic signals converge on metabolic pathways (Schoors et al., 2014). We recently reported that E_2 treatment upregulates the glycolytic enzyme activator phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) in HUVECs in a time- and concentration-dependent manner (Trenti et al., 2017).

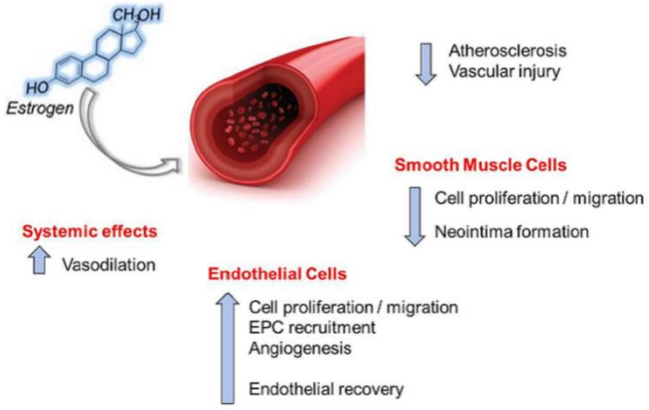

Figure 1.7 Summary of the principal beneficial actions of E2 in the vascular system. EPC, endothelial progenitor cells (Menazza and Murphy, 2016).

In further support of the notion that the macrovascular endothelium is a major direct target for estrogens, testosterone is converted locally to estradiol by the enzyme aromatase expressed in endothelial cells, which also express all known isoforms of estrogen receptors including ERα, ERβ and GPER (Arnal et al., 2010). While ERα is absolutely necessary to several beneficial actions of E_2 in endothelial cells (Bolego et al., 2010), more recently the membrane receptor GPER has been reported to confer cardiovascular benefits, sometimes synergistic with those mediated by ERα (Romano and Gorelick, 2017).

Studies conducted in GPER-knockdown mice showed that the absence of the receptor impairs cardiovascular function (Delbeck et al., 2011; Meyer et al., 2014). For example, the carotid arteries from GPER-deficient mice show increased production of vasoconstrictor prostanoids and response to endothelin-1 (Meyer et al., 2014). Remarkably, GPER hypofunctional polymorphisms have been associated with reduced cardiovascular protection in women (Feldman et al., 2017).

Non-genomic rapid-signaling via GPER is involved in the regulation of vascular biology and tone (Barton, 2018; Prossnitz and Arteburn, 2015). For instance, GPER has been identified as an inhibitory regulator of pro-inflammatory proteins in endothelial cells (Chakrabarti and Davidge, 2012). The ability of GPER to directly induce vasodilatation has been well-estabilished in animal models as well as in human arteries. For example, in vascular preparations from animals and humans the G1 agonist has been found to induce concentration-dependent acute vasodilatation and to counteract the response to a variety of vasoconstrictors (Zimmerman et

al., 2016). Moreover, GPER expression was found to be increased in animal models of spontaneous and secondary hypertension, whereas GPER activation lowered blood pressure, suggesting that the up-regulation of GPER expression may represent an adaptive response to stressful conditions such as hypertension (De Francesco et al., 2013).

The protective effects triggered by GPER activation under both physiologic and pathologic conditions may mimic the beneficial actions of estrogens in the heart and vascular network, suggesting that GPER signaling manipulation could be considered in the clinical management of certain cardiovascular disorders (De Francesco et al., 2017). Consistent with this notion, chronic treatment with the selective GPER agonist G1 improves endothelium-dependent vasomotion in disease conditions associated with vascular inflammation, such as diabetes mellitus (Bologa et al., 2006). Of note, long-term activation of GPER by selective pharmacological agents does not appear to induce undesired uterotrophic effects (Meyer et al., 2014). However, GPER signaling appears to be involved in the proliferation and migration of breast cancer cells. Because the antiestrogen tamoxifen acts a GPER agonist, new compounds acting as ERα and GPER antagonists are required to prevent resistance to endocrine treatment in breast cancer (Lappano and Maggiolini, 2018; Lappano et al., 2012).

Emerging data point to the involvement of GPER in the angiogenic process. It has been demonstrated that GPER signaling stimulates angiogenesis through the engagement of HIF1α/VEGF axis as well as through VEGF-independent signaling (De Francesco et al., 2017). We have recently shown that estrogens trigger angiogenesis via rapid ER signaling that requires GPER and the glycolytic enzyme activator PFKFB3 as a downstream effector, linking for the first time estrogen signaling to the activation of endothelial glycolytic metabolism (Trenti et al., 2017).

1.1.4 Metabolic effects of estrogens

Estrogens play a fundamental role in the control of energy homeostasis, including glucose, by acting on many organs, such as the brain, adipose tissue, pancreas, skeletal muscle and liver. The effect of estrogen on metabolism is ancient; indeed, ancestral ER existed in invertebrates lacking sexual reproductive capabilities, suggesting that they may have played an important role in energy metabolism and survival (Mauvais-Jarvis et al., 2013).

Clinical trials and animal studies have revealed that loss of circulating estrogen induces rapid changes in whole body metabolism, fat distribution and insulin action (Gupte et al., 2015). Indeed, after menopause, women experience a general increase in weight as well as a redistribution of adipose tissue leading to increased abdominal fat deposition (Rettberg et al., 2014). Excess accumulation of adipose tissue in the intra-abdominal region of the body correlates with increased risk of insulin resistance, type 2 diabetes, hypertension and CVD (Mauvais-Jarvis et al., 2013). The mechanisms by which E2 regulates lipid and glucose metabolism have not yet been fully understood (Gupte et al., 2015). E2 suppresses white adipose tissue accumulation by decreasing fatty acid, triglyceride synthesis and lipogenesis. Administration of E2 reduces adipocyte size in ovariectomized (OVX) female mice by reducing fatty acid uptake (down-regulation of lipoprotein lipase) and

lipogenesis (down-regulation of acetyl-coenzyme A carboxylate and fatty acid synthase) (D'Eon et al., 2005; Mauvis-Jarvis et al., 2013). ERs are expressed in adipose tissue, indicative of the potential for estrogen regulation of adipocyte function, and ERα predominantly regulates adipose homeostasis via growth and proliferation of adipocytes (Rettberg et al., 2014). Consistently, ERαKO mice develop severe intra-abdominal obesity and present insulin resistance and impaired glucose tolerance (Heine et al., 2000), supporting a protective role of ERα signaling in the regulation of adipose homeostasis and metabolism.

E2 also has effects on cholesterol metabolism. It has been shown that E2 increases plasma HDL and triglyceride, while reducing LDL and total cholesterol levels, thereby decreasing the risk of onset of dyslipidemia and atherosclerosis. It is also known that liver ERα plays a role in the regulation of lipoprotein synthesis and receptors for cholesterol uptake (Palmisano et al., 2017). Moreover, studies conducted in GPER knockout murine models show the involvement of GPER in cholesterol metabolism (Prossnitz and Arterburn, 2015). In ovary-intact mice, deletion of GPER increased total cholesterol and LDL cholesterol plasma levels (Meyer et al., 2014). Furthermore, humans carrying the hypofunctional P16L genetic variant of GPER have increased plasma LDL cholesterol (Hussain et al., 2015). However, the role of GPER and non-genomic mechanisms in E2 metabolic effects is only beginning to emerge (Sharma et al., 2018),

Among the effects in the setting of energy homeostasis, estrogenic agents regulate glycolytic metabolism (Mauvais-Jarvis et al., 2013; Sun et al., 2014). Estrogens regulate insulin secretion, increase insulin tissue sensitivity and prevent β-pancreatic cells damage. Estrogen receptors are expressed in pancreatic β-cells as well as in many insulin target tissues. In particular, ERα stimulates insulin secretion from β-cells and is involved in the regulation of glucose metabolism in the liver, skeletal muscle and adipose tissue (Bryzgalova et al., 2006). The role of ERα in controlling glucose homeostasis has been evaluated in genetically obese mice in which treatment with the selective ERα agonist PPT enhanced glucose tolerance and insulin sensitivity (Bansal and Chopra, 2018). More recently, the role of the membrane receptor GPER in glycemic control has been highlighted. For example, G1 treatment stimulates insulin secretion, while both G1- and E2-mediated insulin secretion is defective in islets of GPER-knockout mice. In addition, these mice display impaired glucose tolerance, which correlated with decreased insulin release *in vitro* (Sharma and Prossnitz, 2011). The molecular mechanisms responsible for the beneficial role of estrogens in systemic glucose homeostasis involve the regulation of the functional expression of glucose transporters (GLUT), in particular GLUT4, in different insulin target tissues, including skeletal muscle and adipose tissue (Rettberg et al., 2014; Bryzgalova et al., 2006).

A specific aspect of estrogen's metabolic effects occurs in tissues that depend on glycolytic metabolism as a main energy source, including the nervous system and endothelium (Rettberg et al., 2014). In this setting, results from *in vitro* and *in vivo* studies suggest that estrogens play a modulatory role in glucose transport by positively regulating the expression and function of glucose transporter isoform 1, GLUT1 (Shi and Simpkins, 1997; Cheng et al., 2001). GLUT1 belongs to a family of structurally related glycoproteins that mediate facilitated glucose transport. It is a low-affinity transporter, but is highly sensitive to changes in glucose levels

and is thought to contribute mainly to basal glucose uptake. It plays an important role in tissues which depend on glycolytic metabolism as an energy source, including the nervous system and endothelium (Ohno et al., 2011). GLUT1 expression levels are closely regulated by glucose availability and demand; in particular, conditions of hypoglycemia lead to increased blood brain barrier (BBB) GLUT1 expression (Rettberg et al., 2014). Studies in OVX rats showed that E2 treatment increases the functional expression of GLUT1 protein by cerebral microvessels (Shi and Simpkins, 1997). In the frontal cortex of OVX monkeys, GLUT1 protein expression increased at the level of cerebral blood vessels following E2 treatment, while GLUT1 mRNA levels remained unchanged (Cheng et al., 2001).

Based on the knowledge that cancer cells rely on aerobic glycolysis to grow and differentiate, the effect of E2 on glycolytic metabolism has been also tested in different **cancer cell models**. It has been shown that GLUT1 expression is upregulated by 10 nM estrogen after 24 h treatment in the Ishikawa endometrial cancer cell line, leading to increased glucose internalization required for cancer cell uncontrolled growth (Medina et al., 2004). Even in MCF-7 human breast cancer cells, E2 increases GLUT-1 expression thus promoting tumor growth (Imbert-Fernandez et al., 2014; Rivenzon-Segal et al., 2003). Overall, estrogenic agents play a modulatory role in glucose transport by positively regulating the expression and function of GLUT1.
In addition to glucose transporters, E2 regulates a variety of proteins involved in glucose metabolism. For instance, in pathological lung cells, E2 was observed to promote glucose utilization via the pentose-phosphate pathway and increase cellular survival under oxidative stress conditions (Fig. 1.8).

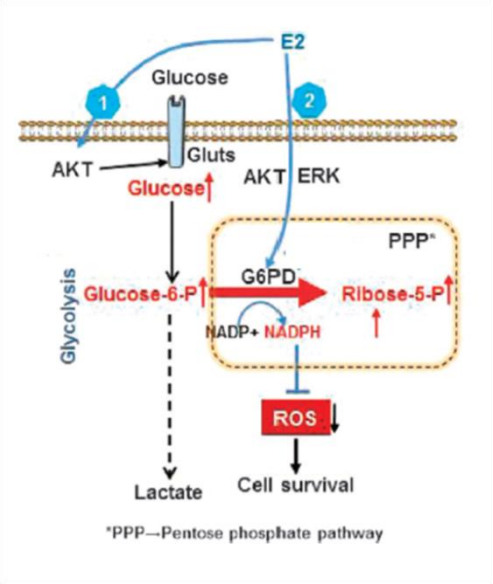

Figure 1.8 E2 activates the pentose-phosphate pathway favoring glucose transport and stimulating glucose-6-phosphate dehydrogenase (G6PD) (Sun et al., 2014).

Mechanistically, E2 activated Akt, induced membrane translocation of glucose transporters (GLUT1 or GLUT4), and increased glucose uptake (Sun et al., 2014). Furthermore, E2 increased transcription of glucose-

6-phosphate dehydrogenase (G6PD), a key enzyme in the pentose-phosphate signaling pathway. A relationship between estrogen and increased G6PD has also been observed in breast cancer cells (Sun et al., 2014).
Another important target of E2 is PFKFB3, a key protein of glycolysis that catalyzes the synthesis of fructose 2,6-bisphosphate (F2,6BP), which is a powerful activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme of glycolysis (Atsumi et al., 2005). Activation of the glycolytic pathway by E2 has been shown to be important for the growth and survival of MCF-7 breast cancer cells. In particular, PFKFB3 and its product, F26BP, are induced by E2 and required for E2-mediated stimulation of glucose uptake and glycolysis (Imbert-Fernandez et al., 2014) (Fig. 1.9).

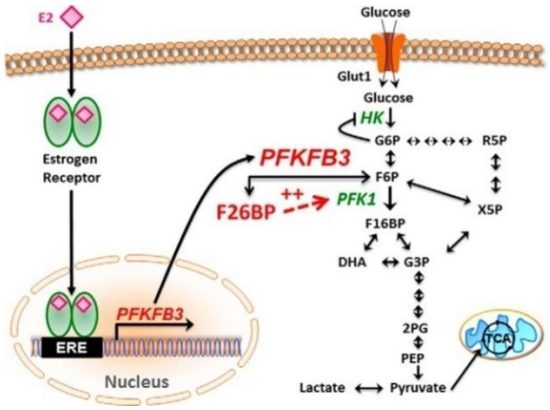

Figure 1.9 Model of PFKFB3 regulation by E2. E2 binds to traditional estrogen receptors (ER) that translocate to the nucleus and promote PFKFB3 transcription. PFKFB3 produces F2,6BP which allosterically activates PFK1 and the entire glycolytic pathway (Imbert-Fernandez et al., 2014).

The authors suggested that E2 increases PFKFB3 transcription by mechanism involving ERα. The observation that the PFKFB3 promoter contains putative ER response elements (EREs) supports the concept that PFKFB3 represents a direct transcriptional target of E2 (Imbert-Fernandez et al., 2014). Therefore, PFKFB3 can be considered as a downstream effector of E2, which serves to activate the energy pathways necessary for the survival and growth of tumor cells in metabolically adverse environments, such as in hypoxic tissues. We recently found that E2 regulates PFKFB3 levels in human endothelial cells. Similarly to E2, treatment with the selective GPER agonist, G1, induced a rapid increase in PFKFB3 amounts, highlighting a metabolic endocrine cross-talk with important implications in endothelial cell function (Trenti et al., 2017).

1.2 REGULATION OF ENDOTHELIAL GLYCOLYTIC METABOLISM

1.2.1 Endothelial cell metabolism

Endothelial cells (ECs), lining the interior surface of blood vessels, have immediate access to oxygen in the blood stream, an ideal environment to accomplish oxidative metabolism. However, ECs rely on glycolysis for generating most of their energy, since 85% of their ATP is produced through this pathway (De Bock et al., 2013). Glycolysis is the first step in glucose metabolism in the cytoplasm of all cells, regardless of aerobic or

anaerobic conditions. In this process a glucose molecule is transformed into two pyruvate molecules, involving different enzymatic reactions. In an aerobic environment, pyruvate is then decomposed into acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) and is completely oxidized to CO_2 through the mitochondrial respiratory chain with oxidative phosphorylation (32 molecules of ATP / glucose molecule) (Vander Heiden et al., 2009). Conversely, in an hypoxic environment, pyruvate is transformed into lactic acid by lactate dehydrogenase (LDH; 2 molecules of ATP / glucose molecule). Usually cells use glycolysis as a source of ATP mainly under anaerobic conditions (e.g. in the muscle in hyper-work). However, similarly to many cancer cells, ECs produce large amounts of lactate even in the presence of ample O_2 (sufficient for oxidative glucose metabolism), a phenomenon called aerobic glycolysis. Aerobic glycolysis has been mainly associated with tumor cell metabolism and described as "Warburg effect". (Potente and Carmeliet, 2017) (Fig. 1.10).

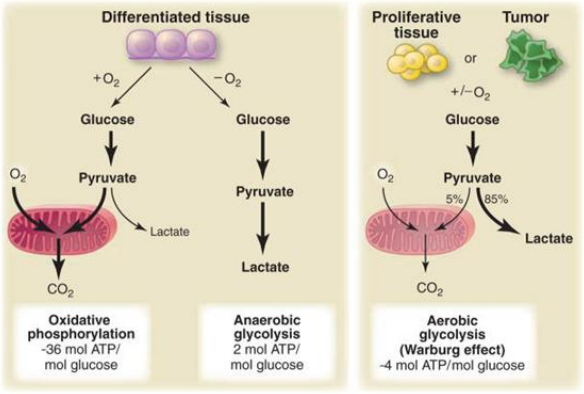

Figure 1.10 Schematic representation of oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Vander Heiden et al., 2009).

Compared with other healthy cell types (including cardiomyocytes, hepatocytes, fibroblasts and macrophages), ECs have higher rates of glycolysis and their glucose consumption is in the same range as that of many cancer cells (Rohlenova et al., 2018; Xu et al., 2014). Even when sufficient oxygen is available for oxidative glucose metabolism, and despite the fact that much less ATP per mole glucose is generated via glycolysis than via glucose oxidation, cancer cells prefer aerobic glycolysis. This allows cancer cells to spare precursors for the generation of biomolecules rather than for energy production (Verdegem et al., 2014).

Despite its low energy yield, this metabolic pathway can be advantageous for both cancer and endothelial cells. High level of glycolytic enzymes can give **cancer cells** the ability to resist hypoxia, a condition frequently found in solid tumors. In this situation glycolysis can be further enhanced in order to compensate for reduced oxygen supply. Overexpression of glycolytic enzymes including PFKFB3, lactate dehydrogenase (LDH), hexokinase (HK) and phosphofructokinase 1 (PFK1) and glucose transporters including GLUT1 (Moreno-Sánchez et al., 2007; Nowak et al., 2018) has been observed in tumor cells and is considered as an essential component of malignant phenotype and a feature of invasive cancers (Gatenby and Gillies, 2004).

Glycolysis represents also an advantage for **endothelial cells** as it produces ATP faster and at greater quantities than oxidative metabolism when available glucose is unlimited. The fast kinetics of glycolysis is beneficial for the sprouting behavior of ECs. Glycolysis also makes ECs more resistant to hypoxia. This is because ECs can use glycolysis anaerobically as long as glucose is available. ECs become sensitive to O_2 deprivation only when glucose is limiting. The use of glycolysis as a primary energy source thus seems like a *bona fide* adaptation for a cell that forms new vessels in hypoxic tissues. Interestingly, ECs can store glucose intracellularly as glycogen, raising the possibility that ECs use this endogenous source to sprout into avascular tissues (Potente and Carmeliet, 2017). A further advantage of glycolysis for ECs is to reduce the formation of reactive oxygen species (ROS). ECs thus minimize the production of ROS that are generated through the respiratory chain, protecting themselves from the high-oxygen environment in which they reside (De Bock et al., 2013; Stapor et al., 2014) and maximizing oxygen transport to perivascular cells and/or energy-consuming tissues (e.g. heart and brain).

A summary of the main steps of endothelial cell metabolism and the enzymes involved is shown in Figure 1.11.

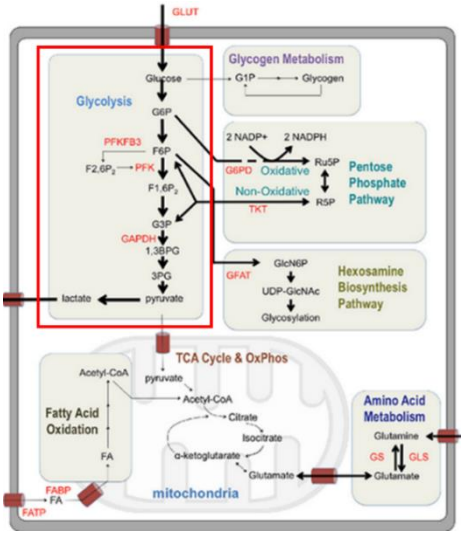

Figure 1.11 Endothelial cell metabolism. Highlights of key metabolic pathways displayed by endothelial cells that mainly rely on glycolysis for energy supply.
1,3BPG: 1,3-bisphosphoglyceric acid, 3PG: 3-phosphoglycerate, Acetyl-CoA: acetyl-coenzyme A, F1,6P2: fructose 1,6 bisphosphate, F2,6P2: fructose 2,6 bisphosphate, F6P: fructose 6-phosphate, FA: fatty acid, FABP: fatty acid binding protein, FATP: fatty acid transfer protein, G1P: glucose 1-phosphate, G3P: glyceraldehyde 3-phosphate, G6P: glucose 6-phosphate, G6PD: glucose-6-phosphate dehydrogenase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GFAT: glutamine-fructose-6-phosphatetransaminase, GlcN6P: glucosamine-6-phosphate, GLS: glutaminase, GLUT: glucose transporter, GS: glutamine synthetase, NADP+/NADPH: nicotinamide adenine dinucleotide phosphate, OXPHOS: oxidative phosphorylation, PFK: phosphofructokinase, PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, PPP: pentose phosphate pathway, R5P: ribose 5-phosphate, Ru5P: ribulose 5-phosphate, TCA: tricarboxylic acid, TKT: transketolase (Verdegem et al., 2014).

Glucose uptake in ECs occurs through facilitated diffusion, an energy-independent process mediated by glucose transporters (GLUT), in particular by GLUT1 (Mann et al., 2003). Glycolytic metabolites are used as a substrate by various biosynthetic pathways, producing macromolecules necessary for the growth, division and migration of ECs (see also Fig. 1.11; Vandekeere et al., 2015).

Glycolytic activity is influenced by the microenvironment in which ECs reside (De Bock et al., 2013). Glycolysis is accelerated during the angiogenic process. In adults, most ECs are quiescent (*phalanx cells*) for long periods of time, but they can quickly begin to proliferate and migrate to form new vessels in response to several pro-angiogenic stimuli including VEGF, estrogen and in response to the hypoxic environment (Verdegem et al., 2014).

In particular, it has been observed that the signaling pathways that control EC metabolism also control the angiogenic switch in tumor settings. Proangiogenic molecules such as VEGF enhance glycolysis by increasing GLUT1 and glucose uptake and driving expression of glycolysis activators such as PFKFB3. A similar induction of glycolysis genes is evoked by hypoxia and involves the activation of HIF-1α (hypoxia inducible factor-1α) (Potente and Carmeliet, 2017). A more detailed description of the functional effects of glycolytic proteins will be given in the next section.

When activated for migration or proliferation, ECs double their glycolytic flux (De Bock et al., 2013). In this setting, the specific localization of glycolytic enzymes ensures rapid local energy production, necessary to support migration. Indeed, it has been observed that glycolytic enzymes are concentrated in the mobile *lamellipodia* and *filopodia* protuberances of endothelial cells and compartmentalized with actin filaments (De Bock et al., 2013). Many glycolytic enzymes are inactive as dimers, but become more active in the tetrameric configuration. These enzymes bind to actin, which stabilizes the tetrameric configuration thus increasing their enzymatic activity (Real-John et al., 2010).

Upon establishment of a newly formed vessel, ECs appear to change their glucose metabolism and downregulate glycolysis. Non-proliferating, contact-inhibited ECs *in vitro*, mimicking quiescent endothelium, have lower glycolytic activity than proliferating ECs (Vandekeere et al., 2015): in homeostatic rest conditions the endothelium needs to maintain a state of quiescence and to facilitate the transport of oxygen and nutrients to the surrounding tissues. In accordance, a recent study reported that, after the formation of a new vessel, this state of reduced metabolic activity is also induced by shear blood flow stress which reduces glucose uptake and, through the transcription factor (KLF2), downregulates the expression of glycolytic enzymes, including HK2, PFKFB3 and PFK1 (Doddaballapur et al., 2015). Overall, metabolism is an important determinant of EC phenotype and a promising target for the control of physiopathological angiogenesis.

1.2.2 Functional effects of PFKFB3 and GLUT1 in the endothelium

Similarly to cancer cells, ECs rely on glycolytic metabolism to perform important functions including proliferation and migration for new vessel formation. Well characterized proteins in the control of endothelial function under physiopathological conditions are the glucose transporter GLUT1 and PFKFB3, the glycolytic enzyme activator. GLUT1 is the most abundant endothelial GLUT isoform and regulates glucose movement between the extracellular and intracellular compartments, maintaining a constant supply of glucose available for cell metabolism (Mann et al., 2003).

Endothelial glycolytic metabolism is upregulated in response to stimuli such as hypoxia, glucose deprivation or growth factors (Loike et al., 1992; Verdegem et al., 2014). For example, mice with endothelial cell-specific

deletion of the oxygen-sensing transcription factor HIF-1α show decreased GLUT1 levels and reduced glucose transport with functional consequences in the heart and brain (Huang et al., 2012). Moreover, in bovine retinal endothelial cells cultured under hypoxic conditions, a time-dependent increase in GLUT1 expression and glucose uptake peaking after 12-24 h was observed, suggesting a possible role for GLUT1 in diabetic retinopathy (Takagi et al., 1998). Recently the role of GLUT1 in brain angiogenesis has been also highlighted. In a study conducted in a mouse model characterized by GLUT1 transporter deficiency an arrest of angiogenesis has been described, with profound diminution of the brain microvasculature, resulting in overt manifestations of brain dysfunction (Tang et al., 2017). Accordingly, a study by Huang et al. (2012) found that decreased GLUT1 expression in the vascular endothelium is determinant for whole-organ metabolism, which could be functionally relevant particularly in fuel-sensitive organs such as heart and brain.

To the best of our knowledge, there are no data on the role of GLUT1 in the angiogenic process in tumors, nevertheless it is conceivable that GLUT1 also plays a role in tumor angiogenesis promoting tumor growth. Indeed, some studies have shown that biopsies of poorly differentiated and more aggressive tumors with a high proliferative index such as breast and ovarian cancer (Ravazoula et al., 2003; Cantuaria et al., 2001) are characterized by higher GLUT1 expression levels with respect to well-differentiated tumors.

The best characterized protein in terms of functional regulation of endothelial cells is PFKFB3, a key glycolytic promoting enzyme that forms fructose-2,6-bisphosphate (F2,6P2). F2,6P2 is a positive allosteric effector of 6-phosphofructo-1-kinase (PFK-1), the enzyme that catalyzes the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6P2), and is one of the rate-limiting checkpoints of the glycolytic flux. Thus PFKFB3 activity is a potent stimulator of glycolysis and dictates the equilibrium between glycolysis and the pentose phosphate pathway (Okar and Lange, 1999) (Figs. 1.11 and 1.12).

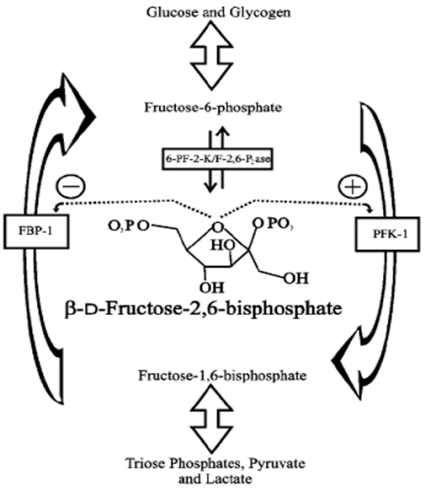

Figure 1.12 Representation of the central role of fructose 2.6 bisphosphate (F2,6BP) in glycolysis. Glucose is transformed into fructose-6-phosphate by hexokinase and is subsequently transformed into fructose 1,6-bisphosphate by PFK1 (6-phosphofructo-1-kinase). PFKFB (6-phosphofructo-2-kinase (6-PF-2-K) / fructose-2,6-bisphosphatase (F-2,6-P2ase)) regulates the formation of F2,6BP (fructose-2,6-bisphosphate), which is not an intermediary metabolite of the glycolytic cascade but an activator of PFK1 (Okar and Lange, 1999).

PFKFBs are a family of bifunctional enzymes that possess both kinase and phosphatase activity. Several PFKFB isoforms are expressed in different tissues including PFKFB1, PFKFB2, PFKFB4 (Yalcin et al., 2009). PFKFB3 is an inducible isoform: its functional expression is upregulated by growth factors and inflammatory cytokines (Ros and Schulze, 2013), implicating its role in setting the glycolytic rate under multiple physiological and pathological conditions. Amongst all PFKFB iso-enzymes, PFKFB3 has a much higher kinase than bisphosphatase activity (700-fold), thus favoring the production of intracellular $F2,6P_2$ levels and controlling its abundance. PFKFB3 is also the most abundant isoenzyme in ECs (De Bock et al., 2013).

Recently, the role of PFKFB3 in the angiogenic process has been highlighted. In particular, PFKFB3 is necessary for the functions of specialized endothelial cells defined as *tip cells* and *stalk cells*, involved in EC migration and proliferation, respectively (De Bock et al., 2013). In line with evidence that endothelial proliferation requires glycolysis (Lunt and Vander Heiden, 2011), the knockdown or overexpression of PFKFB3 suppresses or accelerates EC proliferation in vitro. In particular, PFKFB3 knockdown in ECs results in a decrease in glycolysis (35–40%) and proliferation (50-80%) compared to control cells. The effect on proliferation has been evaluated both under normoxic and hypoxic conditions: in normoxia it is evident after 72 to 96 h (Xu et al., 2014).

In addition, it has been observed that PFKFB3 affects endothelial directional migration regulating the formation of *filopodia* and *lamellipodia*, a feature of tip cells that reduces intercellular adhesion and promotes EC motility. The contraction and protrusion of *filopodia* and *lamellipodia* is based on actin cytoskeletal remodeling and requires a large amount of ATP. Thus, knockdown of PFKFB3 impairs *tip cell* formation. One possible mechanism underlying this effect is that PFKFB3 compartmentalizes with F-actin in motile protrusions to provide ATP. Consistent with these results, a significant defect in the formation of *lamellipodia* in PFKFB3-knockdown ECs was observed under both normoxic and hypoxic conditions (Xu et al., 2014). Staining of retinal ECs *in vivo* showed that in *tip* cells the mitochondria (0.5-4 μm), too large to fit inside the *filopodia and lamellipodia* (0.25 μm), are present in the perinuclear cytosol but were excluded from *filopodia.* The spread of ATP by perinuclear mitochondria may not be sufficient to support the high energy demand needed to support the motor activity of cells involved in migration (De Bock et al., 2013).

In line with what has been observed by silencing PFKFB3, treatment of ECs with the selective PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) leads to a partial (30% -40%) and transient reduction of glycolysis and *in vitro* angiogenesis (Schoors et al., 2014) (Fig. 1.13).

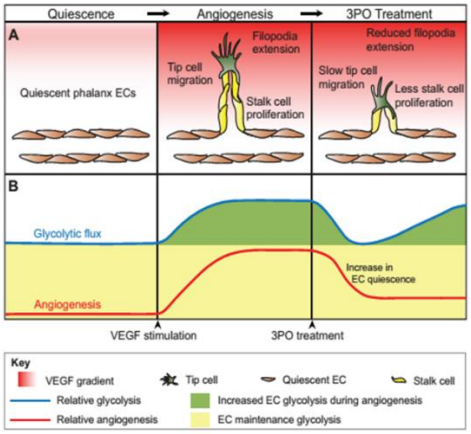

Figure 1.13 Effect of the selective PFKFB3 inhibitor (3PO) on EC migration. ECs increase glycolytic flux during migration and proliferation in the angiogenic process. Treatment with 3PO partially and transiently reduces glycolysis and reduces angiogenesis (Stapor et al., 2014).

The role of endothelial PFKFB3 in the angiogenic process was further supported by *in vivo* studies. In particular, the role of PFKFB3 in promoting neovascularization has been established in a neonatal mouse model of oxygen-induced retinopathy (OIR). Unlike humans, retinal vascular development in mice occurs after birth. In this model, either selective deletion of endothelial PFKFB3 or treatment of control mice with a PFKFB3 inhibitor dramatically suppressed retinal neovascularization. In particular, the density of retinal vessels in newborn mouse pups deficient in endothelial PFKFB3 was lower than that in control pups. Furthermore, in OIR mice, the vascular regrowth in the avascular area of the retina was also relatively delayed in mice deficient in endothelial PFKFB3 compared with control mice, implying that endothelial PFKFB3 plays a role in physiological neoangiogenesis (Xu et al., 2014).

In addition to retinopathy, other pathological conditions are characterized by increased angiogenesis, including intestinal inflammation and psoriasis. In these disease models inhibition of PFKFB3 by 3PO has been shown to reduce angiogenesis (Teuwen et al., 2017).

One of the disorders where angiogenesis contributes substantially to the progression of disease is cancer (Hanahan and Weinberg, 2011). Growing cancer masses need new blood vessels to supply oxygen and nutrients. When vessels grow under pathological conditions, they are often structurally highly abnormal. Disorganized vessels are dilated and tortuous, covered by fewer pericytes, and lined by an irregular endothelium, characterized by hypermotile ECs with excess *filopodia* and deregulated EC rearrangements (Cruys et al., 2016). Accordingly, endothelial PFKFB3 is crucial for tumor angiogenesis. In fact, tumor blood supply and size were decreased in mice deficient in endothelial PFKFB3 compared with control mice, demonstrating that PFKFB3 in endothelial cells is indeed critical for tumor angiogenesis. These observations were collected 15 days after tumor implantation, by quantifying tumor blood flow with *imaging* techniques (Xu et al., 2014). Remarkably, PFKFB3 is also crucial for tumor cell glycolysis. In cells of breast, colon, lung, pancreas, prostate and ovarian cancer increased expression of PFKFB3 associated with increased tumor proliferation has been observed (Yalcin et al., 2014). PFKFB3 has been also found to be overexpressed in

several human cancer tissues, including ovarian cancer, conferring a metabolic profile that predisposes cancer cells to proliferation and tumor progression (Atsumi et al., 2002). Therefore, PFKFB3 inhibition could have relevant effects on tumor progression due to both direct effects on tumor cells and indirect effects on endothelial cells.

The above findings demonstrate that proteins of the glycolytic metabolism, in particular PFKFB3, can represent an interesting pharmacological target for the regulation of tumor growth and angiogenesis. In the setting of tumor angiogenesis or in pathologies characterized by deregulation of the angiogenic process, the major advantage of using PFKFB3 inhibitors is to provide an incomplete block of glycolysis, since the total blockade of this metabolic pathway could have adverse effects (Clem et al., 2013). Furthermore, since the pro-angiogenic action of various growth factors such as VEGF and fibroblast growth factor (FGF) converges on PFKFB3, it can be hypothesized that anti-angiogenic therapy based on targeted PFKFB3 inhibition may be useful to avoid mechanisms of resistance of VEGF-targeting therapy (Fig. 1.14).

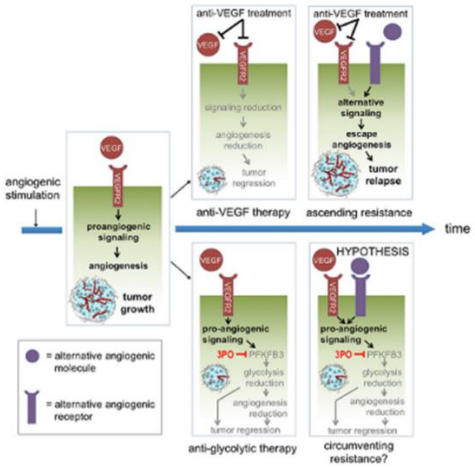

Figure 1.14 Rationale to target EC metabolism, a hypothesis. *Top*: conventional anti-VEGF antiangiogenic treatment suffers from increasing resistance due to a shift towards alternative pro-angiogenic molecules of potentially various origins. *Bottom*: treatment against metabolism bypasses this problem by targeting PFKFB3 protein downstream of angiogenic signaling pathways. Blockage of PFKFB3 with 3PO results in reduced glycolysis levels and a subsequent halt of the angiogenic process. 3PO: 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3, VEGF: vascular endothelial growth factor, VEGFR2: vascular endothelial growth factor receptor 2 (Verdegem et al., 2014).

Overall, the functional role of PFKFB3 has been better characterized than that of GLUT1. However, since these proteins operate in concert to adapt cell metabolism to microenvironmental changes, they are conceivably regulated by common mechanisms.

1.3 MECHANISMS INVOLVED IN THE REGULATION OF GLYCOLYTIC PROTEIN ACTIVITY

1.3.1 Regulation of PFKFB3 and GLUT1 by transcriptional and posttranscriptional mechanisms

The glycolytic pathway is regulated by the availability of substrates, allosteric effectors and the functional expression of transporters and enzymes (Yalcin et al., 2009). The regulation of protein abundance allows metabolic and functional cellular adaptation to environmental changes/demand and several studies, mainly in cancer cells, focused on the mechanisms controlling the amount of PFKFB3 and GLUT1, two major players in glycolysis.

GLUT1 and PFKFB3 are finely regulated at both transcriptional and post-transcriptional levels including changes in the degradation rate to trigger glycolysis in a time- and spatial-specific fashion. The latter aspect will be analyzed in Section 3.2 below. Stimuli including hypoxia (Bartrons and Caro, 2007), E2 (Cheng et al., 2001; Imbert-Fernandez et al., 2014), pro-inflammatory molecules (Chesney et al., 1999), and pro-angiogenetic factors such as VEGF (Xu et al., 2014) regulate transcription of PFKFB3 and GLUT1, increasing protein expression and activity. In mammalian cells, under hypoxic conditions, HIF-1α is stabilized and translocates to the nucleus where it acts as a transcription factor, binding to the promoter of genes of the glycolytic pathway. Of note, PFKFB3 as well as GLUT1 have a HIF–responsive element in their promoter (Obach et al., 2004), and their expression is upregulated under hypoxic conditions such as those associated with ischemic tissue and cancer. For example, GLUT1 expression is upregulated in hypoxic bovine retinal endothelial cells (Takagi et al, 1998). In a hypoxic environment, HIF-1α is required for the upregulation of genes encoding for glycolytic enzymes including PFKFB3 and GLUT1 (Veschini et al., 2007; Xu et al., 2014).

In the cancer setting, the expression of glycolytic proteins is constitutively elevated (Moreno-Sánchez et al., 2007). For example, in biopsies from colon and breast cancer (Bando et al., 2005, Imbert-Fernandez et al., 2014). PFKFB3 mRNA is overexpressed compared to healthy tissues. There is also evidence that its expression is positively regulated in response to hypoxia and during phase S of the cell cycle (Bando et al., 2005; Atsumi et al., 2002). The amount of GLUT1 was also found to be higher in cancer with respect to healthy cells. For example, gene and protein overexpression of GLUT1 has been highlighted in breast cancer cells (MCF7 and MDA-MB231) compared to non-tumorigenic mammary cells (MCF10A; Hamann et al., 2018). In line with these observations, hypoxia increased PFKFB3 and GLUT1 mRNA levels in Hep-3B hepatic cancer cells (Minchenko et al., 2002). Furthermore, in glioblastoma cells under hypoxic conditions there are increased levels of HIF-1α, which moves as a heterodimer into the nucleus and transactivates target genes including GLUT1 and various glycolytic enzymes that help the cells to adapt to hypoxia (Semenza, 2001). Of note, in breast cancer cells a cooperation between ERα and HIF-1α has been also demonstrated under hypoxic conditions. Several E2-regulated glycolytic proteins are also regulated by HIF-1α, suggesting that ER and HIF-1α coordinate a gene expression program via a complex crosstalk (Yang et al., 2015).

Protein amounts can be regulated at post-transcriptional levels by several mechanisms affecting mRNA and protein turnover. Several studies addressed the regulation of mRNA stability of glycolytic proteins in different cell types, mainly in cancer cells (Wang et al., 2014; Qi and Pekala, 1999).

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 the 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 GLUT1 gene expression. In terms of structure, GLUT1 mRNA contains a 3'-UTR of 884 nucleotides and is considered to be A+U rich. Several laboratories have reported alterations in the stability of GLUT1 transcript in various cell lines and tissues under conditions including glucose deprivation, experimental diabetes, hemangioblastoma as well as cytokine, hormone and metabolite stimulation as reviewed by Qi and Pekala (1999).

The amount of PFKFB3 and GLUT1 translated mRNA is also regulated by the action of miRNAs, small single-stranded non-coding RNA molecules that, once bound to complementary mRNA in the 3'-UTR region, cause inhibition of protein translation (Fabian et al., 2010; Oliveto et al, 2017). This topic will be discussed below in Section 3.3.

In addition to the above regulation of transcript stability or quantity, PFKFB3 and GLUT1 activity can be also affected by post-translational modifications. In particular, cancer cells can adjust PFKFB3 activity according to their own metabolic needs both by varying PFKFB3 gene expression and through post-translational protein modifications. For instance, S-gluthationylation of PFKFB3 results in decreased PFKFB3 activity (Seo et al., 2014), whereas PFKFB3 phosphorylation enhances its activity. PFKFB3 phosphorylation is associated with increased glycolysis and cancer progression (Bando et al., 2005). The effect of phosphorylation on PFKFB3 activity has also been studied in non-cancer cells. For example, monocytes exposed to hypoxia rapidly stimulate glycolysis by activating the enzyme PFKFB3 through protein phosphorylation on serine, position 461, located at the protein C-terminal domain (Marsin et al. 2002).

Similarly to PFKFB3, GLUT1 undergoes post-translational modifications such as phosphorylation, which leads to increased protein activity; in particular, phosphorylation of GLUT1 by protein kinase C on serine at position 226 enhanced cell surface localization and glucose uptake in ECs (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).

1.3.2 Mechanisms of ubiquitination and protein degradation

Ubiquitination is a post-translational modification that regulates the expression level of cell proteins, directing them to degradation through the proteasome or lysosomal route. The number of ubiquitin molecules is crucial in conveying the protein towards one or the other way: in the case of lysosomal degradation, mono-ubiquitination is sufficient (Amerik and Hochstrasser, 2004). In eukaryotes the ubiquitin-proteasome system (UPS) is the major intracellular pathway responsible for the degradation of cellular proteins. It has been estimated that 80% -90% of cellular proteins undergo this degradation system (Caldeira et al., 2014).
Polyubiquitylated proteins are recognized and degraded in an ATP-dependent manner by a proteolytic complex known as the 26S proteasome. This system is a protease present in the cytoplasm and in the nucleus of eukaryotic cells, and comprises a catalytic core particle (CP), also termed the 20S proteasome, which is capped at both ends with 19S regulatory particles. These particles recognize ubiquitinated protein and are also implicated in the translocation of proteins within the 20S subunit, where they are degraded to oligopeptides (Fasanaro et al., 2010; Voges et al., 1999).

PFKFB3 is an unstable protein that undergoes degradation by the UPS. Aminoacid sequence analysis reveals that PFKFB3 contains a sequence in the C-terminal domain that is common to many short-lived proteins and reported to be a motif for rapid degradation (Riera et al., 2003). Other target proteins of UPS are the glucose transporters GLUT1 and GLUT4. It has been shown that treatment of HUVECs with low concentrations of the proteasome inhibitor bortezomib indirectly increases GLUT1 expression and promotes angiogensis (Veschini et al., 2007). Moreover, posttranslational modifications of GLUT1, such as monoubiquitinylation or sentrin conjugation, regulate protein stability (Ohno et al., 2011). In particular, the expression levels and membrane localization of GLUT transporters including GLUT1 can be regulated by sentrin, an ubiquitin-like protein also known as SUMO-1. In more detail, mUbc9is, a structural homologue of the E2-ubiquitin-conjugating enzyme, is a sentrin-conjugating enzyme that directs GLUT1 to proteasome- or lysosome-mediated degradation. Augmented mUbc9 expression results in a decreased cellular content of GLUT1 (Giorgino et al., 2000). Overall, the mUbc9 sentrin-conjugating enzyme represents a further regulator of GLUT1 protein levels relevant for basal glucose uptake in normal and pathological states. Interestingly, estrogens reduce ubc9 expression, thus reducing sumoylation (Lai et al., 2017). Other studies have shown that the lysosomal pathway plays a major role in GLUT1 degradation (Ortiz et al., 1992; Rosa et al., 2009). Treatment with chloroquine, an agent that suppresses lysosomal degradation, abolished GLUT1 degradation, whereas the proteasome inhibitor MG132 did not influence GLUT1 expression levels (Rosa et al., 2009).
Overall, there is evidence that the rate of PFKFB3 and GLUT1 proteasomal or lysosomal degradation is involved in the regulation of protein amount.

I will now describe the UPS in more detail. This is the main non-lysosomal intracellular proteolytic system responsible for degradation of thousands of proteins, particularly those of short-lived and regulatory nature (Stangl and Stangl, 2010).
The UPS is part of an elaborate network of protein quality control mechanisms that cells possess to maintain proteome integrity. The proteasome plays an important role in the degradation of abnormal and damaged

proteins, cell cycle regulators, oncogenes, tumor suppressors and transcription factors (Voges et al., 1999). In fact, degradation of polyubiquitinate proteins by the 26S-proteasome complex represents a crucial mechanism for qualitative and quantitative proteome control.

Protein degradation by the UPS can be divided in two steps: 1) covalent attachment of ubiquitin (Ub), an 8 kDa protein, to the substrate protein, and 2) degradation of the polyubiquitinated protein by the 26S-proteasome complex. Three enzyme families are involved in protein ubiquitination:

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

E3: ubiquitin-protein ligase

The Ub moiety is first loaded on the active-site cysteine of E1 enzymes. Charging with Ub induces a conformational change in E1, allowing the recruitment of one ubiquitin-conjugating enzyme E2 that receives the activated Ub, forming another thiolester linkage. E2 enzymes aid in carrying the activated ubiquitin from E1 to the substrate. All E2 enzymes harbor a conserved core that is utilized in E2-E3 binding. E3 Ub-protein ligases are responsible for final target protein selection and specificity. Ub is covalently bound to the target protein by an isopeptide linkage between the carboxy-terminal glycine of ubiquitin and the ε-amino group of lysine in the target protein (Fasanaro et al., 2010; Nandi et al., 2006). The human genome encodes 2 E1, 40 E2 and 650 E3 (Caldeira et al., 2014). Ubiquitin needs to be removed from tagged proteins before they enter the proteolytic core of proteasomes by deubiquitinaing enzymes (DUBs), which are associated with 19S. As ubiquitin is bound to enzymes by thioester linkage, DUBs are thiol proteases (Nandi et al., 2006) (Fig. 1.15).

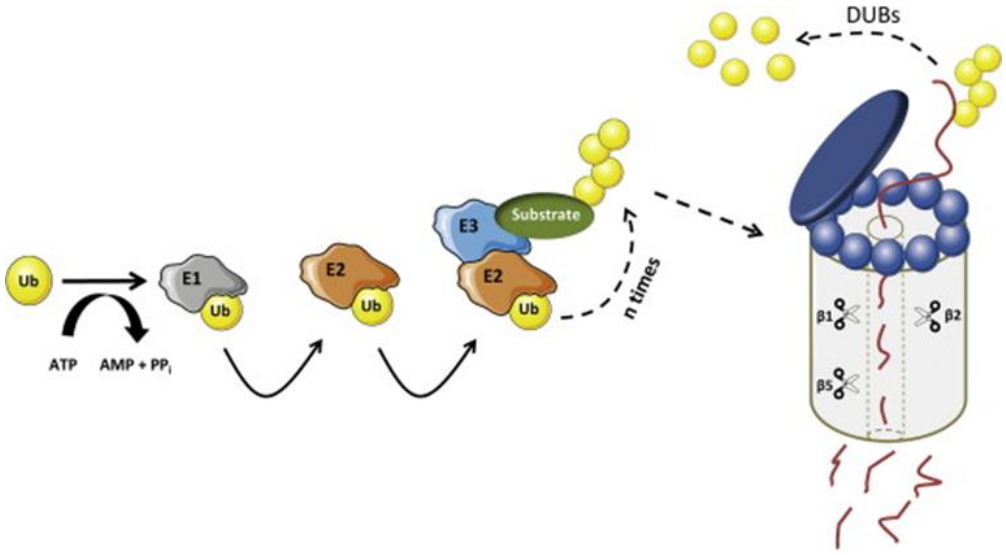

Figure 1.15 Main components of the ubiquitin–proteasome system. In order to be attached to the substrate protein, ubiquitin is first activated by the E1-activating enzyme in an ATP-dependent manner, and further transferred to the E2-conjugating enzyme. The substrate-specificity of this pathway is given by E3 ligase, which allows the ubiquitination of a specific substrate. When a proper chain is assembled, with at least four ubiquitin moieties, it is recognized, unfolded and deubiquitinated by the 19S regulatory particle. This unfolding process allows the protein to enter the 20S catalytic particle where it is degraded by the b1 (trypsin-like activity), b2 (caspase-like activity) and b5 (chymotrypsin-like activity) subunits. Eventually small peptides are generated and ubiquitin is regenerated (Caldeira et al., 2014).

E3 ubiquitin ligases

The substrate-specificity of the proteosomal degradation pathway is given by the E3 ligase, which allows the ubiquitination of a specific substrate. These enzymes are categorized in two major classes, HCT and RING, based on their catalytic domain. The RING-E3 ligase class is further divided into the SKP1-CUL1-F-box-protein complex (SCF) and the Anaphase-Promoting Complex/Cyclosome (APC/C).

These complexes display a similar structure, being composed of three invariable subunits - a catalytic RING protein, a scaffold protein and an adaptor protein - and a variable component, an F-box protein for SCF and the activators Cdh1 and Cdc20 for APC/C, which confer substrate specificity (Fasanaro et al., 2010) (Fig. 1.16).

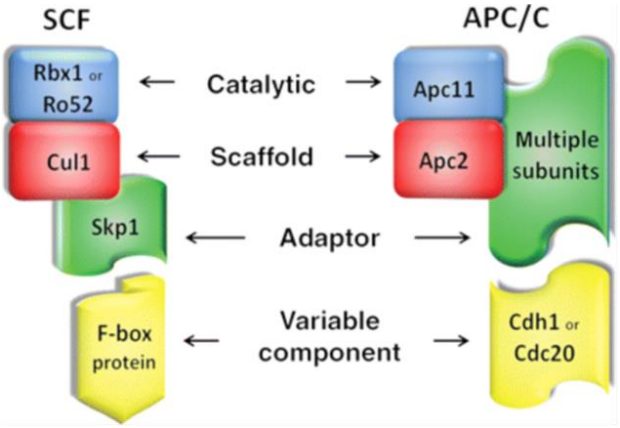

Figure 1.16 Structural similarities between SCF and APC/C E3 Ub-ligase complexes. Both complexes are composed of a catalytic RING protein (blue), a scaffold protein (red), and an adaptor protein (green). Variable components (yellow) give substrate specificity to the complexes (Fasanaro et al., 2010).

Despite structural similarity, these protein complexes have different functional relevance. The SCF complex is always active and its action is dependent on post-translational modifications of substrates, such as phosphorylation. In fact, once phosphorylated, the substrate is recognized by a specific F-box protein of the SCF complex (Fasanaro et al., 2010). Approximately 70 F-box proteins have been identified in humans. For example, estrogen induces phosphorylation and subsequent degradation of p27 by the SCF complex characterized by a specific F-box; accordingly, the SCF-Skp2/Cks E3 ligase inhibitor stabilizes p27 protein in the nucleus and prevents its degradation, thus increasing the half-life of this protein (Pavlides et al., 2013).
The APC/C complex can be activated by phosphorylation (Kraft et al., 2003). In addition, the expression of Cdh1 and Cdc20 APC/C activators can be modulated by protesomal degradation. N2-[(4-methylphenyl) sulfonyl]-L-arginine methyl ester hydrochloride (TAME) is a synthetic inhibitor specific for the APC/C complex; it prevents APC/C activation by Cdc20 and Cdh1, inducing ubiquitination-dependent dissociation of Cdc20 (Zeng and King, 2012). It has been reported that treatment of *Xenopus* oocytes with TAME prodrug (proTAME), a cell-permeable derivative processed by intracellular esterases to yield the parent compound (TAME), efficiently inhibited cyclin B proteolysis. ProTAME was also activated efficiently in HeLa cells causing a robust mitotic arrest (Zeng et al., 2010).

As discussed above, PFKFB3 is an unstable protein that undergoes degradation by the UPS. The first evidence that PFKFB3 is regulated by mechanisms affecting protein stability and subjected to rapid degradation by polyubiquitylation-dependent proteasome comes from a study in neurons (Herrero-Mendez, et al., 2009). For instance, in contrast to other isoenzymes, PFKFB3 contains a region recognized by the E3 ubiquitin ligase APC/C-Cdh1 complex that plays a major role in the rapid degradation of PFKFB3 in neurons, where low PFKFB3 levels are associated with elevated Cdh1 levels. In fact, neuronal PFKFB3 levels are very low because the protein is constantly subject to proteosomal degradation by Cdh1 action (Herrero-Mendez, et al., 2009). Moreover, overexpressing or silencing Cdh1 in neoplastic and non-neoplastic cells, respectively, decreases or activates proliferation and glycolysis by modulating PFKFB3 activity (Almeida et al., 2010). The involvement of UPS in PFKFB3 degradation has also been established in mouse myoblasts (Riera et al., 2003). More recently, two ubiquitin ligases, APC/C-Cdh1 and SCF-β-TrCP (SKP1-CUL1-F-box-protein complex)-(β-transducin repeat-containing protein), have been found to sequentially regulate glycolysis by targeting PFKFB3 in an ovarian cancer cell line. During cell cycle there is an increase in PFKFB3 protein, which follows a decrease in the action of Cdh 1. However, the presence of PFKFB3 is short-lasting since the enzyme is also a substrate for the ubiquitin ligase SCF complexes that mediate the degradation of several cell cycle proteins (Tudzarova et al., 2011).

Overall, the expression of ligases belonging to different families finely tune PFKFB3 levels and glycolysis with functional effects in target cells. Both SCF and APC/C E3 ligases are present in HUVECs (Fasanaro et al., 2010), but the role of the proteasome system in the regulation of glycolytic proteins (e.g. PFKFB3 and GLUT1) stability in endothelial cells is not well understood.

Of note, **estrogens** appear to modulate protein levels by targeting protein translation or proteasomal degradation rate, possibly via specific E3 ligases. In particular, E2 rapidly decreases ERα expression in estrogen target tissues including uterine artery ECs by promoting its ubiquitination (Tschugguel et al, 2003). On the other hand, it has also been demonstrated that estrogen stabilizes K-Ras by inhibiting its polyubiquitylation, thus contributing to endometrial cellular transformation and tumor growth (Koo et al., 2015).

Estrogens also promote the proteasomal degradation of the tumor suppressor p27, which is prevented by inhibition of SCF-Skp2/Cks E3 ligase (Pavlides et al, 2013). Moreover, conjugated estrogens (CE) increased E3 ubiquitin ligase HRD1 levels without increasing HRD1 mRNA levels in pancreatic beta-cells. Through post-translational mechanisms involving the stabilization of the E3 ubiquitin ligase E3, CE promote ubiquitination and proteasomal degradation of misfolded proteins via ERAD (endoplasmic reticulum (ER)-associated protein degradation system), thus preventing ER stress and beta-cell dysfunction (Xu et al, 2018).

Deubiquitylating enzymes

Ubiquitination is a reversible post-translational modification. The hydrolysis of ubiquitin linkages is performed specifically by the DUB protease family. Before substrate degradation, the covalent bond between Ub and the

protein is hydrolyzed by DUBs resident in the 26S-proteasome, allowing the release of Ub moieties and homeostasis of the Ub cell pool (Fasanaro et al., 2010) (Fig. 1.15). Intracellular protein levels depend on the balance between ubiquitination and deubiquitination reactions.

Deubiquitinating enzymes can act at different stages of the protein ubiquitination process: (1) at the "initial" stage, by cleaving the ubiquitin precursors to supply ubiquitin monomers to the ubiquitination enzymes; (2) at the "intermediate" stage, by the regulated removal of ubiquitin moieties from proteins to alter their fate (stabilization, conformational change); and (3) at the "final" stage by the removal of ubiquitin chains from substrates addressed to the proteasome to facilitate their degradation and processing into ubiquitin monomers, free to enter a new ubiquitination cycle (Jacomin et al., 2018) (Fig. 1.17).

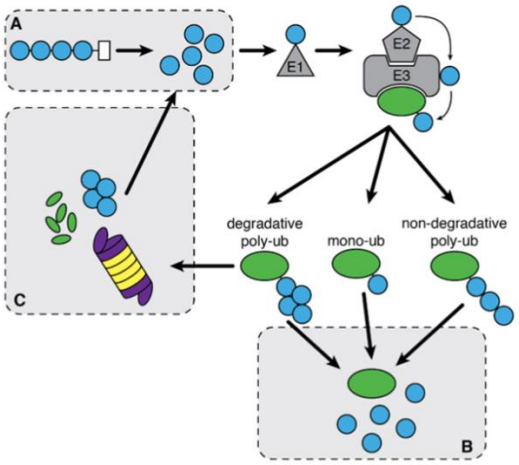

Figure 1.17 Localization of the action of DUBs in the ubiquitination process. Ubiquitination is catalyzed by three types of enzymes: E1, E2, E3. It is a reversible reaction. DUBs process ubiquitin chains at three levels for: (**A**) the generation of ubiquitin monomers from an ubiquitin precursor; (**B**) the selective removal of ubiquitin moieties on ubiquitinated proteins; and (**C**) the recycling of ubiquitin from protein degraded by the proteasome (Jacomin et al., 2018).

The human genome encodes approximately 100 DUBs, which are divided into two main families: the cysteine proteases and the metalloproteases. Cysteine proteases are further divided into five sub-families according to the sequence and structure of their catalytic domain. The most abundant DUB sub-family is the USP with over 50 members (Amerik and Hochstrasser, 2004).

As mentioned above, when deubiquitination occurs before the targeting of a substrate towards proteasomal degradation, it inhibits protein degradation with functional consequences. Notably, USP19 plays a key role in the regulation of HIF-1α levels and, in the absence of this protein, cells fail to mount an appropriate response to hypoxia (Altun et al., 2012).

The relative amounts of DUBs vary in a cell type- and tissue-dependent manner. DUBs and their expression can be induced in a stimulation-dependent manner. Several factors including cytokine and hormones regulate USP activity. For example, USP19 expression has been shown to rise in E2-treated C2C12 myoblasts (Ogawa et al., 2011).

Remarkably, DUBs have been genetically linked to cancer as oncogenes and tumor suppressors, inflammatory diseases, and neurodegeneration. Drugging the UPS has become a major research interest in recent years and several drugs targeting various components of the machinery are currently in clinical and pre-clinical development (Caldeira et al., 2014; Landré et al., 2014; Poondla et al., 2019).

Overall, the amount of glycolytic proteins, namely PFKFB3 and GLUT1, can be modulated through transcriptional and post-transcriptional mechanisms including gene expression, protein translation (see next Section) 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 might vary depending on the stimulus. Whether E2 could regulate cell metabolism and specifically promote glycolysis targeting the stability of glycolytic proteins, and in particular that of PFKFB3 and GLUT1, is yet unexplored.

1.3.3 Regulation of protein translation by miRNA and the role of estrogens

The recent 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. In particular, miRNAs act as negative post-transcriptional regulators of gene expression by targeting multiple mRNAs and triggering translation repression and/or RNA degradation (Chamorro-Jorganes et al., 2013; Iorio et al., 2007; Oliveto et al., 2017) (Fig. 1.18).

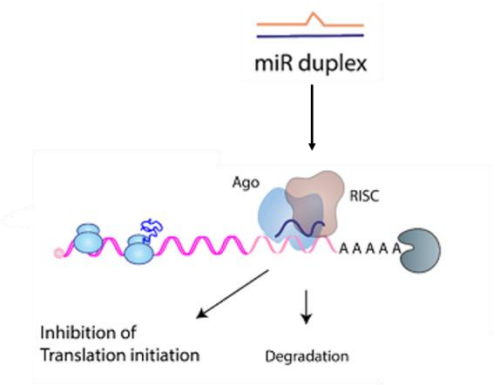

Figure 1.18 Major functional role of miRNAs (Kir et al., 2018).

In order to repress protein translation, it is crucial that the nucleotides in position 2–8 of the miRNA (called the *seed sequence*) are almost perfectly complimentary to regions at the 3′-UTR of their target genes (Chamorro-Jorganes et al., 2013). In mammalian tissues, miRNAs mainly form imperfect hybrids with sequences in the mRNA 3'-UTR, whereas in plants a complete-base-pairing is common, resulting in translational inhibition or RNA degradation, respectively (Gao and Liu, 2011; Fabian et al., 2010). The pathways of miRNA biogenesis and functions are summarized in Fig. 1.19. Once mature miRNAs are formed in the cytosol, miRNAs are incorporated into the RNA-induced silencing complex (RISC), containing Argonaute (AGO) proteins (AGO1-4), which represent the most important and best characterized protein component of the complex. In particular, AGO proteins unwind double-stranded miRNA to form single

stranded miRNAs. One-strand miRNA is preferentially selected to bind one of the AGO proteins and direct translational inhibition (or mRNA degradation) by base-pairing to the 3'-UTR of its target mRNA (Klinge, 2015).

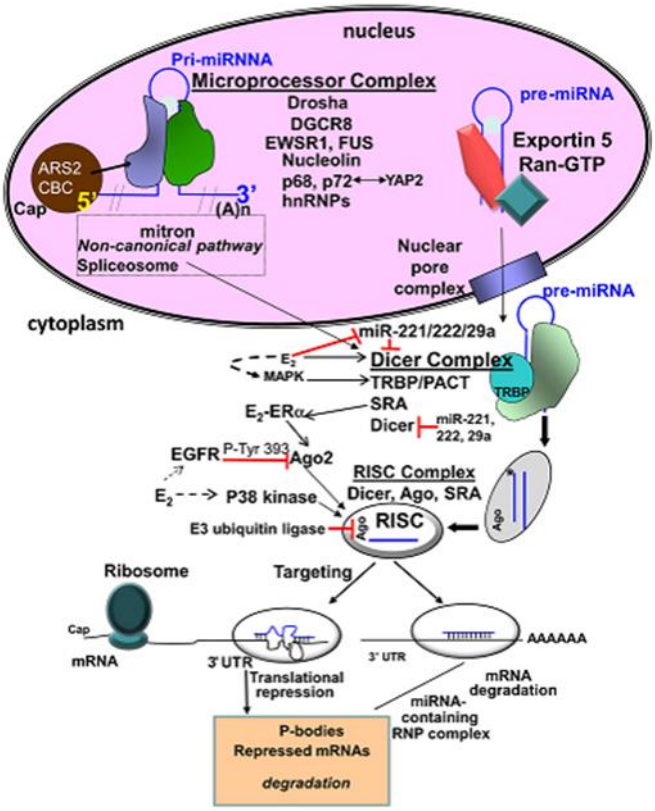

Figure 1.19 Model of canonical miRNA biogenesis and function. Primary transcripts of microRNAs (pri-miRNAs) are transcribed by RNA polymerase II, processed by the RNAse III enzyme, Drosha and its cofactor DGCR8, to precursor microRNAs (pre-miRNAs), which are exported from the nucleus by Exportin/RAN-GTP. In the cytoplasm, pre-miRNAs are processed by the Microprocessor complex that includes Dicer, an RNAse III enzyme, to form mature ~22 nt transiently double-stranded miRNA duplexes that are transferred to Argonaute proteins (most notably AGO2 in the RNA-induced silencing complex (RISC), leading to unwinding of the duplexes to form single stranded miRNAs. The RISC complex binds either to the 3' untranslated region (3'-UTR) or to the open reading frame (ORF) of its target mRNA. Binding of miRNA/RISC complex with the 3'-UTR causes translational repression (Klinge, 2015).

Few studies investigated miRNA expression patterns and function in normal tissues. Kuosmanen et al. (2017) reported a striking change in endothelial miRNA profile between tissue-derived and cultured cells, not surprisingly since the cells adapted from tissue to cell culture environment. They found that the overall miRNA expression decreases significantly in cultured compared to tissue-derived ECs (Kuosmanen et al., 2017). In addition, ECs from different organs show phenotypic, genetic and protein differences as well as a diverse miRNA signature. Over 200 miRNAs have been described in HUVECs (McCall et al., 2011), and some of them regulate key genes and activities including angiogenesis (Kir et al, 2018). The first evidence of the importance of miRNAs in **neovascularization** comes from both *in vitro* and *in vivo* approaches to knockdown enzymes involved in miRNA biogenesis. In particular, mice lacking DICER, a key enzyme in miRNA processing maturation, die due to defective vessel formation (Suarez at al., 2008). More recently, emerging

evidence in post-ischemic tissues suggests a pro- or anti-angiogenic role of specific miRNAs in the regulation of neovascularization (Azzouzi et al., 2015; Kir et al., 2018). For example, it has been reported that miRNA-26a inhibits angiogenesis both *in vitro* and *in vivo*, and the neutralization of miRNA-26a rapidly induces angiogenesis. Pro-angiogenic stimuli such as TNF-α and VEGF reduce miRNA-26a/b expression, with functional readouts in HUVEC growth and migration (Icli et al., 2013). Conversely, miRNA-27 stimulates tip cell specification and endothelial sprouting by targeting negative regulators of angiogenesis, and miRNA-27 mimics injected in mouse ischemic hearts promote neovascularization (Kir et al., 2018). Remarkably, 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 (Wang et al., 2018).

Emerging evidence recognizes miRNAs as novel players in glycolytic **metabolism**, mainly in pathological conditions. For example, miRNAs can act as regulators of metabolic proteins: in several cancer cell types, transfection with specific miRNAs downregulate GLUT1 or PFKFB3 protein synthesis, leading to impaired glycolysis and cell proliferation (Du et al., 2015; Ge et al., 2015; He et al., 2019). The role of miRNAs in cancer cell metabolism has been recently reviewed by Subramanian et al. (2019). To the best of our knowledge, only one study has linked miRNA effects on glycolytic program to the regulation of EC function: in particular, downregulation of miRNA-124 leads to enhanced glycolysis and abnormal proliferation in pulmonary artery ECs (Caruso et al, 2017).

Estrogens may modulate the functional expression of several miRNAs, and deregulation of miRNA expression has been implicated in estrogen-related cancers. In particular, 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). To date, over 200 E2-regulated miRNAs have been identified, and several reports provide evidence that E2 suppresses or stimulates miRNA expression and/or activity acting at multiple levels, including pri-miR maturation and miRNA degradation (Fig. 1.19; Klinge, 2015). For example, E2 reduces endogenous miRNA-26b and miRNA-206 expression in the MCF-7 breast cancer cell line, and this has been correlated with increased cell proliferation or migration. 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. E2 has been shown to inhibit AGO2 expression or phosphorylation by interacting with the EGF pathway. Notably, AGO2 can also be regulated by E3 ubiquitin ligases, which promote its degradation, thereby inhibiting miRNA activity (Klinge, 2015) (Fig. 1.19). The involvement of traditional estrogen receptors (e.g. ERα) in miRNA regulation by estrogens has also been established (Klinge et al., 2015).Recently, in an ER-negative breast cancer cell line, GPER has been shown to mediate the inhibitory effect of E2 on miRNA-338-3p expression. Consistently, miRNA-338-3p mimic decrease E2-induced cancer cell proliferation. This study provides novel evidence on the role of GPER signaling in miRNA regulation (Vivacqua et al., 2018).

Overall, miRNAs are emerging rapid post-transcriptional regulators of protein abundance and function. A single miRNA has the potential to simultaneously regulate multiple proteins. Thus, miRNAs represent novel

and attractive therapeutic targets: several oligonucleotide-based drugs targeting miRNAs are in clinical trials at different phases of clinical development (Matsui and Corey, 2017). The most common strategies to modulate miRNA function are reported in Fig. 1.20. These are oligonucleotide-based approaches to antagonize (e.g. antagomirs, miRNA sponges) or restore (miRNA mimics) miRNA functions.

In addition to these artificial modifications, endogenous molecules including estrogens may contribute to the fine tuning of miRNA activity. Although estrogen can stimulate or suppress miRNA activity in different cancer cell types, their role in healthy tissues and in endothelial cells is largely unknown (Klinge, 2015; McCall et al., 2011). In particular, whether E2-targeted miRNAs participate in the functional regulation of the endothelial glycolytic program remains to be determined.

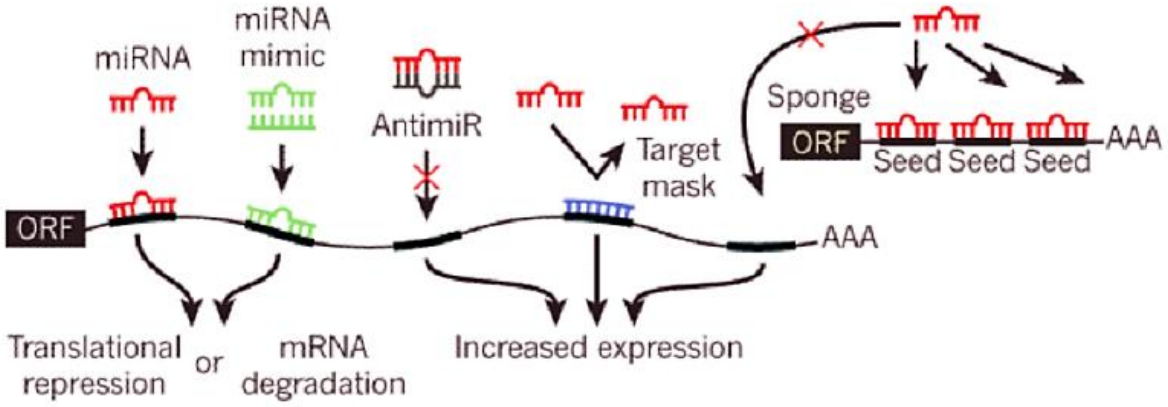

Figure 1.20 The various methods of artificially modulating miRNA expression or activity. Endogenous miRNA (red) binds to complementary sequences in the 3′-UTR of a target gene, resulting in translational repression or mRNA degradation. A miRNA mimic (green) consists of an oligonucleotide duplex of the miRNA and comprises the same nucleotide sequence as an endogenous miRNA, and is designed to target the same mRNAs as that miRNA. An antimiR (antagomir) (grey) is an oligonucleotide that is complementary to an endogenous miRNA, thereby designed to bind and inhibit its function. A target mask (blue) is an oligonucleotide designed to bind to a portion of an endogenous miRNA target without initiating mRNA degradation or translational inhibition. This strategy rescues one particular mRNA from miRNA-mediated repression. MiRNA sponges consist of an open reading frame (ORF) linked to a 3′-UTR that contains several binding sites for a particular miRNA, acting as competitive inhibitors for miRNA binding (Small and Olson, 2011).

2. AIM OF THE WORK

17ß-estradiol (E2), the predominant endogenous estrogen, has proven protective cardiovascular and metabolic effects spanning from endothelial healing and angiogenesis to the control of energy homeostasis including glucose metabolism in insulin-target tissues (Arnal et al. 2010; Cignarella et al., 2010; Mauvais-Jarvis et al., 2013). These effects have been mainly ascribed to the activation of ERα (Bolego et al, 2006), the classic nuclear hormone receptor which acts as a transcription factor. In accordance, ERα knockout mice present a cardiometabolic phenotype similar to that of postmenopausal women (Arao et al., 2018).

More recently, the G protein-coupled estrogen receptor GPER has been identified as a relevant mediator of short-term signaling events in response to E2. Non-genomic rapid signaling via GPER has been shown to mimic several beneficial effects of E2 in the heart and vessels (Barton et al., 2018; Prossnitz and Arteburn, 2015). Remarkably, GPER has been involved in the regulation of hypoxic-ischemic responses requiring rapid metabolic adaptation of endothelial tissue to environmental cues (De Francesco et al., 2017). However, the role of GPER and non-genomic mechanisms in E2 metabolic effects is only beginning to emerge (Sharma et al., 2018), and few studies addressed the mechanisms coupling hormonal signals to metabolic demand in endothelial cells.

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, the glucose transporter GLUT1 and the glycolytic enzyme activator phosphofructokinase-2/fructose-2,6-bisphosphatase-3 (PFKFB3) play a relevant role in the control of endothelial functions, including physiopathological angiogenesis. Recently, it has been shown that GLUT1 protein deficiency arrests angiogenesis, resulting in overt manifestations of brain dysfunction (Tang et al., 2017), and that inhibition of PFKFB3, which plays a key role in EC metabolism, reduces pathologic angiogenesis (De Bock et al., 2013). Interestingly, both GLUT1 and PFKFB3 are finely regulated at both transcriptional and post-transcriptional level by a variety of stimuli including female hormones (Obach et al., 2004; Veschini et al., 2007). For example, E2 enhances GLUT1 mRNA or protein levels in different cell types including microvascular endothelial and cancer cells (Shi and Simpkins, 1997; Imbert–Fernandez et al., 2014). In addition, we recently reported that PFKFB3 is a downstream effector of E2-mediated angiogenesis via endothelial GPER, highlighting that female hormones contribute to adaptable changes of endothelial glycolytic metabolism (Trenti et al., 2017). Notably, E2 rapidly increases PFKFB3 protein levels in a concentration-dependent manner without affecting PFKFB3 mRNA levels, suggesting that non-genomic mechanisms drive estrogen-boosted glycolysis in the vascular endothelium.

PFKFB3 is an unstable protein. In particular, the amount of PFKFB3 is regulated by mechanisms affecting protein stability as it is rapidly degraded through the ubiquitin/proteasome proteolytic pathway (Riera et al., 2003). Proteasomal degradation is a posttranslational mechanism that rapidly adjusts protein concentration in relation to environmental demand. This process is finely tuned by enzymes such as ubiquitin protein ligases (E3), which promotes the attachment of ubiquitin to target proteins with high degree of selectivity, and by ubiquitin specific proteases (USPs), called deubiquitanases (Harrigan et al., 2018). Of note, PFKFB3 is

substrate of two E3 ubiquitin ligases, whose activity controls glycolysis in neoplastic and non-neoplastic cells (Almeida et al., 2010; Tudzarova et al., 2011).

There is limited evidence that E2 modulates protein levels by targeting the rate of protein translation or proteasomal degradation, possibly involving specific E3 ligases (Pavlides et al. 2013; Sudhagar et al., 2011). In particular, E2 rapidly decreases the expression of ERα in estrogen target tissues including uterine artery endothelial cells by promoting its ubiquitination (Tschugguel et al., 2003). On the other hand, estrogen has also been proven to stabilize K-Ras by inhibiting its polyubiquitination, thus contributing to endometrial cellular transformation and tumor growth (Koo et al., 2015). However, whether E2 regulates cell metabolism and specifically promotes glycolysis targeting the stability of glycolytic proteins such as PFKFB3 and GLUT1 in endothelial cells is yet unexplored.

Based on this background, the **general hypothesis** of this thesis project is that female hormones contribute to rapid adaptation of metabolic demand in endothelial tissue by regulating glycolytic protein levels through post-transcriptional mechanisms. Therefore, the **general objective** was to disclose the mechanisms through which estrogenic agents regulate GLUT1 and/or PFKFB3 levels in human endothelial cells (HUVECs), and specifically to assess the role of the membrane receptor GPER in these pathways.

The **first specific aim** as summarized in Diagram 2.1 was to examine whether estrogenic agents rapidly increase PFKFB3 levels by promoting protein stability, namely by decreasing PFKFB3 ubiquitination and proteasomal degradation. For this purpose we investigated: 1) the role of specific E3 ligases in PFKFB3 degradation via the ubiquitin-proteasome system; 2) the effects of E2 and the GPER agonist, G1 on PFKFB3 protein stability; 3) the effect of estrogenic agents on PFKFB3 ubiquitination and more specifically on the regulation of the deubiquitinase USP19.

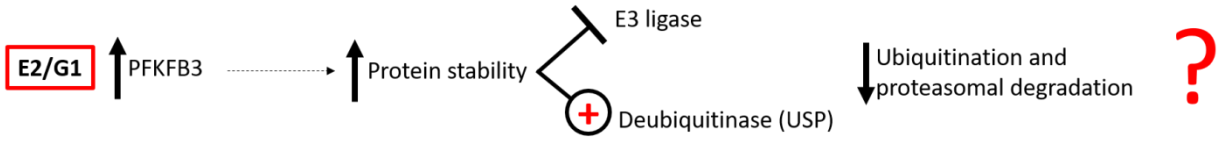

Diagram 2.1. First specific aim.

The **second specific aim** was to investigate if post-transcriptional regulation represents a common mechanism by which female hormones control the functional activation of other proteins of the endothelial glycolytic pathway, in particular GLUT1. For this purpose we assessed the effect of estrogenic agents on: 1) GLUT1 gene and protein expression, and 2) GLUT1 stability (Diagram 2.2).

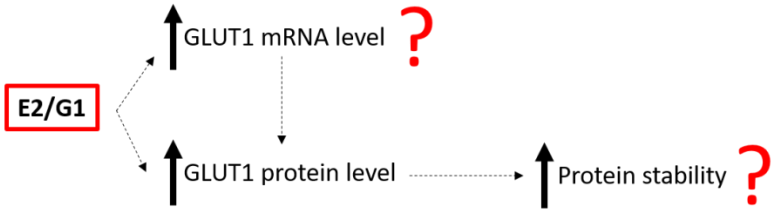

Diagram 2.2 Second specific aim.

MiRNAs are recognized as rapid post-transcriptional regulators of protein abundance and function. They work as a guide by base-pairing with target mRNA to negatively/ultimately regulate protein synthesis. The level of complementarity between miRNA and target mRNA determines whether miRNA triggers endonucleolytic mRNA cleavage and subsequent degradation or translation inhibition. The latter is the most common mechanism in mammals (Diagram 3A; Fabian et al., 2010).

Based on the evidence that in a breast cancer cell line 1) specific miRNAs act as down-regulators of metabolic proteins including GLUT1 and PFKFB3, and 2) E2 reduces the expression of specific miRNAs with functional readouts in cancer cell growth and migration (Ge et al, 2015; He et al, 2019; Tan et al, 2014), **we hypothesized** that E2-targeted miRNAs could participate in the functional regulation of glycolytic programs in cells with highly glycolytic phenotype (Diagram 2.3).

Therefore, **the third specific aim** of this project was to set up a system to appraise the role of specific miRNA in PFKFB3 estrogenic regulation. For this purpose we firstly generated a luciferase reporter vector containing PFKFB3 3'-UTR. Then, to validate miRNAs targeting PFKFB3, we co-transfected SKOV3, an estrogen-dependent ovarian cancer cell line, with the vector containing the 3'-UTR of PFKFB3 and miRNAs. Since we were unable to efficiently deliver the vector to HUVECs, we chose to transfect SKOV3 because similarly to ECs they have high glycolytic reliance. In addition, we examined the effect of validated miRNAs on PFKFB3 protein abundance using miRNA mimics.

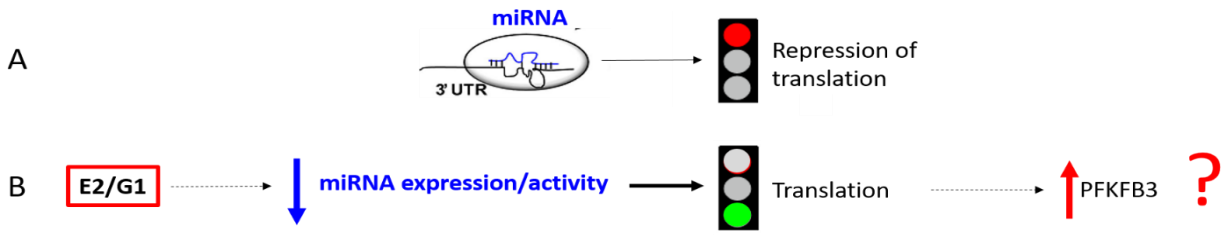

Diagram 2.3 Third specific aim.

Overall, the **main goal** of this project was to define the non-genomic mechanisms involved in the rapid estrogenic regulation of glycolytic protein levels in cells relying on glycolysis, in order to: 1) add knowledge

on the role of the membrane receptor GPER receptor in the metabolic and functional adaptation of cells to environmental changes that might have implications in estrogen's protective and prophylactic effects in vascular ischemic disorders, and 2) improve our understanding on miRNAs as novel targets of non-genomic rapid estrogen signaling.

3. MATERIALS AND METHODS

3.1 Cell Culture

3.1.1 *Human umbilical vein endothelial cells*

Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical cords as previously published (Bolego et al., 2006). Cells were grown in medium M199 (Invitrogen, San Giuliano Milanese, Italy) supplemented with 15% fetal calf serum (FCS, Invitrogen), gentamicin (40 μg/ml, Invitrogen), endothelial cell growth factor (ECGF 100 μg/ml), and heparin (100 UI/ml, Sigma-Aldrich) at 37°C in a humidified 5% CO_2 atmosphere. HUVECs were identified by their morphology and the expression of CD31-related antigen, and used for experiments from passages 2 through 6. For Western blotting experiments, cells were switched to phenol-free M199 supplemented with the same reagents as the standard HUVEC culture medium, 72 h before each assay. The experiments were performed with phenol-free M199 supplemented with 5% FCS, gentamicin (40 μg/ml), ECGF (100 μg/ml) and heparin (100 UI/ml).

3.1.2 *Ovarian cancer cell line (SKOV3)*

The human ovarian cancer cell line SKOV3 was purchased from the American Type Culture Collection (ATCC, USA) and maintained in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin (complete culture medium) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were used from passages 9 through 25. For Western blotting experiments, cells were switched to phenol-free RPMI 1640 supplemented with the same reagents as the standard SKOV3 culture medium 48 h before each assay. The experiments were performed with phenol-free RPMI 1640 supplemented with 2% or 5% FCS for MTT assay and Western blotting respectively.

3.2 MTT Assay. SKOV3 ($2.5x10^3$ cells/well) were seeded in 96-well plates and incubated in complete culture medium. The next day, cells were treated with estrogenic agents as indicated for 72 h. Four h before the incubation end, 10 μL stock solution of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, 5 mg/ml in PBS) was added to each well. Then, the medium was removed and formazan crystals were dissolved in 100 μL dimethylsulfoxide (DMSO). MTT reduction was quantified by measuring light absorbance with a multilabel plate reader (VICTOR2– Wallac) at 570–630 nm. Background absorbance values from control wells (cell-free media) were subtracted. Cell viability is expressed as raw optical density (OD) value.

3.3 Chemotaxis assay. Chemotaxis experiments were performed in a 48-well modifiedmicrochemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) using 8-μm nucleoporepolyvinylpyrrolidine-free polycarbonate filters coated with 10 μg/mL collagen. Lower chambers were filled with RPMI supplemented with 0.1% bovine serum albumin (BSA) in the presence of E2 or G1 (100–1 nM) as chemotactic stimuli, whereas upper chambers were filled with 50 μL SKOV3 cell suspension (1.6×10^5/mL in RPMI supplemented with 0.1% BSA). After 6 h incubation at 37 °C, non-migrating cells on the upper filter surface were removed by scraping. Cells migrated to the lower filter side were stained with Diff-Quick stain (VWRScientific Products, Bridgeport, NJ, USA), and densitometric analysis was performed using the Image J version 1.47

software (National Institutes of Health, NIH, USA). RPMI 1640 with 10% FCS or 0.1% BSA were used as positive and negative control, respectively. Each experiment was performed in sextuplicate. Results are reported as arbitrary units of optical density and represent the mean values of 5 independent assays.

3.4 Western blot. HUVECs (seeded at a density of $3x10^5$ cells/35-mm dish) or SKOV3 cells ($2x10^5$/35-mm dish) were treated as indicated in the Results and lysed with 80 µL lysis buffer (phosphate-buffered saline supplemented with 1.2% Triton X-100, 1X Roche cOmplete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), 2.5 mM NaF, 2 mM $Na_4P_2O_7$ (Sigma-Aldrich), 4 mM Na orthovanadate and 1 mM phenylmethanesulfonylfluoride). After centrifugation at 10000x*g* for 15 min, supernatants were collected for SDS-PAGE and Western blotting. Protein quantification was performed using the bicinchoninic protein assay kit (Euroclone, Milan, Italy). Proteins (20-50 µg) were separated on SDS-PAGE and transferred onto AmershamHybond-P polyvinylidene difluoride membranes. Membranes were then blocked and probed using the following rabbit primary monoclonal antibodies: anti-PFKFB3; anti-USP19; anti-GLUT1; anti-glyceraldehyde-3-phosphate dehydrogenase (all from Abcam, Cambridge, UK). After washing, membranes were incubated with rabbit secondary horseradish peroxidase–conjugated antibodies (Vector Laboratories, Burlingame, CA). Bands were detected by chemiluminescence using the Westernbright™ Quantum (Advansta, Menlo Park, California, USA). Images were acquired with Alliance mini HD9 Imaging System (Uvitec, Cambridge, United Kingdom). Densitometric analysis of bands was performed using Image J. Results are expressed as percentage of controls.

3.5 Indirect immunoprecipitation. HUVECs (seeded at a density of 6 x 10^5 cells/60-mm dish) were treated as indicated in the Results. After treatment, cells were lysed with 200 µL lysis buffer supplemented with 2 mM N-ethyl-maleimide and 2 mM EDTA (both from Sigma) and then quantified as described in Western blot section. An amount (20 µg) of the total lysate was used as input control, while 150 µg of total cell lysate was subjected to immunoprecipitation. Samples were incubated overnight tumbling at 4°C with either a specific PFKFB3 polyclonal antibody (Abcam) or a rabbit pre-immune IgG antibody as a negative control. The following day, protein G-magnetic beads (Millipore, Darmstadt, Germany) were added, and the mixture was incubated for 1 h at room temperature. Beads were recovered, extensively washed with PBS-Tween 1% and aspirated to dryness. PFKFB3 was eluted by heating beads at 80°C in sample loading buffer added with β-mercaptoethanol for subsequent detection by Western blotting. The blots were blocked with StartingBlock T20 (TBS) blocking solution (Clean-Blot IP Detection Kit, Thermo Fisher Scientific, Monza, Italy), probed with mouse anti-ubiquitin (Merk-Millipore) or rabbit monoclonal anti-PFKFB3 (Abcam) antibodies, and then incubated with appropriate secondary HRP-conjugated antibodies (Vector Laboratories). Bands were detected as described in Western blot section. Images were acquired with Alliance HD9Imaging System (Uvitec).

3.6 Real-time PCR. HUVECs (seeded at a density of $3x10^5$ cells/35-mm dish) or SKOV3 ($2x10^5$ cells/35-mm dish) were treated as indicated in the Results in phenol red-free M199 or RPMI 1640 complete culture medium, respectively, added with 5% FCS. Cells were harvested, washed in PBS and total RNA was extracted using

the Total RNA Purification plus Kit (Norgen, Biotek Corp., Ontario, Canada). cDNA was generated from 1 µg total RNA using the Maxima first strand cDNA synthesis kit with dsDNase (Thermo Scientific) according to the manufacturer's instructions. The relative quantification of genes of interest was performed by real-time quantitative PCR (q-PCR) using SYBR Green PCR Master Mix for 40 cycles of denaturation (15 s, 95°C), annealing (30 s, 60°C) and extension (30 s, 72° C) on a QuantStudio 3 Real Time PCR (Applied Biosytems, Thermo Fisher Scientific). Primer sequences were the following: *PFKFB3* (forward) GCGTCCCCACAAAAGTGTTC and (reverse) CCGGACTTTCATGGCTTCCT; *GLUT1* (forward) GATTCCCAAGTGTGAGTCGC and (reverse) GACATCATTGCTGGCTGGAG; *GAPDH* (forward) CACCATCTTCCAGGAGCGAG and (reverse) CCTTCTCCATGGTGGTGAAGAC; *HPRT1* (forward) CCCTGGCGTCGTGATTAGTG) and (reverse) CGAGCAAGACGTTCAGTCCT Target genes were normalized to GAPDH or HPRT1 and analyzed using the $2^{\Delta\Delta Ct}$ method.

3.7 siRNA transfection. HUVECs ($3x10^5$ cells/35-mm dish) were seeded in complete M199 medium. After 24 h, cells were transfected with Lipofectamine3000 (Invitrogen) and 20 nM siRNA (ON-TARGETplus Human GPER (2852) siRNA – SMARTpool, Dharmacon) in complete culture medium with 15% FBS for 72 h. A scrambled siRNA (20 nM) without sequence homology to any known human gene served as negative control. Afterward, medium was replaced with phenol-red free M199 with 15% FCS for 24 h. Cells were then treated in phenol-free M199 with 5% FBS as indicated in the Results. GPER protein levels were measured after 72 h transfection in order to assess knockdown efficiency.

3.8 miRNA Transfection. SKOV3 cells ($2x10^5$ /35-mm dish) were seeded in complete RPMI 1640 medium containing 10% FBS. After 24 h, cells were transfected with miRNAs (75 pmol/well) as detailed below using Lipofectamine3000 for 48 h. A miRNA without any homology to human gene sequences (miRNA-NC) served as a negative control. In order to assess PFKFB3 translational repression, PFKFB3 protein levels were measured 48 h after transfection by Western blot assay. The following miRNA mimics (Sigma-Aldrich) were used:

MISSION® microRNA Mimic miR-26b: UCAAGUAAUUCAGGAUAGGU

MISSION® microRNA Mimic miR-206: UGGAAUGUAAGGAAGUGUGUGG

MISSION® miRNA, Negative Control 2, Sequence from *Caenorhabditis elegans* with no homology to human gene sequences: CGGUACGAUCGCGGCGGGAUAUC

3.9 Vector cloning and cell transfection. The 3′-UTR of human PFKFB3, containing a miR-26b and miR-206 binding site, was PCR-amplified from genomic DNA and cloned between XhoI and XbaI, downstream of firefly luciferase gene in the pmirGLO Dual-Luciferase miRNA Target Expression vector (Promega, Madison, WI, USA). Primer sequences used for cloning were the following:

PFKFB3 UTR fwd 5'-AGCTTACTCGAGGGCAGACGTGTCGGTTCCATTC-3' (XhoI)

PFKFB3 UTR rev 5'-TACTAGTCTAGATTTACTTCGTCCATGTATTTCAACCAGG-3' (XbaI)

HUVECs and SKOV3 were transfected with the vector as follows.

HUVECs ($5x10^4$ or $1x10^5$ cells/well) were seeded in 24-well plates in M199 supplemented with 15% FCS, gentamicin (40 μg/ml), ECGF (100 μg/ml), and heparin (100 UI/ml). After 24 h, cells were transfected with 500 ng/well pmirGLO-3'UTR or pmirGLO (empty vector) in the presence or absence of miRNAs (15 pmol/well miRNA-206, miRNA-26b or miRNA-NC) with either TransIT-X2 (Mirus, TemaRicerca) or Lipofectamine2000 (Life Technologies Inc.) for 24 h.

SKOV3 ($5x10^4$ cells/well) were seeded in 24-well plates in RPMI 1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. After 24 h, the cells were transfected with 500 ng/well pmirGLO-PFKFB3 3'UTR or pmirGLO (empty vector) in the presence or absence of miRNAs (15 pmol/well miRNA-206, miRNA-26b or miRNA-NC) using Lipofectamine3000 (Life Technologies Inc.) for 48 h.

3.10 Luciferase Reporter Assay. 48 h after transfection, cells were lysed in passive lysis buffer (Promega), and luciferase activities were measured with the Dual-Glo® Luciferase Assay System (Promega) using a luminometer (TD 20/20, Promega). Data were normalized as described elsewhere (Campos-Melo et al., 2014). Firefly luciferase signal was normalized to the renilla luciferase signal. To assess luciferase activity changes for each miRNA, normalized values (firefly luciferase activity/renilla luciferase activity) of luciferase activity of pmirGLO-3'UTR were compared to those of the pmirGLO empty Vector. For each transfection condition, luciferase activity was averaged from 4 replicates. Positive values of relative variation in luciferase activity were considered up-regulation while negative values were considered down-regulation. Results are expressed as the mean values of 3 independent experiments.

3.11 Drugs and Chemicals. The following compounds were used for experiments: 17β-estradiol (E2, Sigma), (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (G1, Tocris Bioscience), (3a*S**,4*R**,9b*R**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G-15, Tocris Bioscience), cycloheximide (Sigma Aldrich), 9*H*-Indeno[1,2-*e*][1,2,5]oxadiazolo[3,4-*b*]pyrazin-9-one (SMER-3, Tocris Bioscience), N[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-D-leucinamide (MG132, Sigma Aldrich). Inhibitors were added 30 min before the stimulus.

3.12 Statistical Analysis. All experiments were performed in at least 3 independent replicates; results are presented as mean values, with error bars representing the standard error (S.E.M.) of the average value. Statistical analysis was performed using Graph Pad Prism 5.02 (Graph Pad Software Inc., La Jolla, CA, USA). Student's *t*-test was used to compare the means of two independent groups. Two-way or one-way ANOVA followed by Dunnett's or Bonferroni's *post-hoc* tests were used for multiple comparisons. A P value of <0.05 was considered statistically significant.

4. RESULTS

4.1 *MECHANISMS INVOLVED IN THE REGULATION OF PFKFB3 BY ESTROGENIC AGENTS*

4.1.1 The selective GPER agonist G1 increased endothelial PFKFB3 levels via transcription-independent mechanisms

PFKFB3, the allosteric activator of the key glycolytic enzyme phosphofructokinase 1 (PFK-1), is regulated by different stimuli including inflammatory cytokines and transcription factors, at transcriptional and/or post-transcriptional levels (Doddaballapur et al., 2015; Riera et al., 2003; Almeida et al., 2010). In breast cancer cells, E2-induced changes in glucose metabolism require mRNA synthesis of glycolytic proteins including PFKFB3 (Neeman and Degani, 1989; Imbert-Fernandez et al., 2014). Conversely, we recently established that E2 promotes endothelial cell metabolism by rapidly increasing PFKFB3 protein but not mRNA levels in HUVECs (Trenti et al., 2017).

To further investigate the mechanism by which E2 increases PFKFB3 proteins expression, we initially treated cells with the selective GPER agonist G1 for different time points. Similarly to what previously reported for E2, treatment with G1 (100 nM) increased PFKFB3 protein levels with a peak at 3 h (Fig. 4.1 A). In line with the evidence that PFKFB3 mRNA is unstable (Chesney et al., 1999), very low levels of PFKFB3 mRNA were detected by qPCR, with no statistically significant variation over the time course (15 min – 6 h) of G1 treatment (Fig. 4.1 B). Although a possible contribution of gene expression at different time points (30 min; 2-4 h) cannot be completely ruled out, these data suggest that the increased PFKFB3 abundance elicited by estrogens was largely independent from activation of transcription.

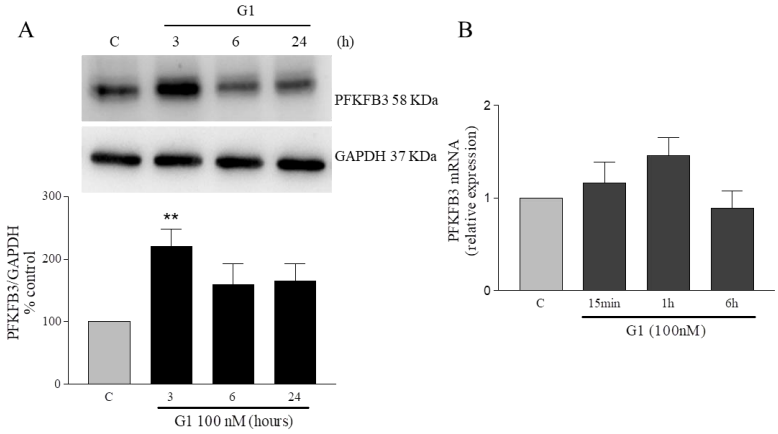

Figure 4.1 The selective GPER agonist G1 increased endothelial PFKFB3 protein levels without affecting PFKFB3 mRNA levels. HUVECs ($3x10^5$ cells/well) were grown in 35-mm dishes in M199 phenol red–free medium with 5% FBS and treated with G1 (100 nM) for the indicated time points. (A) (Upper panel) Representative western blot showing PFKFB3 immunodetection; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (Lower panel) Densitometric analysis of bands, normalized to GAPDH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 8 independent experiments; one-way ANOVA, Dunnett's *post hoc* test: ** $P < 0.01$ *vs.* control. (B) PFKFB3 mRNA levels were measured by q-PCR and normalized to GAPDH. Expression level of control C was assigned a value of 1 and mRNA levels are shown relative to control. Data are expressed as mean ± S.E.M of 3 independent experiments run in triplicate.

Hence, in order to evaluate a potential effect of estrogens on protein stability, HUVECs were treated with the protein synthesis inhibitor cycloheximide (CHX, 35 µM) in the presence or absence of estrogenic agents. Treatment with CHX for different time points (1 to 6 h) resulted in a rapid degradation of PFKFB3, which was counteracted in cells treated with E2 (100 nM; Fig. 4.2 A). Under these experimental conditions, CHX did not alter HUVEC morphology (data not shown). Similarly to E2, treatment with an equimolar G1 concentration (100 nM) blocked PFKFB3 degradation (Fig. 4.2 B), suggesting that estrogenic agents enhanced endothelial PFKFB3 stability via non-genomic mechanisms. It is important to underline that GAPDH protein level was influenced neither by estrogenic agents nor by CHX treatment.

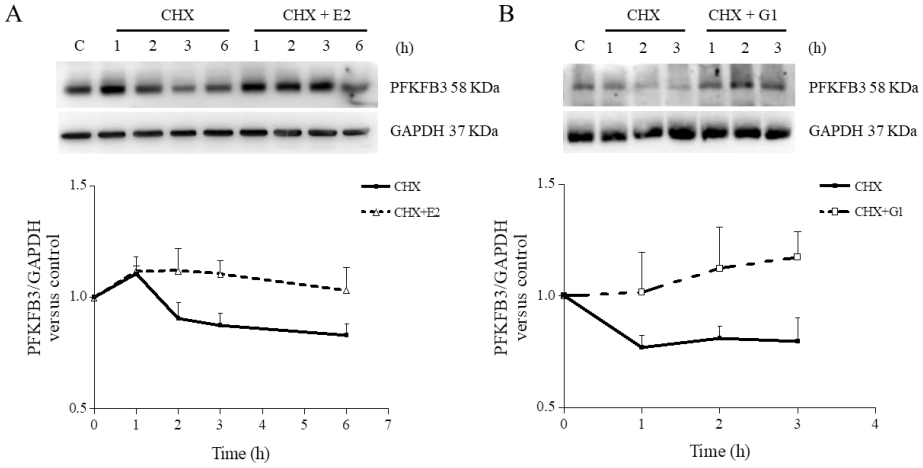

Figure 4.2 17β-estradiol (E2) and G1 promoted PFKFB3 protein stability. (A) HUVECs were grown as described in the legend to Fig. 4.1 and treated with cycloheximide (CHX, 35 µM) in the presence or absence of E2 (100 nM) for 1-6 h. (Upper panels) Representative blot showing PFKFB3 immunodetection; GAPDH was used as a loading control. (Lower panels) Densitometric analysis of bands, normalized to GADPH levels, expressed versus control. Data are expressed as mean ± S.E.M. of 4 independent experiments; two-way ANOVA, $P < 0.05$. (**B**) HUVECs were treated as above in the presence or absence of G1 (100 nM) for 1-3 h. (Upper panels) Representative blot showing PFKFB3 immunodetection; GAPDH was used as a loading control. (Lower panels) Densitometric analysis of bands, normalized to GADPH levels, expressed versus control. Data are expressed as mean ± S.E.M. of 4 independent experiments; two-way ANOVA, $P < 0.05$.

4.1.2 E2 and G1 negatively modulate PFKFB3 protein ubiquitination

Ubiquitination is a finely tuned mechanisms to tag proteins to degradation via proteasome; it involves enzymes acting in sequence and culminating in the attachment of activated ubiquitin to the substrate by combined activity of conjugases and selective ubiquitin ligases (also called E3 ligases). Recent data in different cell types show that PFKFB3 is recognized by the ubiquitin ligase complex APC/C-Cdh1 (Anaphase-Promoting Complex/Cyclosome) and/or by another member of the ubiquitin ligase family, namely SCF-β-TrCP (SKP1-CUL1-F-box-protein complex)-(β-transducin repeat-containing protein). The expression of these ligases finely tunes PFKFB3 levels and glycolysis with functional effects in target cells (Almeida et al., 2010; Tudzarova et al., 2011; Herrero-Mendez et al., 2009).

To test the hypothesis that E2 could rapidly increase PFKFB3 protein levels by inhibiting its proteasomal degradation in HUVECs, we first tested whether the ubiquitin-proteasome system and specific E3 ligases are

involved in PFKB3 degradation. Treatment with either the proteasome inhibitor MG132 (1-10 µM) or the selective inhibitor of the SCF family E3 ubiquitin ligase SMER-3 (0.1-1 µM) for 3-6 h induced a rapid increase in PFKFB3 levels without affecting HUVEC morphology (Fig. 4.3). The amount of PFKFB3 was significantly higher with respect to control already after 3 h of treatment, suggesting that proteasomal degradation was responsible for rapid PFKFB3 turnover. Under our experimental conditions, treatment with the proteasome inhibitor did not affect GAPDH turnover.

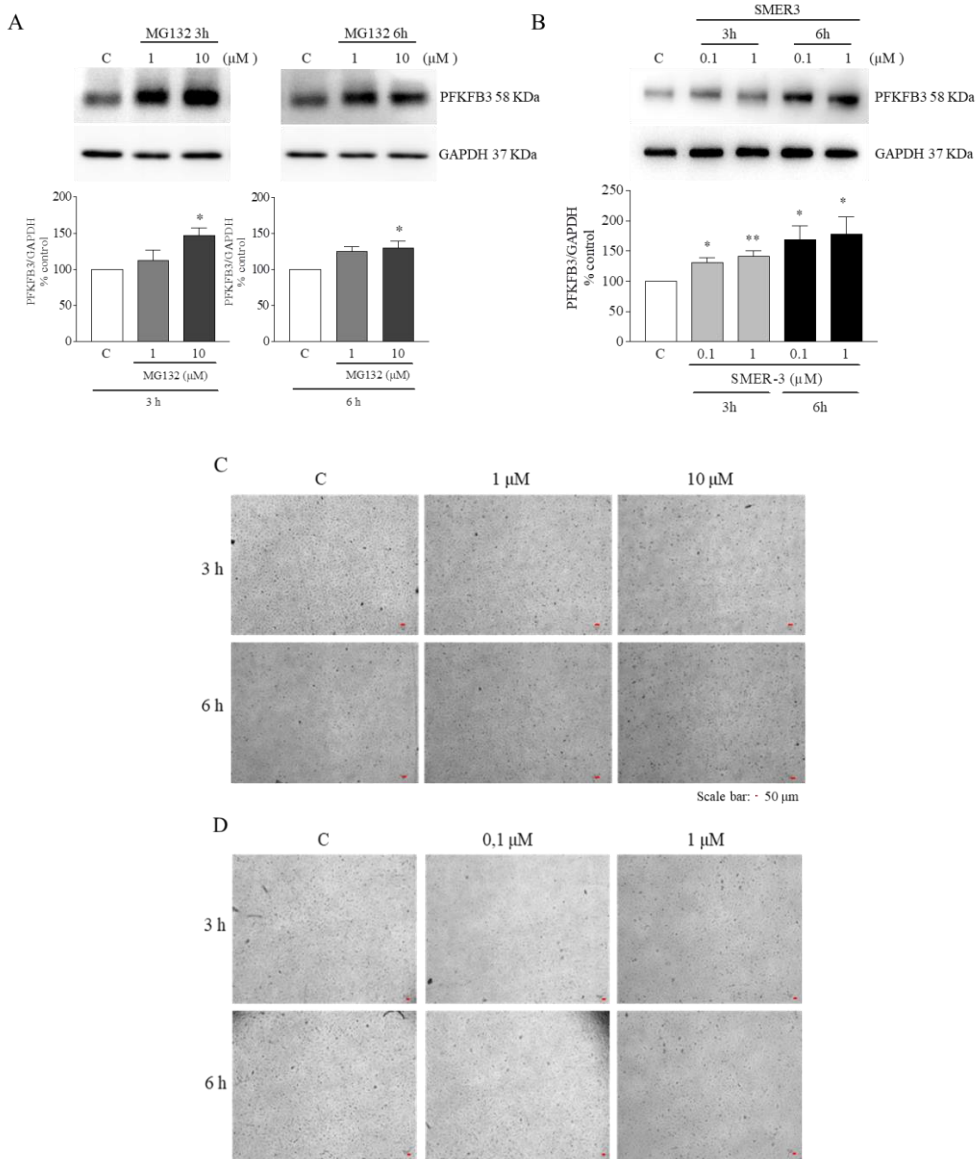

Figure 4.3. The ubiquitin-proteasome system was involved in PFKFB3 degradation. HUVECs were grown as described in the legend to Fig. 4.1 and treated with (**A**) the proteasome inhibitor MG132 (1-10 µM) or (**B**) the SCF E3 ligase inhibitor SMER-3 (0.1-1 µM) for 3-6 h. (Upper panel) Representative western blot showing PFKFB3 immunodetection; GAPDH was used as a loading control. (Lower panel) Densitometric analysis of bands, normalized to GADPH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 4 independent experiments; one-way ANOVA, Dunnett's *post hoc* test: * $P < 0.05$ *vs.* control; ** $P < 0.01$ *vs.* control. (**C-D**) HUVECs ($15x10^4$ cells/well) in 12-well plate were grown in M199 medium supplemented with 5% FCS and treated with (**C**) MG132 (1-10 µM) or (**D**) SMER3 (0.1-1 µM) for 3-6 h. Neither treatment affected cell morphology. Representative images obtained with inverted phase contrast microscope (Nikon Eclipse-Ti) equipped with digital camera (40x); 50 µm scale.

Afterwards, in order to evaluate the effect of estrogenic agents on PFKFB3 proteasomal degradation, we analyzed the ubiquitination of PFKFB3 in HUVECs treated with MG132 in the presence or absence of E2 or G1. As shown in Figure 4.4 A, treatment with E2 (100 nM) for 90 min reduced the ubiquitination level/ amount of polyubiquitination of immunoprecipitate in comparison to that of cells treated with MG132 (10 μM) alone. Similar results were obtained with G1 (100 nM) (Fig. 4.5). These results have been reproduced in at least 3 independent experiments; however due to differences in the yield of immunoprecipitation experiments, quantitative analysis was not performed. The value of this approach has been further confirmed by the inhibition of PFKFB3 ubiquitination induced by treatment with the specific SCF E3 ligase inhibitor SMER-3 (data not shown). Overall, these data clearly indicate that endothelial PFKFB3 underwent rapid proteasomal degradation via SCF family E3 ligases and estrogens/ER agonists increased PFKFB3 levels by inhibiting its degradation via the ubiquitin-proteasome system.

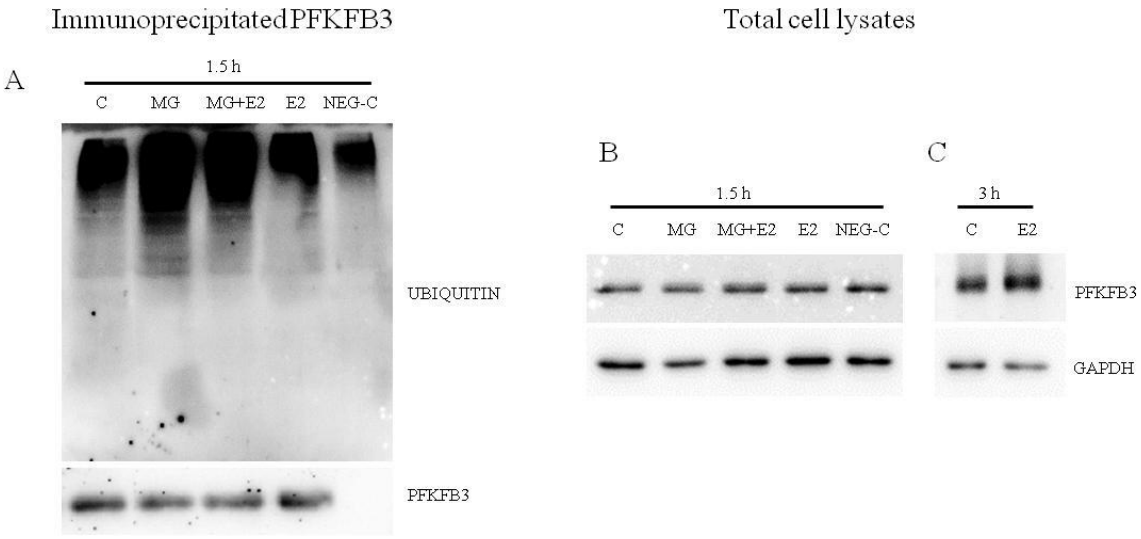

Figure 4.4 E2 decreased PFKFB3 ubiquitination levels. (A) HUVECs ($6x10^5$ cells/well) were grown in 60-mm dishes in M199 phenol-red free medium with 5% FBS and treated with MG132 (10 μM) ± E2 (100 nM) or with E2 alone for 1.5 h. 150 μg of solubilized proteins from total cell lysates was subjected to immunoprecipitation with a specific PFKFB3 rabbit polyclonal antibody. Immunocomplexes were resolved by SDS-PAGE. The ubiquitin-conjugated PFKFB3 was detected with a mouse anti-ubiquitin antibody. As a negative control (NEG-C), 150 μg of solubilized proteins was subjected to immunoprecipitation using an IgG antibody of the same isotype as PFKFB3 (rabbit pre-immune serum; *upper panel*). Immunoprecipitation control of PFKFB3 was assessed using the anti-PFKFB3 rabbit monoclonal antibody *(lower panel*). (**B**) An equal amount of protein from total cell lysates was separated by SDS-PAGE and probed with monoclonal antibodies to PFKFB3 and GAPDH, used as a loading control. A representative experiment out of 3 is shown. (**C**) Internal control of functional response: HUVECs ($3x10^5$ cells /well) were grown in 35-mm dishes in M199 phenol red–free medium with 5% FBS and treated with E2 (100 nM) for 3h. Representative western blot showing PFKFB3 immunodetection; GAPDH was used as a loading control (Trenti *et al*, 2017).

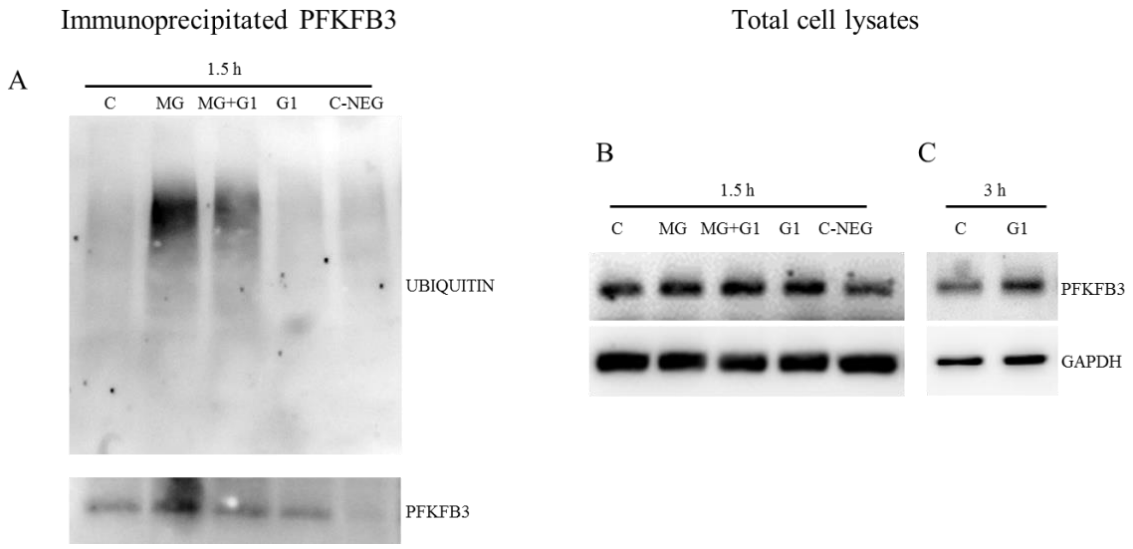

Figure 4.5 G1 decreased PFKFB3 ubiquitination levels. (A) HUVECs ($6x10^5$ cells/well) were grown in 60-mm dishes in M199 phenol-red free medium with 5% FBS and treated with MG132 (10 μM) ± G1 (100 nM) or with G1 alone for 1.5 h. 150 μg of solubilized proteins from total cell lysates was subjected to immunoprecipitation with a specific PFKFB3 rabbit polyclonal antibody and immunocomplexes were resolved by SDS-PAGE. The ubiquitin-conjugated PFKFB3 was detected with a mouse anti-ubiquitin antibody. As a negative control (NEG-C), 150 μg of solubilized proteins was subjected to immunoprecipitation using an IgG antibody of the same isotype as PFKFB3 (rabbit pre-immune serum; *upper panel*). Immunoprecipitation control of PFKFB3 was assessed using the anti-PFKFB3 rabbit monoclonal antibody *(lower panel*). (**B**) An equal amount of protein from total cell lysates was separated by SDS-PAGE and probed with monoclonal antibodies to PFKFB3 and GAPDH, used as a loading control. A representative experiment out of 3 is shown. (**C**) Internal control of functional response: HUVECs ($3x10^5$ cells /well) were grown in 35-mm dishes in M199 phenol red–free medium with 5% FBS and treated with G1 (100 nM) for 3 h. Representative western blot showing PFKFB3 immunodetection; GAPDH was used as a loading control (Trenti *et al*, 2017).

4.1.3 Estrogenic agents enhanced PFKFB3 protein levels by regulating USP19 levels via GPER

Estrogenic agents regulate protein ubiquitination and degradation by affecting either specific E3 ligases or deubiquitinases (Pavlides et al., 2013; Ogawa et al., 2011). In particular, E2 increased the deubiquitinase USP19 levels in a myogenic cell line, leading to reduced protein degradation via the ubiquitin-proteasome system (Ogawa et al., 2011).

Based on this evidence, we analyzed the effect of E2 and G1 on the levels of USP19. As shown in Fig. 4.6 and 4.7 A, treatment with E2 as well as G1 increased USP19 protein levels in a time-dependent manner. The amount of USP19 significantly differed in comparison to control value already after 1 hour of treatment with estrogenic agents and remained higher up to 6 h.

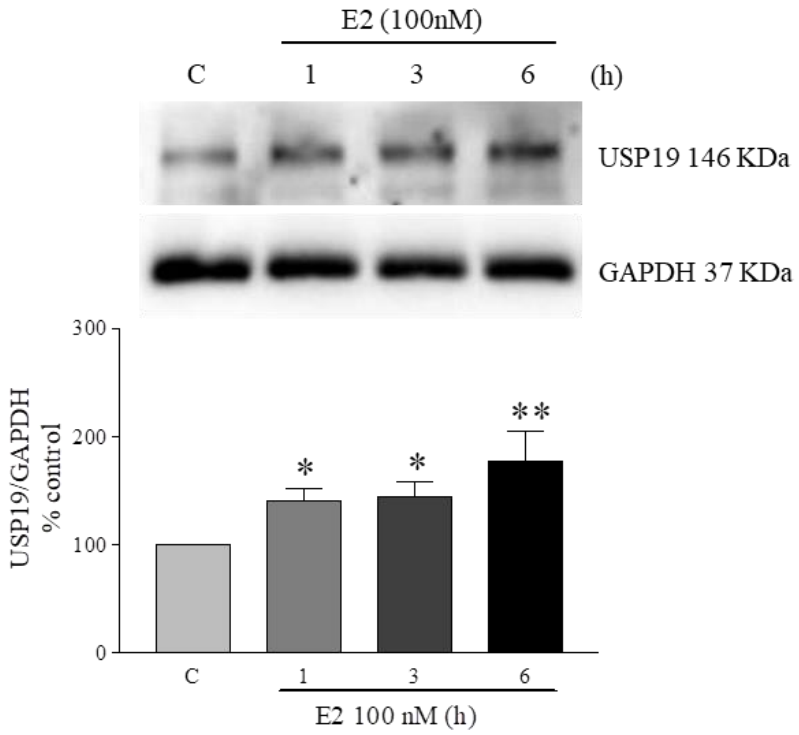

Figure 4.6 E2 treatment increased USP19 protein levels. HUVECs were grown as described in the legend to Fig. 4.1 and treated with E2 (100 nM) for 1-6 h. (*Upper panel*) Representative western blot showing USP19 immunodetection; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GADPH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 4 independent experiments; one-way ANOVA, Dunnett's *post hoc* test: * $P < 0.05$, ** $P < 0.01$ *vs.* control.

To provide further evidence for the involvement of GPER in E2-mediated inhibition of PFKFB3 degradation, we performed experiments using GPER1 specific siRNA. As a result, treatment of HUVECs with GPER siRNA for 72 h led to a significant reduction in USP19 as well as GPER protein levels (Fig. 4.7 B, C).

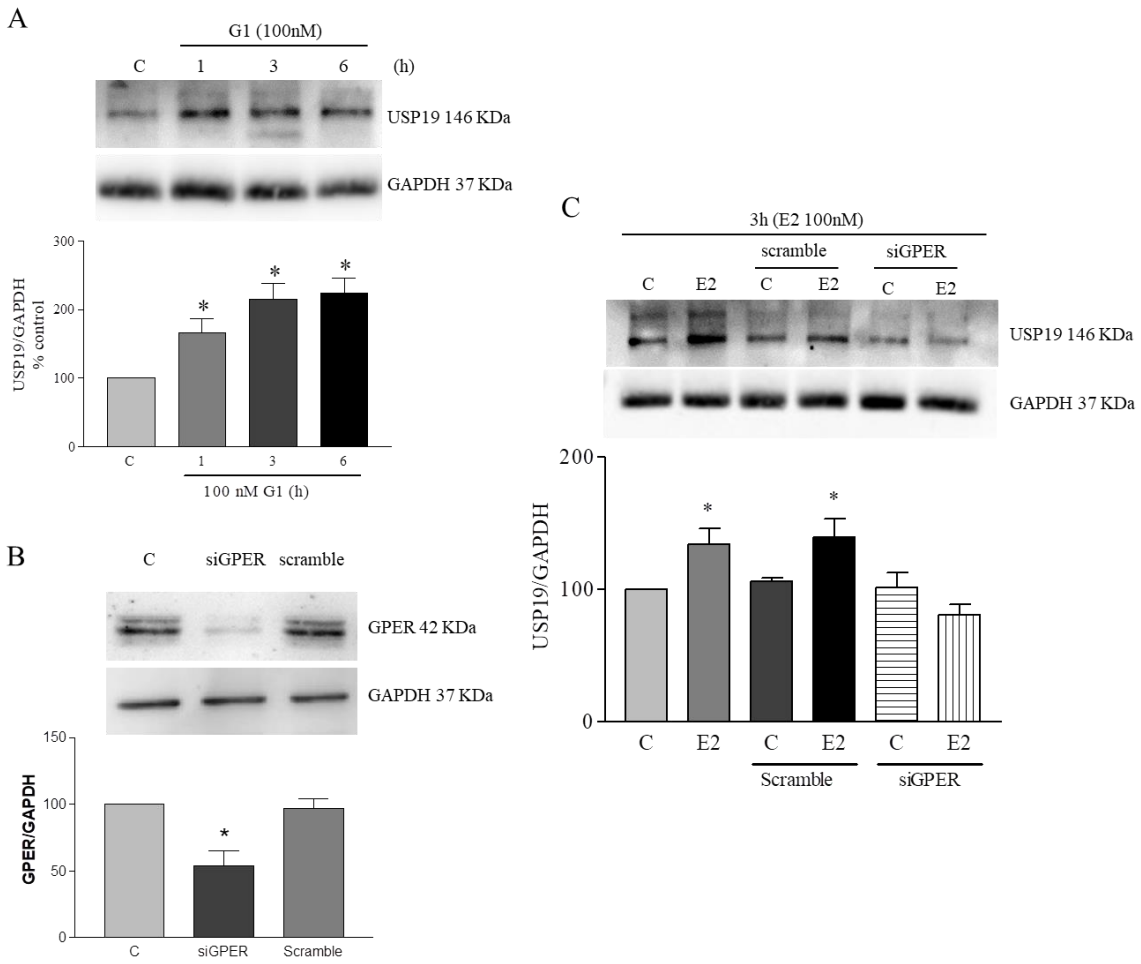

Figure 4.7 GPER mediates the increase in USP19 protein levels induced by E2. (A) HUVECs were grown as described in the legend of Fig. 4.1 and treated with G1 (100 nM) for 1-6 h. (*Upper panel*) Representative western blot showing USP19 immunodetection; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GADPH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 4 independent experiments; one-way ANOVA, Dunnett's post hoc test: * $P < 0.05$ *vs*. control. (**B** and **C**) HUVECs ($2x10^5$/well) were seeded in 35-mm dishes in complete M199 medium, and after 24 h, were transfected with scramble or GPER siRNA (siGPER) for 72 h. After transfection, medium was replaced with phenol-red free M199 supplemented with 15% FBS. After 24 h medium was replaced with M199 supplemented with 5% FBS and cells were treated with E2 (100 nM) for 3 h. (*Upper panel*) Representative western blot showing GPER and USP19 immunodetection, respectively. (*Lower panel*) Densitometric analysis of bands, normalized to GAPDH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 3 independent experiments. One-way ANOVA, Dunnett's *post hoc* test: *$P < 0.05$ *vs.* control.

4.2 *MECHANISMS INVOLVED IN THE REGULATION OF THE GLUCOSE TRANSPORTER GLUT1 BY ESTROGENIC AGENTS*

4.2.1 Estrogenic agents increased endothelial GLUT1 protein expression without affecting GLUT1 mRNA levels

The glucose transporter GLUT-1 is the first rate-limiting step in glucose utilization in tissues, such as the brain, that rely on glycolysis as a source of energy (Ohno et al., 2011), and is the most abundant isoform in endothelial cells (Mann et al., 2003). In addition, *in vivo* data demonstrated that E2 regulates GLUT1 levels at transcriptional or posttranscriptional levels (Shi and Simpkins, 1997; Cheng et al., 2001). However, the effect of E2 or selective ER agonists on endothelial GLUT1 levels has not been assessed yet.

We observed an increase in GLUT1 expression in cells treated with E2 (100 nM) for 1 h and this effect was still detectable after 24 h (Fig. 4.8 A). Similarly, treatment with G1 induced an increase in GLUT1 levels with a comparable time course (Fig. 4.8 B).

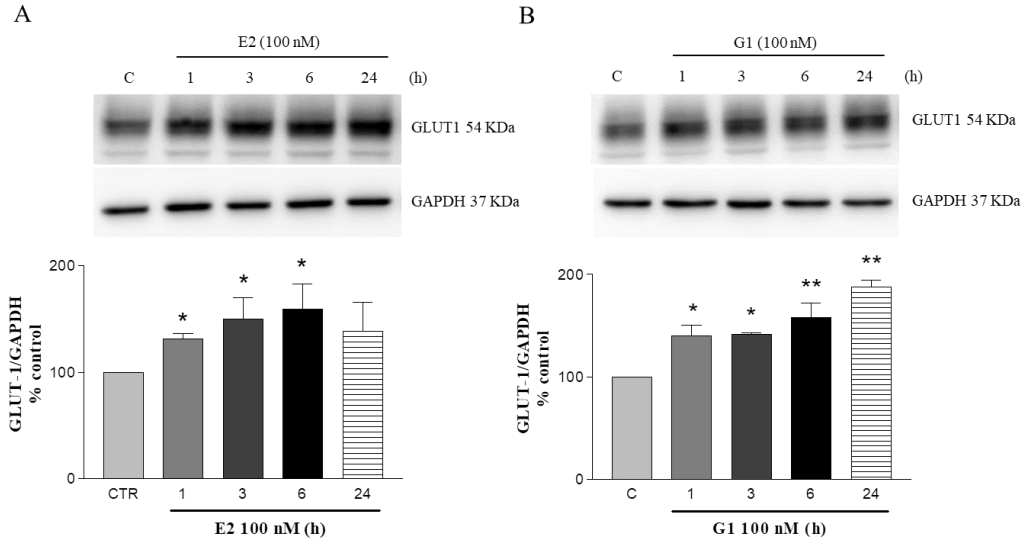

Figure 4.8 E2 and G1 increased GLUT1 protein levels. HUVECs were grown as described in the legend to Fig. 4.1 and treated with E2 (**A**) or G1 (**B**, both 100 nM) for 1-24 h. (*Upper panel*) Representative western blot showing GLUT1 immunodetection; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GADPH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 4 independent experiments; one-way ANOVA, Dunnett's *post hoc* test: * $P < 0.05$; ** $P < 0.01$ *vs.* control.

However, no changes in GLUT1 mRNA expression were observed in HUVECs treated with either E2 or G-1 for up to 6 h (Fig. 4.9).

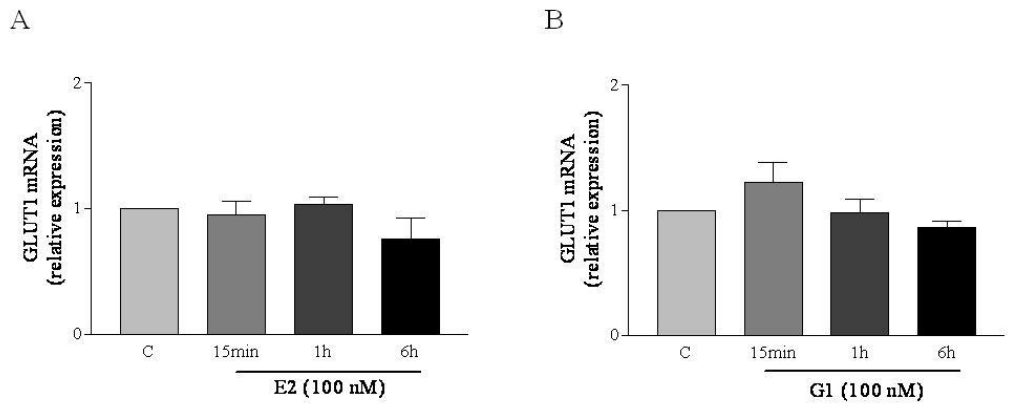

Figure 4.9 E2 and G1 treatment did not affect GLUT1 mRNA levels. HUVECs were grown as described in the legend to Fig. 4.1 and treated with E2 (**A**) or G1 (**B**, both 100 nM) for 15 min to 6 h. GLUT1 mRNAlevels were measured by q-PCR and normalized to GAPDH. Expression level of control was assigned a value of 1, and mRNA levels are shown relative to control. Data are espressed as mean ± S.E.M of 4 independent experiments. One-way ANOVA, ns.

4.2.2 E2 did not affect GLUT1 protein stability

Furthermore, in the attempt to analyze if posttranscriptional regulation represents a common mechanism by which female hormones regulate proteins of the glycolytic pathway in the endothelium, we checked GLUT1 protein stability in the presence or absence of E2 under conditions of protein neo-synthesis blockade. To this end, HUVECs were treated with E2 and CHX (10 µM) at various time points. Differently from what observed for PFKFB3, CHX did not significantly affect GLUT1 stability (Fig. 4.10) within the considered time frame (1-6 h).

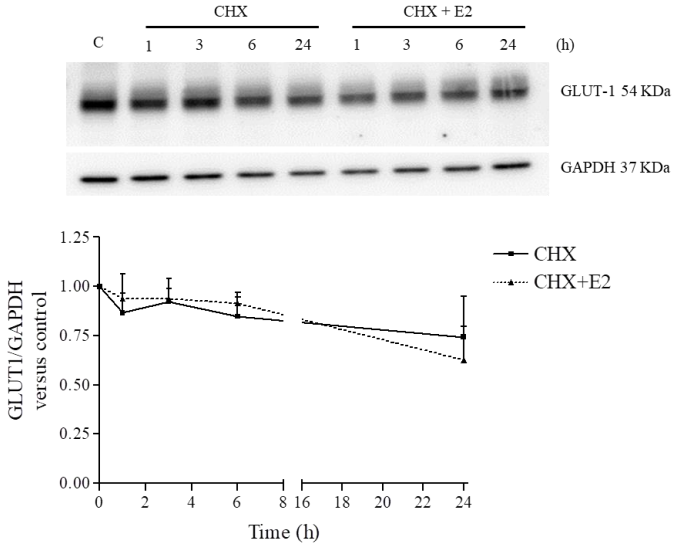

Figure 4.10 E2 did not enhance GLUT1 protein stability. HUVECs were grown and treated as described in the legend to Fig. 4.2 for 1-24 h. (Upper panel) Representative western blot showing GLUT1 immunodetection; GAPDH was used as loading control. (Lower panel) Densitometric analysis of bands, normalized to GADPH levels, expressed versus control. Data are expressed as mean ± S.E.M. of 3 independent experiments. Two-way ANOVA, *ns*.

Accordingly, HUVEC treatment with the proteasome inhibitor MG132 for 3-6 h did not change GLUT1 levels (Fig. 4.11), suggesting that E2 increased GLUT1 levels by mechanisms other than impaired degradation, most likely by promoting protein translation.

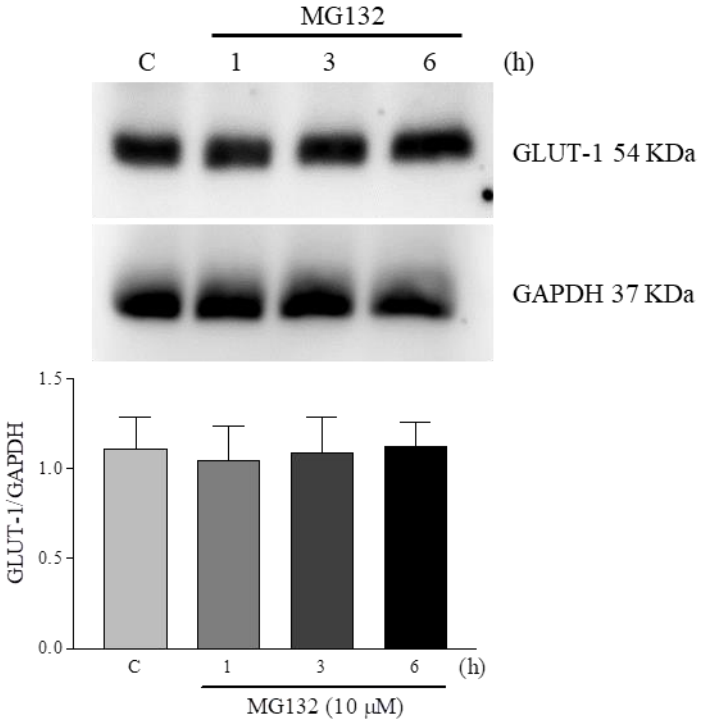

Figure 4.11 The proteasome inhibitor MG132 did not affect GLUT1 protein levels. HUVECs were grown as described in the legend to Fig. 4.1 and treated with the proteasome inhibitor MG132 (10 μM) for 1-6 h. (*Upper panel*) Representative blot showing GLUT1 immunodetection; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GADPH levels. Data are expressed as mean ± S.E.M. of 3 independent experiments; one-way ANOVA, ns.

Overall, these data show that estrogenic agents rapidly fostered endothelial glycolysis by modulating GLUT1 and PFKFB3 levels through different mechanisms. Based on the data from the literature showing that E2 increases the levels glycolytic protein targeting selected miRNAs, we hypothesized that estrogenic agents would regulate the glycolytic program via specific miRNAs.

4.3 *ROLE OF miRNA IN PFKFB3 ESTROGENIC REGULATION IN CELLS WITH HIGH GLYCOLYTIC RELIANCE*

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 established that miRNAs are novel players in the regulation of glycolytic metabolism in cancer cells, and deregulation in miRNA expression has been linked to increased PFKFB3 and GLUT1 protein levels (Subramanian et al., 2019).

Therefore, considering the difficulties in transfecting HUVECs, we chose the highly invasive ovarian cancer cell line SKOV3 to investigate the relationship between estrogens, miRNAs and glycolytic protein levels.

4.3.1 E2 increased PFKFB3 levels via a transcription-independent mechanism in SKOV3 cells

PFKFB3 and GLUT1 have been found to be overexpressed in several human cancer tissues including ovarian cancer (Atsumi et al., 2002; Cantuaria et al., 2001), and E2 further increases PFKFB3 protein and mRNA levels in MCF-7 cells (Imbert-Fernandez et al., 2014). However, the effect of estrogens on glycolytic proteins in ovarian cancer cells has not been tested.

We first analyzed the effect of E2 on PFKFB3 protein expression in SKOV3. E2 treatment increased PFKFB3 levels in a time-dependent manner over 24 h. This effect was already detectable after 3 h at the lowest concentration tested (1 nM, Fig. 4.12). Interestingly, no changes in PFKFB3 mRNA levels were observed following E2 treatment for 1-6 h (Fig. 4.13).

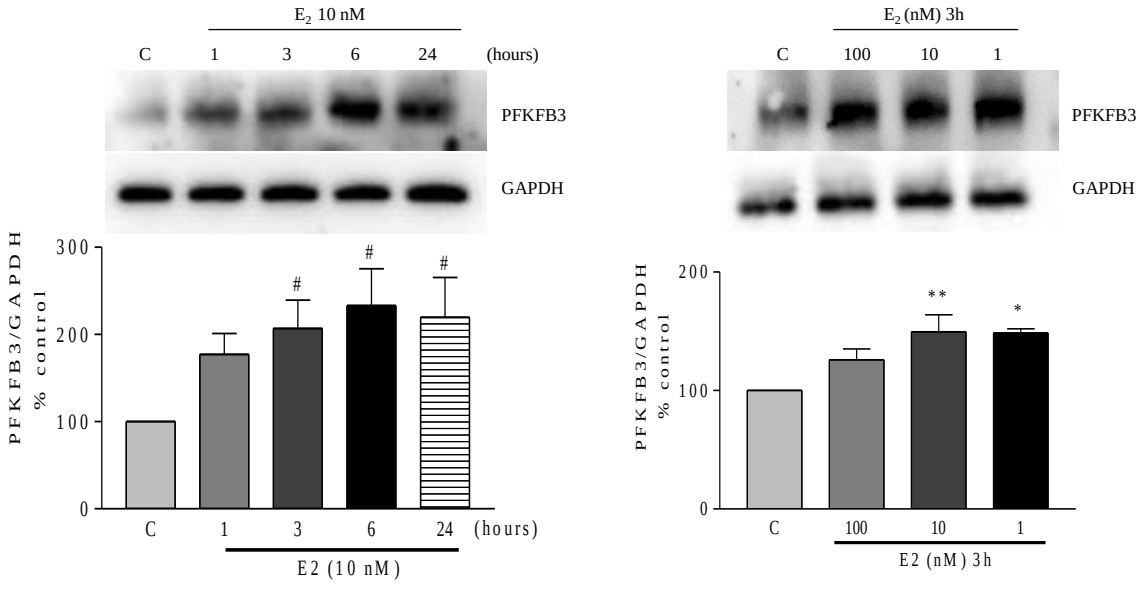

Figure 4.12 E2 induced PFKFB3 protein levels in SKOV3 cells. SKOV3 ($2x10^5$ cells/well) were grown in 35-mm dishes in RPMI 1640 phenol red–free medium with 5% FBS and treated with E2 (1-100 nM) for the indicated time. (*Upper panel*) Representative blot showing PFKFB3 immunodetection; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GAPDH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 3 independent experiments; one-way ANOVA, Dunnett's *post hoc* test: * $P < 0.05$, ** $P < 0.01$ *vs.* control; *t*-test # $P < 0.05$ *vs.* control.

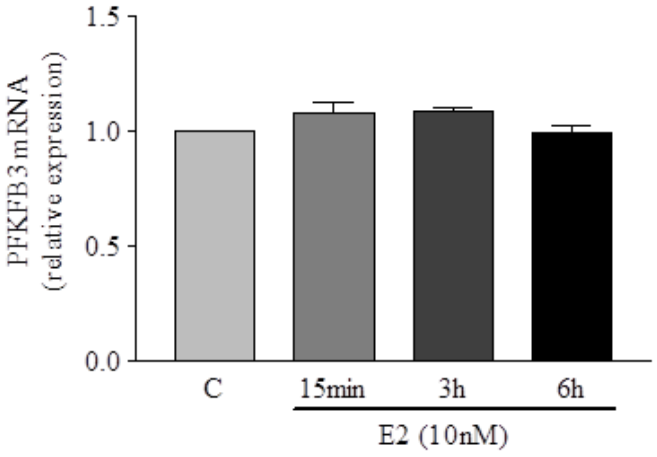

Figure 4.13 E2 treatment did not affect PFKFB3 mRNA levels. SKOV3 cells were grown as described in Fig. 4.12 and treated with E2 (10 nM) for the indicated time. PFKFB3 mRNA levels were measured by Q-PCR and normalized to GAPDH. Expression level of control C was assigned a value of 1 and mRNA levels are shown relative to control. Data are expressed as mean ± S.E.M of 3 independent experiments. One-way ANOVA, *ns*.

Overall, these results suggest that, similarly to what observed in HUVECs, E2 increased PFKFB3 levels in a transcription-independent manner.

4.3.2 Selective agonists and antagonists of ERα and GPER did not influence SKOV3 proliferation

We then assessed the effect of estrogenic agents on SKOV3 proliferation. SKOV3 ovarian carcinoma cells express all ER subtypes: ERα, ERβ (Chan et al., 2014), as well as the membrane GPER (Wang et al., 2013). In ovarian cancer, ERα levels are high and predominate over ERβ, and SKOV3 reproduce this pattern (Chan et al., 2014). There are few and conflicting results on the effect of ER agonists on SKOV3 proliferation (Hua et al., 1995; Chan et al., 2014; Yan et al., 2015; Wang et al., 2013). In particular, the role of GPER in the setting of ovarian cancer is still unclear and there is growing interest in the development of novel ER-selective hormone-based therapies.

SKOV3 proliferation, evaluated by the MTT assay, did not increase in cells challenged with either E2 or G1 (both 100 nM) for 72 h (Fig. 4.14). Antiestrogens inhibit the growth of ER positive cancer cells even in the absence of estrogens by inhibition of growth factor induces proliferation (Hua et al., 1995; Vignon et al., 1987). Of note, the non-selective nuclear ER antagonist tamoxifen induces opposite effects on ERα and GPER (Lappano et al., 2014). We therefore treated ovarian cancer cell with MPP or G15, highly selective ERα and GPER antagonists, respectively. As reported in Figure 4.14, neither compound affected SKOV3 proliferation over a 72-h incubation. Thus, SKOV3 cells did not exhibit a proliferative response to E2 and G1, nor was proliferation inhibited by the antiestrogens MPP and G15.

Surprisingly, estrogen treatment increased PFKFB3 levels in the absence of a proliferative response. However, similar findings were already reported by Hua and coworkers (1995), who found that the E2-dependent

increase in mRNA levels of oncogenes such as *c-myc* did not correlate with the growth response of cancer cells.

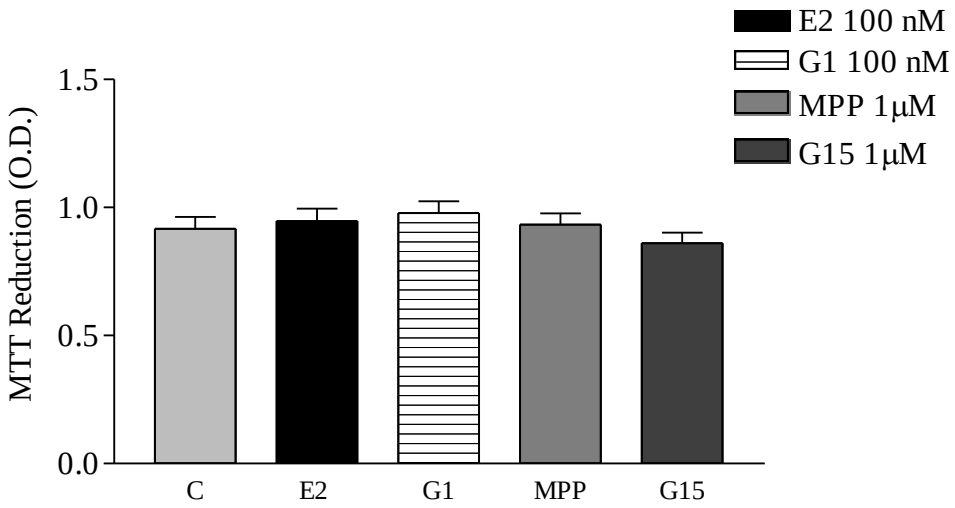

Figure 4.14 Estrogen receptor agonists and antagonists did not affect SKOV3 proliferation. SKOV3 ($2{,}5x10^3$ cells/well) were seeded in 96-well plates and treated for 72 h with E2, G1 (both 100 nM), the selective ERα antagonist MPP or the selective GPER antagonist G15 (both 1 μM) in phenol-red free RPMI 1640 medium supplemented with 2% FBS. Control (C) was 2% FBS. Cell proliferation was measured by MTT assay. The results are expressed as absolute values of optical density (O.D.) and represent the mean ± SEM of 3 independent experiments in quintuplicate. One-way ANOVA, *ns*.

4.3.3 E2 and G1 treatment promoted SKOV3 migration

Cell migration is required for tumor invasion, and estrogen promotes cancer cell invasiveness *in vitro* and *in vivo* (Tsai et al., 2013). There is also evidence for a role of GPER in estrogen-mediated cancer cell migration (Rigiracciolo et al., 2019; Flamini et al., 2011; Yan et al., 2013).

In order to investigate the effect of estrogenic agents on ovarian cancer cell invasiveness, we performed a chemotaxis assay to assess SKOV3 migration in response to E2 or G1 treatment. As shown in Fig. 4.15, the number of migrating cells significantly increased in response to increasing concentrations of E2 as well as G1 (1-100 nM), as evaluated after 6 h. At the highest concentration tested, the effect of estrogenic agents on migration was comparable to that of the positive control (10% FBS). These results suggest that 1) SKOV3 cells are responsive to estrogenic agents, and 2) estrogen-mediated SKOV3 migration involves the membrane receptor GPER. Experiments with the selective PFKFB3 inhibitor 3-PO will clarify the role of PFKFB3 in E2-mediated SKOV3 invasiveness.

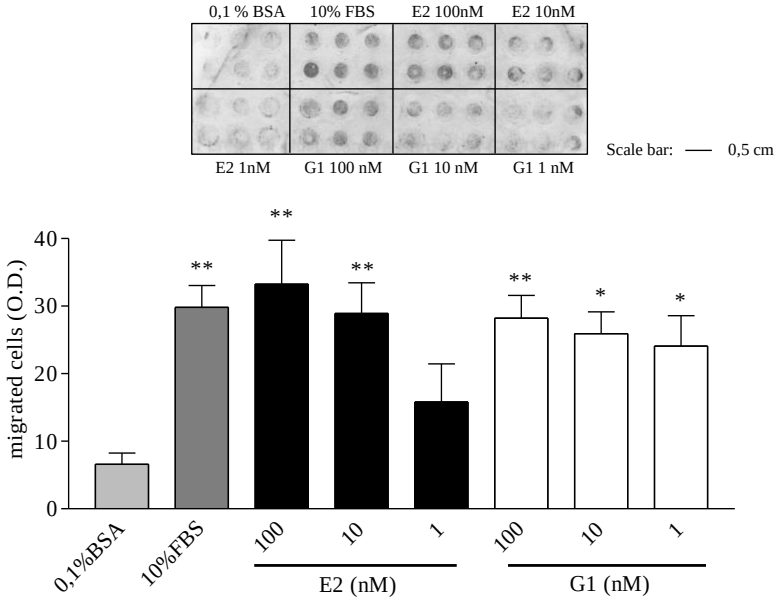

Figure 4.15 Estrogenic agents promoted SKOV3 migration. SKOV3 migration in response to increasing concentrations of E2 or selective GPER agonist G1 (1-100 nM) was measured in a micro-chemotaxis chamber after 6 h. Negative control of migration: 0.1% BSA. Positive control of migration: 10% FBS. *(Upper panel*) Representative images of migrated cells on the bottom of a filter membrane as detailed in Materials and Methods; scale bar: 0.5 cm. (*Lower panel*). Data are expressed as mean ± SEM of 5 independent experiments performed in sextuplicate. One-way ANOVA, Dunnett's *post hoc* test: * $p < 0.05$; ** $p < 0.01$ *vs.* control.

4.3.4 A luciferase assay validated the direct binding of miRNA-26b and miRNA-206 to the 3'-UTR of PFKFB3 in SKOV3 cells

MiRNA-206 and miRNA-26b have been shown to inhibit the proliferation and migration of tumor cells by decreasing PFKFB3 levels (Du et al., 2015; Ge et al., 2015). Moreover, E2 reduced the expression of miRNA-206 and miRNA-26b in the MCF-7 breast cancer cell line, thus promoting cell proliferation (Ge et al., 2015; Tan et al., 2014).

In order to assess the role of miRNAs in the regulation of PFKFB3, we first worked at the in-house generation of the vector containing the 3'-UTR sequence of human PFKFB3 linked with luciferase in the pmirGLO Dual-Luciferase miRNA Target expression Vector (pmirGLO-3'UTR). After co-transfection of SKOV3 with pmirGLO-3'UTR or pmirGLO and miR-26b or miR-206 mimics, we found that miR-206 and miRNA 26b significantly downregulated the luciferase activity with respect to miRNA-negative control (NC, $p<0.05$; Fig. 4.16). It is important to underline that miRNA-NC did not affect the luciferase activity of SKOV3 transfected with pmirGLO-3'-UTR. This supports the specificity of the effect of miRNA 26b and 206 in significantly down-regulating luciferase activity ($p<0.05$).

Using the luciferase assay we validated the direct binding of miRNA 206 and 26b in the 3'UTR of PFKFB3. The vector generated will also be used for further studies of the regulation of endogenous miRNAs by estrogenic agents as well as for identifying other miRNAs targeting PFKFB3.

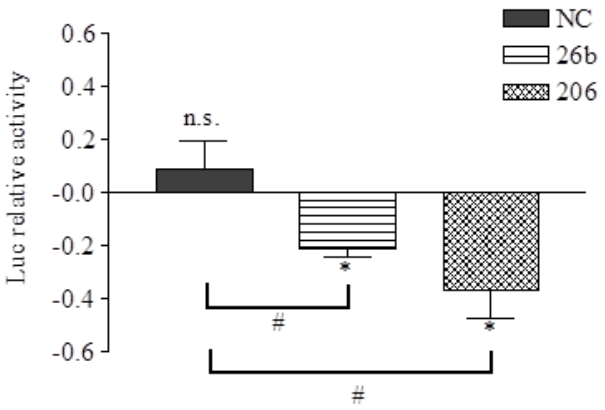

Figure 4.16 MiRNA-26b and miRNA-206 negatively regulated PFKFB3 gene expression in SKOV3 cells. SKOV3 ($5x10^4$ cells/well) were seeded in 24-well plates and, after 24h, co-transfected with a luciferase reporter vector encoding the 3'-UTR of human PFKFB3 along with miRNA-26b, miRNA-206 or miRNA-NC mimics. After 48 h transfection, luciferase activity was measured. Data are shown as positive values for up-regulation and negative values for down-regulation. The experiments were run in quadruplicate. Data are expressed as mean ± SEM of 3 independent experiment. One-way ANOVA, Dunnett's *post hoc* test: # $p < 0.05$. *t*-test was performed to compare the effect of each miRNA on the normalized luciferase activity of pmirGLO-3'UTR. (* $p < 0.05$; ns *vs.* pmirGLO-3'UTR).

4.3.5 MiRNA-26b and miRNA-206 mimics decreased basal and E2-induced PFKFB3 protein levels in SKOV3

Having established that miRNA 26b and 206 bind the PFKFB3 3'-UTR (Fig. 4.16), we transfected SKOV3 cells with miRNA-26b and miRNA-206 mimics to examine the effect of miRNAs on PFKFB3 protein abundance. In a preliminary set of experiments, we established that basal PFKFB3 protein levels were significantly downregulated in SKOV3 transfected with miRNA-26b and miRNA-206 mimics with respect to miRNA-NC (Fig. 4.17).

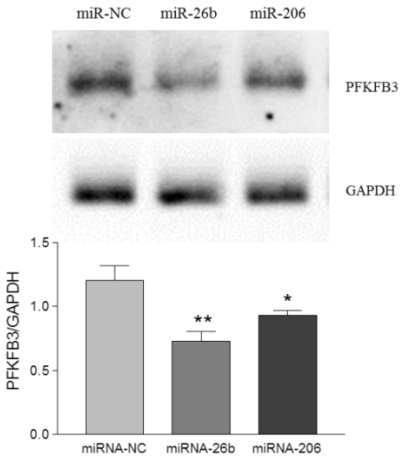

Figure 4.17 MiRNA-26b and miRNA-206 directly modulated PFKFB3 protein levels in SKOV3 cells. SKOV3 ($2x10^5$/well) were grown in 35-mm dishes in complete RPMI medium. Cells were transfected with exogenous miRNA (miRNA-26b, miRNA-206 or miRNA-NC) for 48 h. (*Upper panel*) Representative blot showing the expression of PFKFB3; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GAPDH levels, expressed as percentage of control. Data are expressed as mean ± S.E.M. of 3 independent experiments. One-way ANOVA, Dunnett's *post hoc* test: *$P < 0.05$ and **$P < 0.01$ *vs.* control.

Similar results were obtained when treating cells with E2 (10 nM) in the presence of miRNA-26b, miRNA-206 for 48 h. As reported in Fig 4.18, miRNA-26 and miRNA-206 mimics decreased basal and estrogen-stimulated increase in PFKFB3 amount. As expected, under these conditions, E2 treatment was not able to counteract the effect of exogenous miRNAs.

Whether E2 and the selective GPER agonist G1 negatively regulate endogenous miRNA206 or 26b levels and the potential functional consequences remain to be determined.

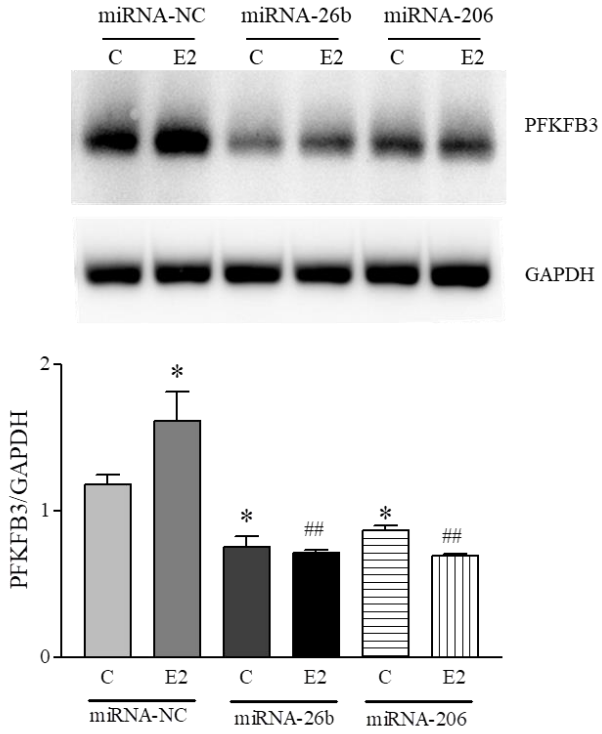

Figure 4.18 MiRNA-26b and miRNA-206 mimics abrogated E2-induced increase in PFKFB3 amount. SKOV3 ($2x10^5$/well) were grown in 35-mm dishes in RPMI 1640 phenol red–free medium with 5% FBS. Cells were pre-treated with 10 nM E2 for 30 min and then transfected with exogenous miRNAs (miRNA-26b, miRNA-206 or miRNA-NC) for 48 h. (*Upper panel*) Representative blot showing the expression of PFKFB3; GAPDH was used as a loading control. (*Lower panel*) Densitometric analysis of bands, normalized to GAPDH levels. Data are expressed as mean ± S.E.M. of 3 independent experiments. One-way ANOVA, Dunnett's *post hoc* test: *P < 0.05 *vs.* C-(miRNA-NC); ##P < 0.01 *vs.* E2-(miRNA-NC).

5. DISCUSSION

It is widely recognized that estrogens play a fundamental role in the control of energy homeostasis and glucose metabolism in health and disease (Mauvais-Jarvis et al., 2013); however, little is known about female hormone control on endothelial glycolytic metabolism. Similarly to cancer cells, ECs rely on glycolysis for ATP generation since high glycolytic flux can provide more ATP in a shorter time with respect to oxidative phosphorylation (Eelen et al., 2015), thus allowing a rapid functional adaptation to environmental demand not only in hypoxic but also in normoxic conditions. We previously established that E2 promotes angiogenesis via GPER-mediated rapid signaling and up-regulation of the key glycolytic protein PFKFB3 as a downstream effector (Trenti et al., 2017).

In this thesis work, we first showed that treatment with the GPER agonist G1 increased PFKFB3 protein levels in a time-dependent manner without affecting mRNA levels. The expression of PFKFB3 is tightly controlled at genomic and non-genomic levels to trigger glycolysis in a time- and spatial-specific way. Indeed, PFKFB3 mRNA and protein are unstable (Chesney et al., 1999; Riera et al, 2003), and PFKFB3 protein activity is controlled by post-translational mechanisms (Bando et al., 2005). Hence we postulated that estrogens would rapidly increase PFKFB3 levels by affecting protein stability. To pursue this hypothesis, cells were treated with cycloheximide (CHX) at concentrations inhibiting ribosome-dependent *de novo* protein synthesis (Horwitz and McGuire, 1980). We showed that the regulation of PFKFB3 by the natural estrogen 17β-estradiol (E2) and G1 occurred at the level of protein stability rather than at transcriptional level, given that both agents abolished PFKFB3 degradation unmasked by CHX treatment. Although E2 regulates PFKFB3 transcription in a breast cancer cell line (Imbert-Fernandez et al., 2014), the underlying mechanisms may not be the same in healthy and cancer cells, and/or vary depending on the stimulus. Accordingly, PFKFB3 expression levels differ considerably among cell types, being generally higher in cancer cells (Atsumi et al., 2002). On the other hand, although *Pfkfb3* mRNA is present in neurons, PFKFB3 protein is absent, suggesting that the enzyme is downregulated post-transcriptionally in these cells. Unlike other isoenzymes, PFKFB3 contains a region that is recognized by the E3 ubiquitin ligase complex APC/C-Cdh1 (Anaphase-Promoting Complex/Cyclosome), which is expressed in neurons and plays a major role in the rapid degradation of PFKFB3 (Herrero-Mendez, et al., 2009). Accordingly, silencing of this ligase activates proliferation and glycolysis in neoplastic and non-neoplastic cells (Almeida et al., 2010). More recently, it has been shown that the combined action of two ubiquitin ligase complexes, APC/C-Cdh1 and SCF-β-TrCP (SKP1-CUL1-F-box-protein complex-β-transducin repeat-containing protein), sequentially regulates PFKFB3 appearance during the cell cycle in the cancer-derived HeLa cell line (Tudzarova et al., 2011). In line with this evidence, we found increased PFKFB3 levels in HUVECs treated with the proteasome inhibitor MG132 or the SCF ubiquitin ligase complex inhibitor SMER3 (Aghajanyy et al., 2010).

These findings prompted us to investigate whether ER ligands induced a rapid increase in PFKFB3 by targeting its ubiquitination. Of note, we show that E2 as well as G1 treatment increased PFKFB3 amount by reducing its polyubiquitynation. To the best of our knowledge, this is the first evidence for a non-genomic effect of E2 and even more so of the selective GPER agonist on the stability of endothelial glycolytic proteins. Few studies

focused on rapid effects of estrogen on post-transcriptional events regulating protein amount. For example, E2 rapidly regulates ERα expression via proteasome-mediated proteolysis in estrogen target tissues including uterine artery endothelial cells (Tschugguel et al., 2003). More recently, it has also been shown that estrogen promotes K-Ras stabilization by inhibiting its polyubiquitination in ovarian cancer cells (Koo et al., 2015). Finally E2 and G1 rapidly stabilize HIF-1α protein (with peak effect at 15 min) without affecting its mRNA levels (Zhang et al, 2017). However, a role for GPER agonists in protein ubiquitination/ proteasomal degradation has not been disclosed previously.

Overall, adjustment of protein abundance due to changes in the degradation rate affords rapid adaptation to environmental demand/changes; thus, key players in essential cellular pathways are often subjected to rapid ubiquitin-proteasome degradation (Stangl and Stangl, 2010). This process is finely tuned, and intracellular levels of ubiquitinated proteins at any given time depend on the balance between ubiquitination and deubiquitination reactions in the ubiquitin-proteasome system. Interestingly, estrogenic agents target both specific E3 ubiquitin ligases and ubiquitin specific proteases (USPs) with opposite effects in different 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). Conversely, E2 increases USP19 protein levels leading to reduced degradation of specific proteins involved in the regulation of myogenesis (Ogawa et al., 2011). Overall, very little is known about the complex interplay between estrogen, estrogen receptors and proteasome regulation, although estrogenic agents might have a relevant role in this process with consequences in health and disease (Xu et al., 2018).

Here we show for the first time that treatment with E2 and G1 increased USP19 levels in a time-dependent manner. The effect was already detected after 1 hour treatment, consistent with the rapid increase in PFKFB3 protein levels and the involvement of a membrane receptor. Interestingly, GPER silencing abolished the E2-mediated increase in USP19 levels, further supporting the role of this membrane receptor in the rapid post-transcriptional regulation of endothelial PFKFB3.

To further explore the effects of estrogenic agents on the glycolytic program in endothelial cells, we also focused on the glucose transporter GLUT1 for the following reasons: 1) diffusion of glucose across the plasma membrane is the first rate-limiting step for subsequent glucose metabolism and energy production; 2) altered GLUT1 expression has been correlated with several disease states (Seidner et al., 1998; Winkler et al., 2015); 3) vascular GLUT1 levels have the potential to regulate whole-organ glucose metabolism and functions in heart and brain (Huang et al., 2012; Tang et al., 2017). In the present study we found that treatment with E2 or G1 significantly enhanced GLUT1 levels at early time points and for up to 24 h, suggesting the involvement of transcriptional and/or post-transcriptional mechanisms. In contrast to PFKFB3, GLUT1 levels were unchanged following CHX treatment in the time frame analyzed. In line with this, the proteasome inhibitor MG132 also failed to affect GLUT1 protein levels. Notably, previously published data show that GLUT1 has a longer half-life with respect to that of PFKFB3 (Heilig et al., 2003; Riera et al., 2003). We were unable to

confirm this finding since CHX treatments longer than 6 h in our hands reduced HUVEC viability as assessed by MTT assay.

It appears that GLUT1 levels are regulated by transcriptional and post-transcriptional mechanisms depending on the context (Imbert-Fernandez et al., 2014; Qi and Pekala, 1999). In particular, there is evidence that E2 increases GLUT1 mRNA (Shi and Simpkins, 1997) or protein levels (Cheng et al., 2001). Therefore, we analyzed the time course of GLUT1 expression in ECs. Our data show that treatment with neither E2 nor G1 affected GLUT-1 mRNA levels at any time points. Hence, further studies are needed to test alternative mechanisms by which estrogens regulate endothelial GLUT-1. For example, there is evidence that GLUT1 amount can be regulated by post-transcriptional modifications through lysosomal degradation (Ohno et al., 2011) or sumoylation (Giorgino et al., 2001). Moreover, based on the evidence that E2 downregulates the expression of miRNA 26b and 206 resulting in increased PFKFB3 abundance (Du et al., 2015; Ge et al., 2015; Tan et al., 2014), it is possible to hypothesize that estrogenic agents posttranscriptionally regulate further glycolytic proteins including GLUT1 via shared miRNAs in endothelial cells.

Overall, in the first part of this thesis work we showed that the membrane receptor GPER mediated the post-transcriptional regulation of endothelial GLUT1 and PFKFB3 abundance. We add knowledge to the mechanisms coupling hormonal signals with metabolic demand in healthy cells that rely on glycolysis to exert their functions, showing that estrogenic agents enhanced the amount of a) GLUT1 protein but not mRNA and b) PFKFB3 protein by increasing deubiquitinase USP19 levels, thereby reducing its ubiquitination and further degradation.

Post-transcriptional protein regulation includes several mechanisms affecting mRNA and protein turnover, which are not mutually exclusive. Among the post-transcriptional mechanisms involved in the regulation of protein levels, miRNAs mediate translational repression through direct binding to the 3'-UTR of target mRNA. Since miRNAs are involved in glycolytic protein regulation (Subramanian et al., 2019) and estrogen signaling (Vrtacnik et al., 2014), we tested the role of miRNAs in PFKFB3 estrogenic regulation. There is strong evidence, at least in estrogen-dependent cancer cells, for a role of miRNA-206 and miRNA-26b in the down-regulation of PFKFB3 protein levels, which impacts on cell growth and migration (Du et al., 2015; Ge et al., 2015). Moreover, E2 reduces the expression of miRNA-26 and miRNA-206 in MCF-7 breast cancer cells (Ge et al., 2015; Tan et al., 2014). Hence, we postulated that estrogens would regulate PFKFB3 protein levels by affecting protein translation via miRNA.

In order to explore the role of specific miRNAs, i.e. miRNA-26b and miRNA-206, in PFKFB3 turnover and the interplay with estrogenic agents in HUVECs, we set up a reporter gene assay. We initially attempted to transfect HUVECs, primary cells notoriously difficult to transfect and susceptible to toxicity of transfection reagents. So far, we have not managed to efficiently transfect HUVECs and we are currently testing different methods to optimize transfection. Therefore, we decided to use SKOV3, a highly invasive ovarian cancer cell

line expressing all estrogen receptor subtypes. Similarly to ECs, cancer cells have high glycolytic reliance; in particular, tumors metabolize about 10-fold more glucose than normal tissues regardless of the cell type or organ. For instance, PFKFB3 and GLUT1 have been found to be overexpressed in several human cancer tissues including ovarian cancer (Atsumi et al., 2002; Cantuaria et al., 2001). In addition, exposure of ER-positive breast cancer cells to E2 increases PFKFB3 protein and mRNA levels via ERα, thereby sustaining tumor growth (Imbert-Fernandez et al., 2014).

The functional role of ER subtypes and specifically GPER in ovarian cancer cell is only partially understood (Hua et al., 1995; Chan et al., 2014; Yan et al., 2015). Although several studies identified a role of GPER in the progression of estrogen-related cancers, reports regarding ovarian cancer have provided conflicting findings. In particular, high concentrations (> 1 μM) of G1 have been shown to exert an inhibitory action on the proliferation of different ovarian cancer cell lines (Ignatov et al., 2013; Wang et al., 2013). Conversely, G1 (100 nM) as well as E2 induce the proliferation of two different ovarian cancer cell lines through the cooperation between ER and GPER (Albanito et al., 2007), and GPER mediates the effects of estrogenic agents on cell proliferation in ERα-negative ovarian cancer cells (Liu et al., 2014). Another study showed that GPER regulates in SKOV3 cell functions (proliferation, migration and invasion) in a ligand-independent manner (Yan et al., 2015).

Based on this background, in order to use an estrogen-responsive line to explore the effect of miRNAs on PFKFB3 levels, we first tested the effect of estrogenic agents on SKOV3 functional responses, namely cell proliferation and migration. Under our experimental conditions (low-serum medium for 72 h) we were unable to detect a proliferative effect of E2 or G1. In addition, we found that SKOV3 proliferation was not inhibited by the selective ERα and GPER antagonists MPP or G15, respectively. This is in contrast to what previously demonstrated by other authors showing that antiestrogens alone were able to reduce cancer cell growth (Vignon et al., 1987). This discrepancy could be explained by the different cell culture conditions used or, alternatively, specifically ascribed to SKOV3 cells displaying growth resistance to estrogenic agents, in spite of the expression of functional estrogen receptors (Hua et al., 1995). Analyzing the effect of estrogenic agents on SKOV3 function, we found that treatment with G1 as well as E2 induced SKOV3 migration in a concentration-dependent manner. Of note, Yan and coworkers (2015) reported that GPER regulates SKOV3 cell functions including migration in a ligand-independent manner, whereas both G1 and E2 promote OVCAR5 cell migration (Yan et al., 2015). On the whole, these results suggest that 1) SKOV3 cells are responsive to estrogenic agents, and 2) estrogen-mediated SKOV3 migration involves the membrane receptor GPER.

It is known that estrogen play a crucial role in the development of ovarian cancer; however, the signal transduction pathways involved in the hormone action are poorly defined (Albanito et al., 2007). In particular, the effects of estrogens on glycolytic proteins in ovarian cancer cell lines have not yet been investigated. Hence, we asked whether estrogenic agents would regulate SKOV3 proliferation and migration via PFKFB3. We found that E2 treatment increased PFKFB3 protein levels already at early time points (3-6 h) and for up to 24

h. PFKFB3 increase occurred without changes in mRNA levels, in line with what observed in HUVECs. Surprisingly, the E2-dependent increase in PFKFB3 protein levels did not correlate with growth response. In line with our observation, however, Hua showed that in SKOV3 E2 treatment increased c-*myc* and c-*fos* mRNA levels in the absence of a proliferative response (1995). It is interesting to note that in several ovarian cancer cancer cell lines estrogen-induced cancer cell growth has been associated with increased c-*fos* expression (Liu et al., 2014), suggesting that the functional activation of estrogen receptors depends on the specific cellular context. It is possible to hypothesize that estrogenic agents promoted SKOV3 migration by increasing PFKFB3 levels and fostering glycolysis. Future experiments using the selective PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO) will test this hypothesis.

Having demonstrated that E2 treatment increased PFKFB3 protein levels via a non-genomic mechanism, we assessed the involvement of specific miRNAs likely to target PFKFB3 3'-UTR. We succeeded in transfecting SKOV3 cells with the vector containing human PFKFB3 3'-UTR with high transfection efficiency. Using the reporter gene assay, we found that both miRNA-26b and miRNA-206 reduced luciferase activity with respect to miRNA negative controls, suggesting that these miRNAs negatively regulate PFKFB3 expression. In the attempt to link PFKFB3 protein expression to miRNA translational inhibition, we tested the effect of exogenous miRNAs on PFKFB3 protein levels. Transfection of SKOV3 cells with miRNA-26b and miRNA-206 significantly reduced PFKFB3 protein expression, compared to miRNA-negative control. We also found that the effect of miRNA-26b and miRNA-206 was not reverted by E2 pretreatment. This is in line with findings by Vivacqua et al., who showed that in breast cancer cells transfected with a miRNA-338-3p mimic treatment with E2 or G1 was no longer able to induce c-fos mRNA and protein levels (Vivacqua et al., 2018). Conversely, other authors showed that miR-206 directly interacts with the 3′-UTR of PFKFB3 mRNA in breast cancer cells lines, and that E2 treatment was partially able to revert this miRNA's effect on PFKFB3 levels (Ge et al., 2015). Overall, we speculate that estrogenic agents are able to inhibit the functional role of endogenous miRNAs but are unable to counteract the effect of long term exposure to exogenous miRNAs.

Notably both miRNA-206 and miRNA-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 may express lower levels of specific miRNAs. In addition, miR-206 expression is reduced in ERα-positive with respect to ERα-negative ovarian cancer cell lines (Li et al., 2014), further suggesting a role for estrogenic agents in regulating endogenous miRNA expression.
In order to establish whether estrogenic agents up-regulate PFKFB3 by inhibiting miRNA functional activity, it would be important to assess the effect of estrogens on endogenous miRNAs by performing q-PCR or luciferase reporter assays in estrogen-treated cells at different time points. We expect that the increased PFKFB3 amount following estrogenic treatment would be at least in part due to decreased expression of endogenous miRNAs. It is also conceivable that E2 reduces miRNA activity by interfering with the process of miRNA maturation (Klinge, 2015).

Overall, we showed that estrogenic agents affect tumor cell function by increasing the migration but not proliferation of SKOV3 cells. Additionally, estrogens control PFKFB3 protein levels in SKOV3 via post-transcriptional mechanisms, likely involving miRNA regulation, in line with the observation that miRNA-206 and miRNA-26b down-regulate PFKFB3 translation in SKOV3 cells.

IMPLICATIONS

This research adds knowledge to the non-genomic mechanism of action of estrogen, where the recently discovered membrane receptor GPER plays a prominent role. We believe that slowing down glycolytic protein turnover via GPER signaling allows estrogens to rapidly and finely tune the function of several cell types including cancer, endothelial and immune cells that rely on glycolysis to rapidly adapt to environmental cues.These results might have implications in estrogen's protective and prophylactic effects in vascular ischemic disorders where rapid metabolic and functional adaptation to environmental changes is required. In addition, the identification of E2-targeted miRNAs in cancer cells, which in turn regulate glycolytic protein levels and thus likely cell growth and invasiveness, will pave the way to the development of miRNA-based treatments for blocking adverse hormone functions such as pathological angiogenesis in E2-dependent cancers.

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