The glycolytic enzyme PFKFB3 is involved in estrogen-mediated angiogenesis via GPER1[1]

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JPET #238212

Running title: Estrogen activates endothelial PFKFB3 via GPER1

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Number of text pages: 25

Number of tables: 0

Number of figures: 8

Number of references: 59

Number of words in the Abstract: 234

Number of words in the Introduction: 736

Number of words in the Discussion: 1225

List of nonstandard abbreviations

E2, 17β-estradiol; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase 3; ER, estrogen receptor;

GPER1, G protein-coupled estrogen receptor; HUVEC, human umbilical vein endothelial cells; 3PO, 3-(3-

pyridinyl)-1-(4-pyridinyl)-2-propen-1-one.

Section assignment: Cardiovascular

Abstract

The endogenous estrogen 17β-estradiol (E2) is a key factor in promoting endothelial healing and angiogenesis. Recently, proangiogenic signals including vascular endothelial growth factor and others have been shown to converge onto endothelial cell metabolism. Because inhibition of the glycolytic enzyme activator phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) reduces pathological angiogenesis and estrogen receptor (ER) signaling stimulates glucose uptake and glycolysis by inducing PFKFB3 in breast cancer, we hypothesized that E2 triggers angiogenesis in endothelial cells via rapid ER signaling that requires PFKFB3 as a downstream effector. We report that treatment with the selective G protein-coupled estrogen receptor (GPER1) agonist G-1 (10^{-10}-10^{-7} M) mimicked the chemotactic and proangiogenic effect of E2 as measured in a number of short-term angiogenesis assays in human umbilical vein endothelial cells (HUVECs). In addition, E2 treatment upregulated PFKFB3 expression in a time- and concentration-dependent manner. Such an effect peaked at 3 h, was also induced by G-1 and abolished by pretreatment with the GPER1 antagonist G-15 or GPER1 siRNA, consistent with engagement of membrane ER. Experiments with the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) showed that PFKFB3 activity was required for estrogen-mediated HUVEC migration via GPER1. In conclusion, E2-induced angiogenesis was mediated at least in part by the membrane GPER1 and required upregulation of the glycolytic activator PFKFB3 in HUVECs. These findings unravel a previously unrecognized mechanism of estrogen-dependent endocrine-metabolic crosstalk in HUVECs and may have implications in angiogenesis occurring in ischemic or hypoxic tissues.

Introduction

The effects of female hormones, in particular the most abundant endogenous estrogen 17β-estradiol (E2), go beyond the well-established targets in reproductive organs and placenta. Estrogen exerts a protective role in the cardiovascular system by interacting with multiple cell types in the vessel wall including immune cells (Bolego et al., 2013; Abu-Taha et al., 2009) as well as resident smooth muscle (Maggi et al., 2003; Geraldes et al., 2002) and endothelial cells, the latter representing a major target of estrogen action (Morales et al., 1995; Holm et al., 1999; Rubanyi et al., 2002). In particular, E2 contributes to endothelial-mediated vasodilation by enhancing nitric oxide production (Bolego et al., 2010) and promotes endothelial repair in case of arterial injury (such as occurring in angioplasty procedures), also fostering endothelial progenitor cell mobilization (Toutain et al, 2009; Fadini et al., 2008). Thus, available evidence points to E2 as a key factor in promoting endothelial healing, as well as angiogenesis (Arnal et al., 2010; Iwakura et al., 2006).

In adult organisms, angiogenesis is virtually absent under normal conditions, except in the female reproductive tract, supporting a relevant role for sex steroids in the context of neovascularization processes (Losordo and Isner, 2001; Reynolds et al., 1992). This also applies to the setting of cardio- and cerebrovascular diseases, where a protective role of E2 in ameliorating ischemic damage has been demonstrated in models of ischemia-reperfusion injury (Fadini et al., 2008; Favre et al., 2010; Murata et al., 2013). Conversely, pathological estrogen-dependent capillary growth is associated with conditions such as hemoangiomas (Conter and Longmire, 1988), endometriosis (Laschke and Menger, 2012) and breast cancer growth, which mainly correlates with nuclear estrogen receptor (ER) expression (Haran et al., 1994; Horak et al., 1992).

Estrogen effects are mediated by ERα and ERβ, classical nuclear hormone receptors acting as transcription factors. Recently, a membrane-located ER belonging to the G protein-coupled receptor superfamily dubbed GPER1 has been discovered. This receptor is expressed in endothelial cells, and mediates non-genomic rapid effects including calcium influx or kinase activation that are involved, among others, in the regulation of vascular tone (Prossnitz and Arterburn, 2015). Of note, long-term activation of GPER1 by selective pharmacological agents does not appear to induce uterotrophic effects (Meyer et al., 2014). ERα has been shown to mediate most beneficial cardiovascular effects of E2 (Bolego et al., 2010; Favre et al., 2010;

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Billon-Galés et al., 2009; Pare et al., 2002), but is also involved in pathological cell proliferation (Harris et al., 2002). The proangiogenic effects of E2 have been mainly ascribed to ERα activation (Sanchez et al., 2011); accordingly, angiogenesis is impaired in ERα knock-out mice (Johns et al., 1996). The possible contribution of the membrane GPER1 to such responses, however, remains to be determined.

The mechanisms downstream ER activation involved in E2-mediated angiogenesis include increased production of proangiogenic factors, changes in the expression of adhesion molecules as well as direct effects on EC proliferation and migration (Geraldes et al., 2002; Morales et al., 1995; Arnal et al., 2010; Losordo and Isner, 2001). An emerging regulatory mechanism is suggested by the observation that angiogenic signaling pathways converge onto metabolism (Treps et al., 2016). Accordingly, it has been recently reported that pathological angiogenesis can be inhibited by blocking the glycolytic enzyme activator phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3; Schoors et al., 2014), The PFKFB3 product fructose-2,6-bisphosphate is the allosteric activator of the key glycolytic enzyme phosphofructokinase 1 (PFK-1), and PFKFB3 represents the most abundant PFKFB isoenzyme in ECs (De Bock et al., 2013), which largely rely on glycolysis for ATP generation (Culic et al., 1997). Because PFKFB3 plays a role in nuclear signaling and its product increases the expression and activity of cyclin-dependent kinase-1 (Yancin et al, 2009), PFKFB3 represents a novel pharmacological strategy for targeting angiogenesis and tumor cell growth/migration.

While E2 effects on several aspects of cell metabolism are well characterized (Mauvais-Jarvis et al., 2013; Della Torre et al., 2016), one report found that ER signaling stimulates glucose uptake and glycolysis by inducing PFKFB3 expression and activity in a breast cancer cell line (Imbert-Fernandez et al., 2014). Whether PFKFB3 is a molecular target of E2 in non-tumor cell types such as endothelial cells, however, is as yet unknown. In order to further explore the mechanisms underlying the pro-angiogenic effect of estrogen, we set out to assess whether a) the glycolytic activator PFKFB3 plays a role in E2-mediated angiogenesis, and b) GPER1 is involved in this process.

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Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from normal-term umbilical cords as previously published (Bolego et al., 2006). Briefly, cells were grown in medium M199 (Invitrogen, S. Giuliano Milanese, Italy) supplemented with 15% FCS (Invitrogen), gentamicin (40 μg/ml, Invitrogen), endothelial cell growth factor (ECGF 100 μg/ml), and heparin (100 UI/ml Sigma-Aldrich, Saint Louis, Missouri, USA), at 37°C in a humidified 5% CO_2 atmosphere. HUVECs were identified by their morphology and detection of CD31-related antigen, and used for experiments from passages 2 through 6. For western blotting experiments, cells were switched to a 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).

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 (G-1, Tocris Bioscience, Bristol, UK), (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), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO, Merck Millipore, Darmstadt, Germany). Inhibitors were added 30 min before the stimulus.

MTT Assay. HUVECs (2 x 10^4 cells/well) were seeded in 96-well culture plates and incubated in complete culture medium. The next day, the medium was replaced with a fresh one containing the test agents and cells were incubated for 24 h. Four hours before the incubation end 10 μL stock solution of MTT (5 mg/ml in PBS) was added to each well. At the end the incubation medium was removed and formazan crystals were dissolved in 100 μL DMSO. MTT reduction was quantified by measuring light absorbance with a multilabel plate counter (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.

Wound Healing Assay. HUVECs (1 x 10^5 cells) were seeded in complete culture medium on 1% gelatin-coated 24-well culture plates and allowed to reach confluence. One scratch was made and the medium was replaced with fresh complete medium containing test compounds. Three images for each sample were captured at 40X under a phase contrast inverted microscope (Nikon Eclipse Ti) equipped with a digital

camera at different time points: immediately after the scratch was made (time 0) and after 6 h (end of incubation time). The wound area of each image was measured using the ImageJ 1,47v software (NIH, USA) and the average wound area of 3 images was determined for each sample. Quantitative analysis of cell migration was performed as percentage of area change using the following formula: % change= (average wound area at time 0 h) - (average wound area at time 6 h)/ (average wound area at time 0 h) $\times$ 100. Values are expressed as percent change from control cells.

Chemotaxis Assay. Chemotaxis experiments were performed in a 48-well modified Boyden chamber (Neuro Probe) using 8-µm Nucleopore polyvinylpyrrolidine-free polycarbonate filters coated with 10 µg/ml collagen. Lower chambers were filled with phenol-free M199 supplemented with 1% FBS and 100 U/ml heparin in the presence or absence of 10 ng/ml VEGF or G1 (10^{-9}-10^{-7} M), whereas upper chambers were filled with 50 µL cell suspension (1.6×10^5 cells in phenol-free M199 supplemented with 1% FBS and 100 U/ml heparin). After 6 h incubation at 37°C, non-migrating cells on the upper filter surface were removed by scraping. The cells that had migrated to the lower side of the filter were stained with Diff-Quick stain (VWR Scientific Products, Bridgeport, NJ, USA), and densitometric analysis was performed using the Image J 1,47v software (NIH, USA). Each experiment was performed in sextuplicate and results are shown as mean values of 5 independent assays.

Capillary–Like Tube Formation Assay. HUVECs (2×10^4 cells/well) were plated onto a thin layer (140 µl) of basement membrane matrix (Matrigel[TM], Becton Dickinson, Waltham, MA, USA) in 48-well plates, and incubated at 37°C for 4 h in cell culture medium in the presence or absence of test compounds as indicated in the Results section. Two images per well were captured at 40X under a phase contrast inverted microscope (Nikon Eclipse Ti) equipped with a digital camera. Images were analyzed using Angiogenesis Analyzer, a plugin developed for the ImageJ software (Carpentier et al., 2012). Data on topological parameters (number of junctions, master segment meshes and total mesh area) of the capillary-like network were analyzed in each well. Junctions are measured as pixels with 3 neighbors and master segments define segments delimited by two junctions (Guidolin et al., 2004). Data are expressed as either absolute values or % of control.

Western Blot Analysis. HUVECs (3×10^5 / 35-mm dish) were seeded in complete culture medium in 1% gelatin-coated dishes. Upon confluence the medium was replaced. Cells were treated as indicated in the

Results section and lysed with 100 μL lysis buffer (PBS supplemented with 1.2 % Triton X-100, Roche cOmplete™ inhibitor cocktail 1X, NaF 2.5 mM, $Na_4P_2O_7$ 2 mM, Na orthovanadate 4 mM, PMSF 1 mM). After centrifugation at 10000 x g for 15 min, supernatants were collected for SDS-PAGE and western blotting. Protein quantification was performed by the BCA assay (Sigma). Proteins (40-60 μg) were separated on SDS-PAGE and transferred onto PVDF membranes (Hybond-P, Amersham). Membranes were then blocked and probed using the following rabbit primary antibodies: anti-PFKFB3 (Proteintech, Manchester, UK); anti-GPER1 (Abcam, Cambridge, UK); anti-GAPDH (Santa Cruz, Dallas, Texas; USA). After washing, membranes were incubated with appropriate secondary HRP-conjugated antibodies (Vector Laboratories, Burlingame, California; USA) at 1:2500 dilution. Bands were detected by chemiluminescence using the LiteAblot Turbo (Euroclone, Pero, Italy). Images were acquired with VersaDoc™ 4000 Imaging System (Bio Rad, Hercules, CA, USA). Densitometric analysis of bands was performed with Image J 1,47v (NIH, USA). Results are expressed as percent of controls.

RNA Interference. HUVECs were seeded at a density of 2×10^5 cells per well into 35-mm dishes in complete medium. After 24 h, they were transfected with siLentFect™ (Bio-Rad, Hercules, CA, USA) and 20 nM siRNA (ON-TARGETplus Human GPER11 (2852) siRNA – SMARTpool, Dharmacon, Lafayette, CO, USA) per well in complete culture medium with 15% FBS. A scrambled siRNA without sequence homology to any known human gene served as negative control. After 72 h, fresh complete medium with 15% FBS was added, and pharmacological treatments were started as indicated for migration, Matrigel and immunoblotting assays. GPER1 protein levels were measured after 72 h transfection in order to assess knockdown efficiency.

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 GraphPad Prism 5.02 (GraphPad Software Inc., La Jolla, CA, USA). Student's t-test was used to compare the means of two independent groups to each other, whereas one-way analysis of variance followed by Dunnett's or Bonferroni's *post-hoc* tests was used for multiple comparisons. A P value of <0.05 was considered statistically significant.

Results

The Proangiogenic Effect of Estrogen Involves the Membrane GPER1. Although the pro-angiogenic effect of E2 has already been described (Morales et al., 1995), the role of the membrane receptor GPER1 in mediating this effect has not been assessed. For this purpose, we used a variety of *in vitro* experimental approaches mimicking the major steps of the angiogenic process (Rubanyi et al., 2002; Simons et al., 2015). We first performed a chemotaxis assay to assess HUVEC migration in response to the selective GPER1 agonist G-1 at early time points (4 h). As shown in Fig. 1A, the number of migrating cells significant increased in response to increasing concentrations of G-1 (10^{-10}-10^{-7} M). At the highest concentration, the effect of G-1 on migration was comparable to that of the positive control VEGF (10 ng/ml). Throughout the concentration range of this assay, the selective GPER1 agonist did not significantly affect HUVEC viability over the 24 h-incubation (Fig. 1B).

In order to assess the effect of G-1 on collective HUVEC migration, we used a wound healing migration assay. Similarly to equimolar E2 concentration, G1 (10^{-7} M) promoted wound closure (Figs. 2A and 2B). Pre-treatment of E2-stimulated cells with the selective GPER1 antagonist G-15 (10^{-6} M) appeared to counteract this effect, even though in a statistically non-significant manner.

Finally, we analyzed the effect of G-1 on tubularization, the process of organization of HUVECs into capillary tube-like structures when cultured onto extracellular matrix proteins (Fig. 3A). As shown in Fig. 3B, treatment with 10^{-7} M G-1 increased specific parameters of capillary-like tube formation such as junction number, mesh area, mesh number and master segment number of HUVECs seeded onto Matrigel™ and incubated in phenol red-free medium containing 5% FCS and ECGF (100 µg/ml). The effect of the selective GPER1 agonist was similar to that induced by E2 and was antagonized by G-15 (10^{-6} M), further suggesting that the proangiogenic effect of estrogens was mediated, at least in part, by the membrane GPER1.

17β-Estradiol Increases PFKFB3 Expression in Endothelial Cells Via GPER1. In order to assess a possible contribution of the glycolytic activator PFKFB3 to HUVEC migration in response to ER agonists, we first analyzed the effect of E2 on PFKFB3 expression. E2 enhanced PFKFB3 expression in a time- (1-24 h; Fig. 4A) and concentration- (10^{-9}-10^{-7} M; Fig. 4B) dependent manner. The increased expression of PFKFB3 in response to E2 peaked at 3 h (Fig. 4A) and was also detectable at the lowest E2 concentration tested (10^{-9} M; Fig. 4B). No changes in *PFKFB3* mRNA levels were observed following E2 treatment

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(Supplemental Figure 1). The selective GPER1 antagonist G-15 abolished the E2-mediated effect on PFKFB3 expression (Fig. 5A). We also performed experiments with the ER antagonist ICI 182,780, which failed to abolish the effect of E2 on PFKFB3 protein amount, and even turned out to behave as an agonist by enhancing PFKFB3 expression (Supplemental Figure 2) in line with previous evidence (Thomas et al, 2005). Moreover, similarly to E2, treatment with the selective GPER1 agonist G-1 (10^{-9}-10^{-7} M) induced a concentration-dependent increase in PFKFB3 levels (Fig. 5B), which reached statistical significance at the highest concentration tested, further supporting a role for GPER1 in ER signaling in ECs.

To provide additional evidence for the involvement of GPER1 in E_2-mediated angiogenesis we performed additional experiments using a GPER1-specific siRNA. Treatment of HUVECs with GPER1 siRNA for 72 h led to a significant reduction in GPER1 protein amount compared with control (Fig. 6A). Consistent with a role for GPER1 in E_2-mediated angiogenesis, no significant changes in HUVEC migration in response to E_2 were observed in GPER1 knock-out in contrast to control HUVECs (Fig. 6B). Similarly, an impaired response in capillary-like network formation was observed in HUVECs treated with GPER1 siRNA with respect to scramble-treated cells (Fig. 6C). Finally, we demonstrated that E_2 treatment failed to induce a significant increase in PFKFB3 level in GPER1 knock-out cells (Fig. 6D).

PFKFB3 is Required for Estrogen-Mediated Endothelial Cell Migration Via GPER1. Given that both E2 and G-1 enhanced accumulation of PFKFB3, which is known to play a key role in the angiogenic process (Schoors et al., 2014), we tested the effect of E2 in HUVECs pretreated with the PFKFB3 inhibitor, 3PO (40 μM). As shown in Fig. 7A, 3PO alone inhibited the migratory potential of ECs grown in the presence of 5% FCS and ECGF (100 μg/ml), and abolished the wound-closure effect elicited by E2. In addition, treatment with 3PO for 24 h did not affect MTT reduction (Fig. 7B), suggesting that under these experimental conditions and in this time frame inhibition of glycolysis did not influence cell viability and/or proliferation. Further, 3PO inhibited the ability of E2-challenged ECs to form capillary-like structures. Similar to what observed for E2, 3PO also abolished the pro-angiogenic potential of the selective GPER1 agonist G-1 (Fig. 8), thereby reinforcing the involvement of this membrane receptor in the formation of tube-like structures. To further support the consequences of PFKFB3 inhibition on the angiogenic process, we performed additional experiments using a selective PFKFB3 inhibitor, PFK15, at a concentration lower than that of 3PO. The data showed that treatment with 1 μM PFK15 counteracted ER ligand-mediated

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angiogenesis similar to 3PO (Supplemental Figure 3A) without affecting HUVEC viability (Supplemental

Figure 3B).

Discussion

In the present study we provide evidence for the membrane GPER1 and the glycolytic activator PFKFB3 playing a role in estrogen-mediated angiogenesis, thereby highlighting for the first time such an estrogen-dependent endocrine-metabolic crosstalk in HUVECs. These data relay estrogen with glycolysis and energy production with angiogenesis, a critical step of tumor growth/metastasis.

Angiogenesis is a tightly regulated process comprising several sequential steps, which involve mediators produced by both normal tissues under physiological conditions and in pathological settings. Female reproductive organs (ovary, uterus and placenta), well-recognized estrogen targets, exhibit periodic growth and regression, supporting a role of female hormones, in particular the most abundant endogenous estrogen E2, in physiological angiogenesis (Reynolds et al., 1992). Moreover, several studies suggest a beneficial effect of female hormones in the regeneration of ischemic tissues, where estrogen-mediated angiogenesis may improve outcomes (Fadini et al., 2008; Iwakura et al., 2006). The pro-angiogenic effect of E2 has been also established in pathological conditions characterized by a pro-inflammatory environment, including endometriosis and tumors of reproductive organs, where the proangiogenic effect is considered deleterious (Laschke and Menger, 2012; Haran et al., 1994). The opposite role of estrogen-mediated angiogenesis in cardiovascular disease as opposed to cancer is in line with the knowledge that antiangiogenic therapies are associated with deleterious cardiovascular effects (Daher and Yeh, 2008). In addition, E2 accelerates cutaneous wound healing, a process closely associated with angiogenesis (Hardman and Ashcroft, 2008), and protects against skin ischemia (Toutain et al., 2009).

The mechanisms responsible for the pro-angiogenic effect of E2 have been widely characterized including an effect on endothelial cytoskeletal architecture via ERα (Geraldes et al., 2002; Arnal et al., 2010; Sanchez et al., 2011). However, there are few and inconsistent data on the role of the membrane GPER1 (Holm et al., 2012; Prossnitz and Barton, 2014), a G-protein coupled ER expressed by a number of tissues including the vascular endothelium, where it mediates rapid hormone effects (Revankar et al., 2005). We here demonstrate that the selective GPER1 agonist G1 induced a concentration-dependent increase in HUVEC migration, which was already detectable at concentrations in the low nM range. More important, we found that G1 promoted the morphological differentiation of HUVECs into capillary-like structures, thus

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mimicking *in vitro* some of the steps involved in angiogenesis *in vivo* (Rubanyi et al., 2002). This effect was prevented by selective antagonists, further supporting a role for GPER1 in E2-mediated angiogenesis. We also show that the selective GPER1 agonist did not affect EC viability/proliferation over 24 h, consistent with the idea that previously described effects on cell migration and tubularization are independent from HUVEC proliferation. It is possible though that at later time points G1 could enhance proliferation and/or inhibit HUVEC apoptosis, in line with studies using the endogenous ligand E2 (Morales et al., 1995; Fadini et al, 2008; Spyridopoulos et al., 1997).

Overall, our data point to GPER1 as a promising target in promoting angiogenesis associated with beneficial cardiovascular effects such as those observed in the setting of ischemic damage (Fadini et al, 2008; Iwakura et al., 2006). In fact, consistent with a major role in hypoxia-mediated signaling, GPER1 regulates the hypoxic-ischemic response in both the cardiovascular and central nervous system (Murata et al., 2013; Patel et al., 2010; Lappano et al., 2016). Notably, pharmacological GPER1 activation does not appear to induce undesired effects on reproductive tissues such as the uterus (Meyer et al., 2014). In fact, no changes in uterine growth were detected in two GPER1 knockout mouse models (Otto et al., 2009; Windahl et al., 2009). Despite the lack of a general agreement on the impact of GPER1 selective agonists in non-vascular tissues (Prossnitz and Barton, 2014), several studies conversely point to a predominant role for ERα in estrogen and tamoxifen uterotrophic effects (Bolego et al., 2010; Nasu et al., 2008). Hence, pharmacological activation of GPER1 may be safer than estrogen-based therapeutic regimens in relation to the female genital tract.

Based on the knowledge that HUVECs resemble cancer cells in their preferential use of glycolysis (Verdegem et al., 2014) to generate most of their ATP for functional responses including angiogenesis, we focused on the glycolytic activator PFKFB3, a downstream effector of several proangiogenic growth factors including VEGF (Goveia et al., 2014), to further explore the mechanisms involved in E2-mediated angiogenesis via GPER1. Of note, PFKFB3 has an HIF-responsive element in its promoter (Obach et al., 2004), and its expression is upregulated under hypoxic conditions such as those associated with ischemic tissue and cancer (Tawakol et al., 2015; Cordero-Espinoza and Hagen, 2013; Xu et al., 2014). It is therefore not surprising that multiple pro-angiogenic factors rely on PFKFB3-mediated glycolysis for their effect.

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Pharmacological inhibition of PFKFB3 leads to a partial reduction of glycolysis, which in turn negatively affects cell proliferation and migration in endothelial cells. Therefore, PFKFB3 targeting represents a promising antiangiogenic strategy overcoming resistance and insufficient efficacy of classical antiangiogenic therapies (Schoors et al., 2014).

Recently, it has been demonstrated that E2 increases PFKFB3 expression in breast cancer cells and thereby sustains tumor growth (Imbert-Fernandez et al., 2014). We therefore hypothesized that the increased angiogenic response in E2-exposed HUVECs was mediated by PFKFB3. In line with this hypothesis, we demonstrated that: 1) E2 enhanced PFKFB3 expression in a time- and concentration-dependent manner; and 2) the selective PFKFB3 inhibitor 3PO abolished the wound healing closure as well as the morphological differentiation of E2-treated HUVECs to form capillary-like structures. Interestingly, the increase in PFKFB3 levels upon E2 treatment compared with control was detectable already after 1 h and peaked after 3 h, consistent with the activation of a membrane receptor. Such a rapid response was not due to *PFKFB3* transcriptional activation (Supplemental Figure 1). It has been described that PFKFB3 expression can be regulated at post-translational levels by several mechanisms including mRNA or protein stability (Chesney et al., 1999; Riera et al., 2003; Almeida et al., 2010). We are currently investigating the mechanism by which estradiol increases PFKFB3 proteins expression without affecting gene transcription. However, since a role for ERα and downstream intracellular kinases including FAK has been suggested in angiogenesis (Sanchez et al., 2011; Gopal et al., 2012), and E2 decreases miR-206 and increased PFKFB3 expression in breast cancer cells through ERα (Imbert-Fernandez et al., 2014; Ge et al., 2015), we cannot exclude a receptor cross-talk and/or functional redundancy with ERα in HUVECs (Prossnitz and Barton, 2014). Similar to E2, treatment with the selective GPER1 agonist G1 increased PFKFB3 expression in a concentration-dependent manner (Fig. 5B). Taken together, the observations that treatment with GPER1 siRNA reduced E2-mediated HUVEC migration, tube-like structure formation and PFKFB3 expression support the notion that PFKFB3 is required for estrogen-mediated EC migration through GPER1 signaling.

Several studies provide evidence for E2 effects in the control of energy balance in different cells and tissues (Mauvais-Jarvis et al., 2013; Della Torre et al., 2016). For instance, E2 is known to foster glucose uptake and utilization in several cancer cells (Sun et al., 2014). However, to the best of our knowledge, this

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is the first report linking hormonal effects to glycolysis activation in endothelial cells with a special focus on the role of PFKFB3 in this process.

In conclusion, the present study showed that GPER1 is involved in E2-mediated angiogenesis. We also add knowledge to the mechanisms involved in E2-mediated angiogenesis by demonstrating that the glycolytic activator PFKFB3 is a downstream effector of E2 action in this process. These findings may have implications in angiogenesis occurring in ischemic or hypoxic tissues.

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Acknowledgments

Authorship Contributions

Participated in research design: Bolego, Trevisi

Conducted experiments: Trenti, Tedesco, Boscaro, Ferri

Performed data analysis: Trenti, Cignarella, Bolego

Wrote or contributed to the writing of the manuscript: Cignarella, Trevisi, Bolego

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Footnotes

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Legends for Figures

Figure 1: Estrogen-mediated HUVEC migration involves the membrane receptor GPER1. **A)** HUVEC migration in response to increasing concentrations of the selective GPER1 agonist G-1 (10^{-7}-10^{-10} M) was measured in a microchemotaxis chamber after 6 h. *Upper panel:* data are expressed as mean ± S.E.M. of 5 independent experiments performed in sextuplicate (one-way ANOVA, Dunnett's *post-hoc* test: *, $P < 0.05$; **, $P < 0.01$ *vs.* control). *Lower panel*: representative images of migrated cells on the bottom of filter membrane as detailed in Methods. Scale bar: 0.5 cm. **B)** HUVECs plated in 96-well plates (10^4 cells/well) were incubated in phenol red-free complete culture medium with 5% FBS in the presence of G-1 (10^{-11}-10^{-7} M) for 24 h. Cell viability was measured by MTT assay. Data are expressed as mean ± S.E.M. of 3 independent experiments performed in quadruplicate.

Figure 2: E2 promotes wound healing through GPER1. HUVECs were seeded in 24-well plates (10^5 cells/well), wounded and treated with G-1 or E2 (10^{-7} M). The selective G1 antagonist G-15 (10^{-6} M) was added 30 min before E2. **A)** Representative image (scale bar: 100 μm); **B)** Quantitative analysis of wound-healing experiments. Wound closure was calculated as described in Methods. Bars show the mean ± S.E.M. of 4 independent experiments. One-way ANOVA, Bonferroni's post-hoc test: **, $P < 0.01$ *vs* control.

Figure 3: **The selective GPER1 agonist G1 promotes the formation of tube-like structures, while the GPER1 antagonist G15 counteracts E2-mediated effects**. HUVECs (2×10^4 cells/well) were seeded onto 48-well plates coated with Matrigel in phenol red-free complete culture medium with 5% FBS and treated with G-1 or E2 (both 10^{-7} M) in the presence or absence of G-15 (10^{-6} M). **A)** Representative phase contrast microphotographs (scale bar: 100 μm); **B)** Quantitative analysis of specific parameters of capillary tube formation after 4 h incubation on Matrigel as determined using Angiogenesis Analyser (ImageJ). Data are expressed as mean ± S.E.M. of 5 independent experiments. One-way ANOVA, Bonferroni's post-hoc test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs* control; ##, $P < 0.01$ *vs* E2.

Figure 4: E2 induces PFKFB3 expression in a time- and concentration-dependent manner. **A)** HUVECs (3×10^5/well) were grown in 35-mm dishes and, after reaching confluence, treated with 10^{-7} M E2 in phenol red-free complete medium with 5% FBS for the time indicated. *Upper panel:* representative blots showing the expression of PFKFB3; GADPH expression was used as loading control. *Lower panel*:

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densitometric analysis of bands, normalized to GADPH levels, expressed as % of control. Data are expressed as mean ± S.E.M. of 5 independent experiments. *t*-test: *, $P < 0.05$ *vs.* control. **B)** HUVECs (3×10^5/well) were grown in 3.5-cm dishes and, after reaching confluence, treated with E2 (10^{-9}-10^{-7} M) in phenol red-free complete medium with 5% FBS for 3 h. *Upper panel*: representative blots showing PFKFB3 detection; GADPH expression was used as a loading control. *Lower panel*: densitometric analysis of bands, normalized to GADPH levels, expressed as % of control. Data are expressed as mean ± S.E.M. of 5 independent experiments. One-way ANOVA, Dunnett's post-test: **, $P < 0.01$ *vs.* control.

Figure 5. **The selective GPER1 agonist G1 enhances the expression of PFKFB3 while the GPER1 antagonist G15 blocks E2-mediated PFKFB3 expression**. **A)** HUVECs (3×10^5/well) were grown in 35-mm dishes and, upon confluence, treated with 10^{-7} M E2 in phenol red-free complete medium with 5% FBS for 3 h. The selective GPER1 antagonist G-15 (10^{-6} M) was added 30 min before E2. *Upper panel*: representative blots showing the expression of PFKFB3; GADPH expression was used as a loading control. *Lower panel*: densitometric analysis of bands, normalized to GADPH levels, expressed as % of control. Data are expressed as mean ± S.E.M. of 5 independent experiments. One-way ANOVA, Bonferroni's post-test: **, $P < 0.01$ *vs.* control; [###], $P < 0.001$ *vs.* E2). **B)** HUVECs (3×10^5/well) were grown in 3.5-cm dishes and, after reaching confluence, treated with G-1 (10^{-9}-10^{-7} M) in phenol red-free complete medium with 5% FBS for 3 h. *Upper panel*: representative blots showing PFKFB3 immunodetection; GADPH was used as a loading control. *Lower panel*: densitometric analysis of bands, normalized to GADPH, expressed as % 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.

Figure 6. Treatment with GPER1 siRNA reduces E2-mediated HUVEC migration, tube-like structure formation and PFKFB3 expression. HUVECs (2×10^5/well) were seeded in 35-mm dishes and, after 24 h, transfected with siRNA for 72 h. After transfection, fresh complete medium was added and pharmacological treatments were started as indicated. **A)** and **D)** Cell lysates were subjected to Western blotting as described in the legend to Fig. 5. *Upper panel*: representative blots showing the expression of GPER1 and PFKFB3, respectively; GADPH was used as loading control. *Lower panel*: densitometric analysis of bands, normalized to GADPH levels, expressed as % 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.
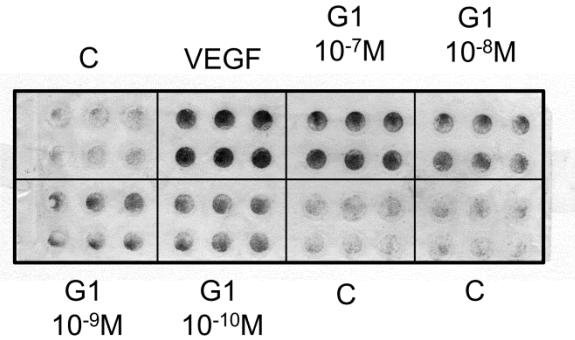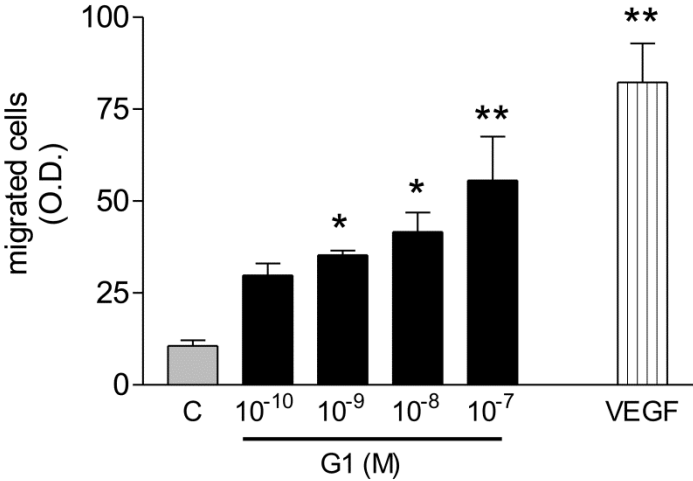
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B) HUVEC migration in response to E2 (10^{-7} M) was measured in a microchemotaxis chamber as described in the legend to Fig. 1. Data are expressed as mean $\pm$ S.E.M. of 5 independent experiments performed in sextuplicate. One-way ANOVA, Dunnett's post-hoc test: **, $P < 0.01$ *vs.* control. **C)** HUVECs (2 x 10^4 cells/well) were seeded onto Matrigel-coated 48-well plates in phenol-red free complete culture medium with 1% FBS containing E2 (10^{-7} M). Capillary tube formation on Matrigel was assessed after 4 h incubation using Angiogenesis Analyser (ImageJ) as described in the legend to Figure 3. Data are expressed as mean $\pm$ S.E.M. of 3 independent experiments. One-way ANOVA, Dunnett's post-hoc test, * $P < 0.05$ *vs.* control.

Figure 7: The PFKFB3 inhibitor 3PO inhibits E2-mediated collective HUVEC migration without affecting cell viability. HUVECs (10^5 cells/well) were plated in 24-well plates and the assay was performed in confluent cells as described in Methods. **A)** Representative images of a wound-healing experiment after 6-h incubation in phenol-red free complete culture medium with 5% FBS. HUVECs were treated with 10^{-7} M E2; 3PO (40 μM) was added 30 min before E2 (t0: upon wounding; t6: 6-h incubation, scale bar: 100 μm). **B)** Quantitative analysis of wound closure as described in Methods. Data are expressed as mean $\pm$ S.E.M. of 5 independent experiments. One-way ANOVA, Bonferroni's post-hoc test: *, $P < 0.05$ *vs.* control; ###, $P < 0.001$ *vs.* E2. **c)** HUVECs (10^4 cells/well) were plated in 96-well plates and incubated in phenol red-free complete culture medium with 5% FBS in the presence of 3PO (10 - 40 μM) or E2 (10^{-7} M). Cell viability was measured by MTT assay. Data are the mean $\pm$ S.E.M of 3 independent experiments performed in quadruplicate.

Figure 8: The PFKFB3 inhibitor 3PO blocks the formation of tube-like structures induced by E2 via GPER1. HUVECs (2×10^4 cells/well) were seeded onto Matrigel-coated 48-well plates in phenol red-free complete culture medium with 5% FBS containing E2 (10^{-7} M) or G-1 (10^{-7} M) in the presence or absence of 3PO (40 μM). Quantitative analysis of specific parameters of capillary tube formation after 4 h incubation on Matrigel was performed using Angiogenesis Analyser (ImageJ). Data are expressed as mean $\pm$ S.E.M of 5 independent experiments. One-way ANOVA, Bonferroni's post-hoc test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; ###, $P < 0.001$ *vs.* E2; §, $P < 0.05$; §§, $P < 0.01$ *vs.* G1.
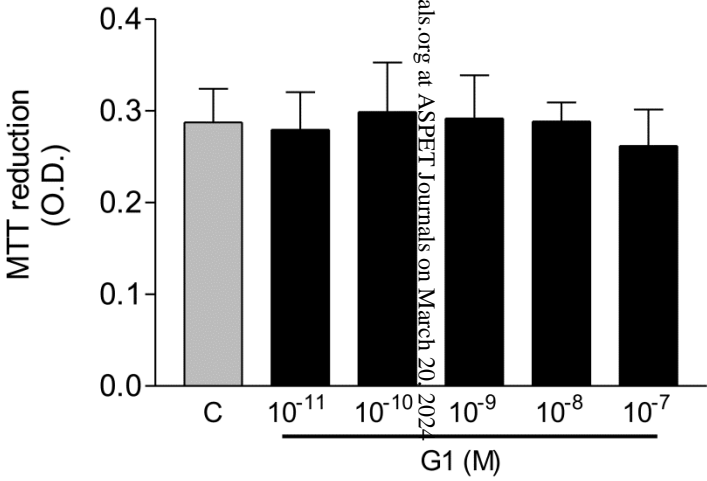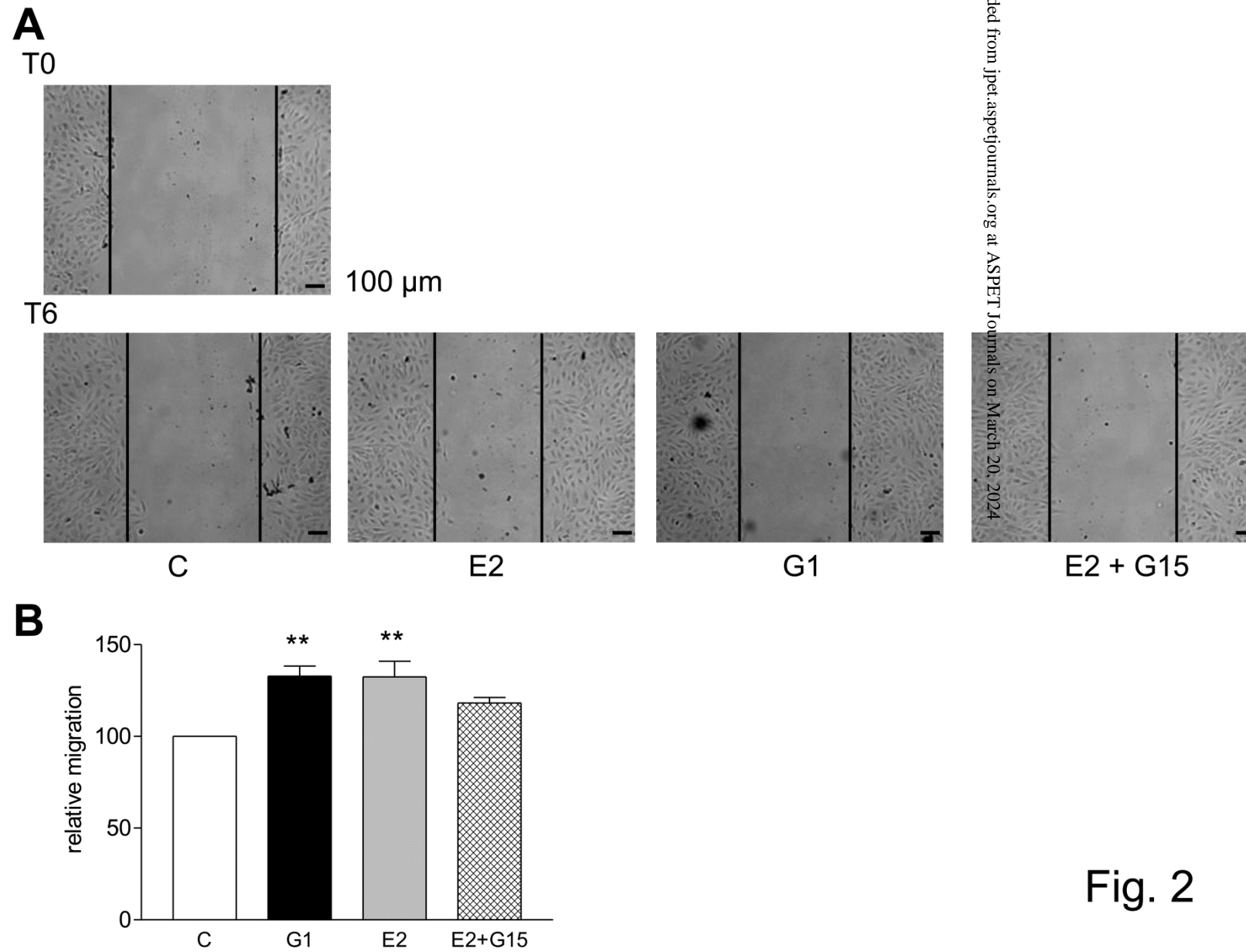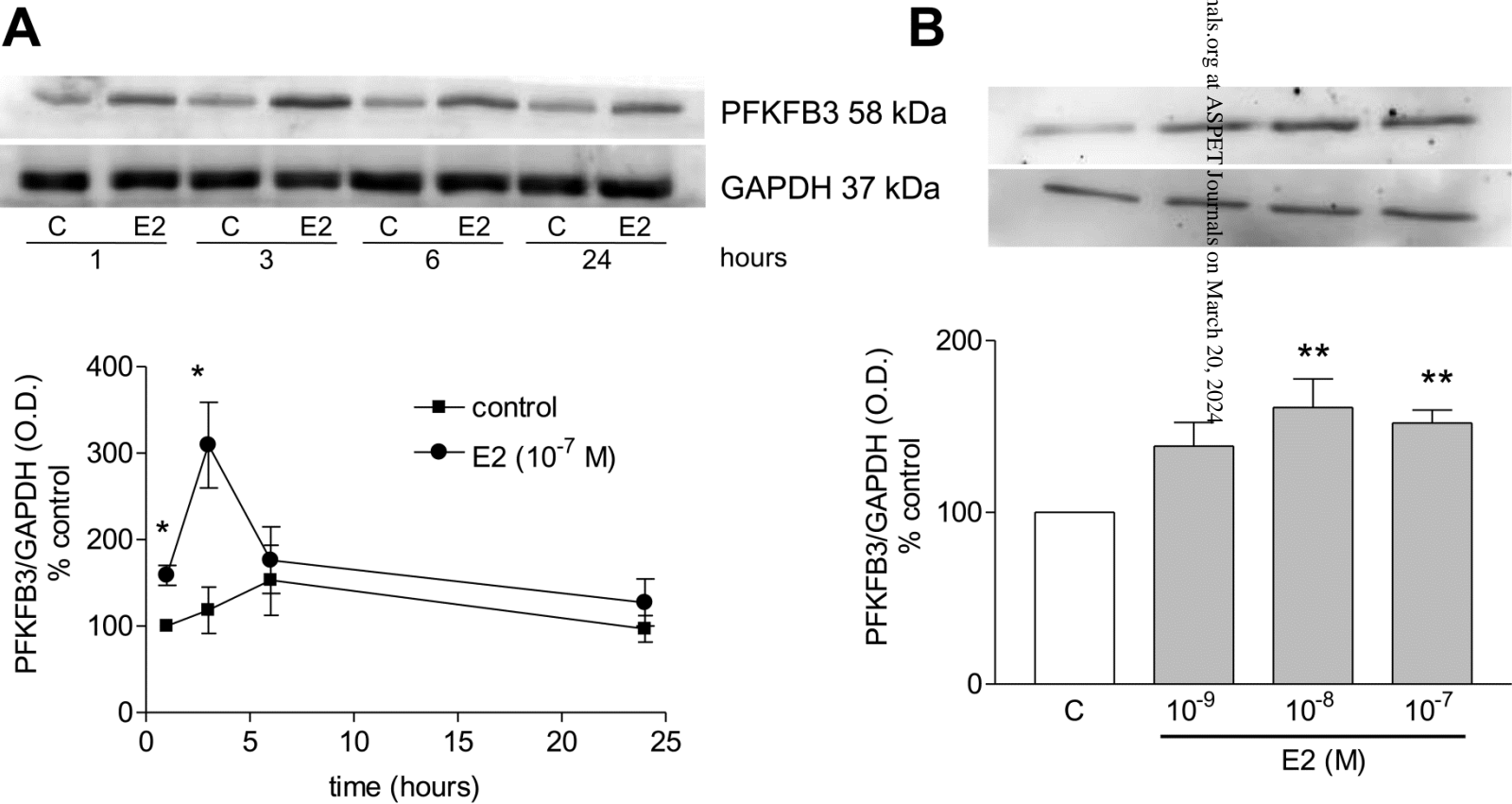
A

migrated cells (O.D.)

100
75
50
25
0

C 10^{-10} 10^{-9} 10^{-8} 10^{-7} VEGF

G1 (M)

B

MTT reduction (O.D.)

0.4
0.3
0.2
0.1
0.0

C 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}

G1 (M)

C   VEGF   G1 10^{-7}M   G1 10^{-8}M

G1 10^{-9}M   G1 10^{-10}M   C   C

Scale bar: ━ 0.5 cm

Fig. 1

29

A

T0

T6

C   E2   G1   E2 + G15

100 µm

B

Fig. 2

A

CTR   E2   G1   G15   G15+ E2

100 μm

B

Fig. 3

31

A

PFKFB3 58 kDa

GAPDH 37 kDa

C E2 C E2 C E2 C E2 hours
1 3 6 24

B

Fig. 4

Fig. 5

A

GPER 42 kDa

GAPDH 37 kDa

GPR30/GAPDH (O.D.) % control

150

100

50

0

control   scramble   siRNA

*

B

Fig. 6

cell migration (O.D.)

60

40

20

0

C   E2   C   E2

scramble   siGPER

**

C

nb master segments (% control)

300

200

100

0

C   E2   C   E2

scramble   siGPER

**

nb meshes (% control)

300

200

100

0

C   E2   C   E2

scramble   siGPER

**

mesh area (% control)

400

300

200

100

0

C   E2   C   E2

scramble   siGPER

nb junctions (% control)

250

200

150

100

50

0

C   E2   C   E2

scramble   siGPER

*

D

PFKFB3   58 kDa

GAPDH   37 kDa

PFKFB3/GAPDH % control

200

100

0

C   E2   C   E2

scramble   siGPER

*

34

Fig. 7

100 μm

A

T0

T6

C   E2   3PO   3PO+E2

B

relative migration

200

150

100

50

0

C   E2   C   E2
            3PO

*
***
###
***

C

MTT reduction (O.D.)

0.3

0.2

0.1

0.0

C   10   20   40   E2
     3PO (μM)

Fig. 8