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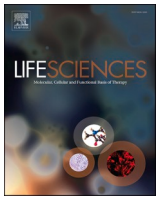

MiR-206 inhibits estrogen signaling and ovarian cancer cell migration without affecting GPER

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ABSTRACT

Aims: Estrogen-regulated pathways are involved in the etiology and progression of epithelial ovarian cancer (EOC), but the relative contribution of estrogen receptor isoforms is unclear. Only a subset of patients responds to antiestrogens including tamoxifen. Based on our previous evidence that miR-206 behaves as an oncosuppressor in EOC, we hypothesized that miR-206 would interfere with G protein-coupled estrogen receptor (GPER)-mediated signaling and cell motility.

Main methods: PFKFB3 and FAK proteins from OC cells challenged with selective estrogen receptor agonist and antagonist were measured by Western blotting. Cell proliferation and motility were analyzed by MTT and Boyden chamber, respectively. Estrogen-dependent cells were transfected with miR-206 mimic or control using Lipofectamine.

Key findings: The migration of SKOV3 and OVCAR5 cells significantly increased following treatment with 17β-estradiol (E2) and the selective GPER agonist G1. However, tamoxifen failed to inhibit E2 effect and even promoted SKOV3 cell migration. Estrogen receptor ligands did not affect SKOV3 proliferation. The GPER antagonist G15 significantly prevented E2-mediated upregulation of PFKFB3 expression, while G1 concentration-dependently upregulated PFKFB3 levels. Consistent with the functional link between PFKFB3 and FAK activation, E2 and G1 increased FAK phosphorylation at Tyr397. Transfection with miR-206 abolished estrogen-induced EOC migration and down-regulated PFKFB3 protein levels. Notably, miR-206 transfection reduced ERα protein abundance, whereas GPER amount was unchanged.

Significance: By blocking estrogen signaling and G1-induced EOC cell invasiveness with no direct interference with GPER levels, miR-206 mimics have the potential to act as pathway-selective antagonists and deserve further testing as RNA therapeutics in estrogen-dependent EOC.

1. Introduction

Preclinical and clinical evidence supports a role of estrogen-regulated pathways in the etiology and progression of epithelial ovarian cancer (EOC), which represents the most common and aggressive ovarian cancer (OC) subtype. Consistently, hormone replacement therapy use correlates with increased risk of developing EOC [1]. In contrast to breast cancer, however, anti-estrogen therapies have proven effective only in a small subset of patients and largely failed to prolong overall survival (OS) of ovarian cancer patients, mainly due to the development of resistance [2,3]. In this setting, the role of estrogen receptor (ER) status in the identification of responders as well as the resistance mechanisms have not been fully elucidated [2–5].

Estrogen signaling is mediated by two different nuclear receptor isoforms, namely ERα and ERβ. In particular, a role for ERα in tumor growth and invasiveness has been reported in several ovarian cancer cell lines and *in vivo* models [1]. Little is known about the effects of the more recently identified membrane G protein-coupled estrogen receptor

Abbreviations: E2, 17β-estradiol; EOC, epithelial ovarian cancer; FAK, focal adhesion kinase; FBS, fetal bovine serum; GPER, G-protein coupled estrogen receptor; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; SERM, selective estrogen receptor modulators.

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(GPER) in female reproductive tumors. Specifically, GPER is broadly expressed in high-risk ovarian cancer associated with lower survival rates, and its expression is higher in both advanced stages and patients with recurrence [6,7]. However, the prognostic value of GPER in OC remains controversial [8]. Indeed, GPER has been shown to mediate both tumor-suppressor and tumor-promoting actions [9], and a study reported the relevance of an interplay between GPER and ERα in OC [10]. Of note, therapeutic antiestrogens including the selective estrogen receptor modulator (SERM) tamoxifen act not only as ERα antagonist but also as GPER agonists [11,12]. This may impact the long-term response to therapeutic antiestrogens [13].

The main effects of estrogenic agents mediated by their receptors are related to cell survival, migration and proliferation, but little is known about the signaling proteins involved. We and others have previously shown that the glycolytic protein 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3, EC 2.7.1.105) and focal adhesion kinase (FAK, EC 2.7.10.2) are overexpressed and play a major role in OC cell proliferation and migration [14–16]. Recently, it has been shown that pharmacological or genetic inhibition of PFKFB3 decreases FAK phosphorylation and activation, thereby affecting cancer cell stemness or migration [17–19]. Both proteins are known to be up-regulated by estrogens in breast cancer cell lines. In particular, PFKFB3 is induced by 17β-estradiol (E2) via ERα and is required for E2-mediated proliferation of MCF-7 cells [20], whereas FAK activation by estrogens occurs in ERα-negative breast cancer cells via GPER [21]. However, little is known about the estrogenic functional regulation of ovarian cancer cell proliferation and motility via PFKFB3 or FAK [22]. Of note, both E2 and tamoxifen induce FAK phosphorylation and activation, promoting cell motility in endometrial cancer [23,24]. Overall, there is limited evidence on the signaling proteins involved in estrogen-induced OC progression and on the role of ERs including GPER in the response to estrogens and tamoxifen.

Based on this background as well as our recent observation that miRNA (miR)-206 behaves as an oncosuppressor endowed with anti-migratory properties in several EOC cell models via inhibition of PFKFB3 and downstream FAK [25], we hypothesized that miR-206 could represent an alternative strategy to control GPER-mediated estrogen signaling and ovarian cancer cell motility. In particular, the present study aimed to assess: 1) the role of GPER in the estrogenic regulation of PFKFB3 and FAK proteins and the functional consequences in EOC cells; and 2) the regulatory action of miR-206 on the functional expression of estrogen-modulated proteins.

2. Materials and methods

2.1. Drugs and chemicals

The following compounds were used for experiments: 17β-estradiol (Sigma Aldrich); (±)-1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[*c*]quinolin-8-yl]-ethanone (G1); (3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[*c*]quinoline (G15); all from Tocris Bioscience (Bristol, UK).

2.2. Cell culture

The human ovarian cancer cell lines SKOV3, OVCAR5 and CAOV3 were purchased from the American Type Culture Collection (ATCC, USA) and maintained in RPMI 1640 (Sigma) medium supplemented with 10 % fetal calf serum (FCS, Gibco, Thermo Fisher Scientific Italia, Rodano, Milan Italy), 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 to 25. For experiments with estrogenic agents, cells were switched to phenol red-free RPMI 1640, 24 h before each assay.

2.3. MTT assay

SKOV3 (2.5×10^3 cells/well) were seeded in 96-well plates and treated with estrogenic agents as indicated in the Results section for 24-72 h in phenol red-free RPMI 1640 supplemented with 2 % FCS. Four hours before the incubation end, a 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 dimethyl sulfoxide (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 the raw optical density value and represents the mean value of three independent assays, performed in quadruplicate.

2.4. miRNA transfection

SKOV3, OVCAR5 and CAOV3 (1×10^5 cells/ml) were seeded in complete culture medium. The next day (70 % confluent), cells were transfected with miR-206 (37 nM) using Lipofectamine3000 (Life Technologies Inc.) for 72 h in phenol red-free RPMI 1640 with 5 % FCS. At the end of transfection period, Western Blot or chemotaxis assays were performed. A miRNA without any homology to human gene sequences served as negative control (miR-NC, 37 nM). The following miRNAs (purchased from Sigma-Aldrich) were used:

MISSION® microRNA Mimic miR-206: cat. Nr. HMI0364.

MISSION® microRNA Negative Control 2, Sequence from *Caenorhabditis elegans* with no homology to human gene sequences: cat. Nr. HMC0003.

2.5. Chemotaxis assay

Chemotaxis experiments were performed in a 48-well modified microchemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) using 8-μm nucleopore polyvinylpyrrolidone-free polycarbonate filters coated with 10 μg/mL collagen. Upper chambers were filled with 50 μL cell suspension (1.6×10^5 cells/mL) in phenol red-free RPMI 1640 supplemented with 0.1 % bovine serum albumin (BSA), whereas the lower chambers were filled with phenol red-free RPMI 1640 supplemented with 0.1 % BSA, corresponding to basal migration, in the presence or absence of E2 (1–100 nM) or G1 (1–100 nM), or with 10 % FCS (positive control). ER antagonists as detailed in the Results section were added both in the upper and lower compartment. Selected experiments were performed using cells transfected with miR-206 (37 nM). 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 (VWR Scientific Products, Bridgeport, NJ, USA), and densitometric analysis was performed using the ImageJ version 1.47 software (National Institutes of Health, NIH, USA). Each experiment was performed in sextuplicate. Results are reported as arbitrary units of optical density and represent the mean values of three/five independent experiments.

2.6. Western blot

SKOV3, OVCAR5 or CAOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes and treated with estrogenic agents as indicated in the Results section or transfected with miR-206 (37 nM). In selected experiments, the GPER antagonist G15 was added 30 min before the stimulus. At the end of treatments, cells were lysed with 80 μL lysis buffer (RIPA buffer, 1× 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 10,000 ×*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 (20–40 μg) were separated on SDS-PAGE and transferred onto AmershamHybond-P polyvinylidene difluoride membranes. Membranes were then blocked and probed using the following primary monoclonal antibodies: 1:5000 rabbit anti-PFKFB3; 1:1000 mouse anti-FAK; 1:10,000 rabbit anti-GAPDH (all from Abcam, Cambridge, UK); 1:1000 rabbit anti-phospho-FAK^{Tyr397} (Cell Signaling Technology, Danvers, MA, USA). After washing, the membranes were incubated with rabbit or mouse secondary horseradish peroxidase–conjugated antibodies (Vector Laboratories, Burlingame, CA). Bands were detected by chemiluminescence using the Clarity western ECL substrates (Biorad, Segrate, Milano, Italy). Images were acquired by Azure C400 (Azure biosystem, Dublin, CA). Densitometric analysis of bands was performed using Image J version 1.47 software. Results are expressed as absolute values and represent the mean values of 3–4 independent experiments.

2.7. RNA extraction and real-time qPCR

SKOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes in complete culture medium. Cells were washed in PBS and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentrations were determined using a NanoDrop™ One Microvolume UV–Vis spectrophotometer (Thermo Fisher Scientific). cDNA was generated from 1 μg total RNA using the Maxima first strand cDNA synthesis kit with dsDNase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The relative quantification of genes of interest was performed by qPCR 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 Biosystems, Thermo Fisher Scientific). Primer sequences were the following: PFKFB3 (forward) GCGTCCCCACAAAAGTGTTC and (reverse) CCGGACTTTCATGGCTTCCT; GAPDH (forward) CACCATCTTCCAGGAGCGAG and (reverse) CCTTCTCCATGGTGGTGAAGAC. Target genes were normalized to GAPDH and analyzed using the $2^{-\Delta Ct}$ method.

2.8. 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 6 (Graph Pad Software Inc., La Jolla, CA). Student's *t*-test was used to compare the means of two independent groups. One-way ANOVA followed by Tukey's or Dunnett's post hoc tests were used for multiple comparisons. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Estrogenic agents increased SKOV3 cell migration but not proliferation via GPER

Most human ovarian cancers express estrogen receptors, including GPER [1], whose function in the setting of ovarian cancer is not fully characterized. Cell migration is a crucial attribute of cancer cells that influences the tumor invasive potential. The pro-migratory effect of E2 has already been described in SKOV3 [26] and ERα-negative OC cell lines [27]. However, the role of GPER in estrogen-induced migration has not been fully defined in earlier studies [28,29].

We first confirmed that SKOV3 cells express ERα as well as the membrane receptor GPER (Supplementary Fig. S1). Then, we explored the functional role of estrogenic agents, evaluating the effect of E2 and the selective GPER agonist G1 on cell proliferation and migration. As shown in Fig. 1A, the number of migrating cells significantly increased in response to increasing concentrations of E2 (1–100 nM). Treatment with G1 induced an effect comparable to that of E2 on SKOV3 cell migration. To further evaluate the role of GPER in cell migration, we treated SKOV3 cells with the selective GPER antagonist G15 (1 μM) in the presence or absence of E2. E2-stimulated migration was almost completely prevented by G15 (Fig. 1B), highlighting the involvement of GPER in this process. We also performed experiments with tamoxifen (1 μM), which is used as an anti-estrogen in ERα-positive cancer treatment. Tamoxifen treatment failed to inhibit the effect of E2 and even promoted SKOV3 cell migration with respect to control, in line with its GPER

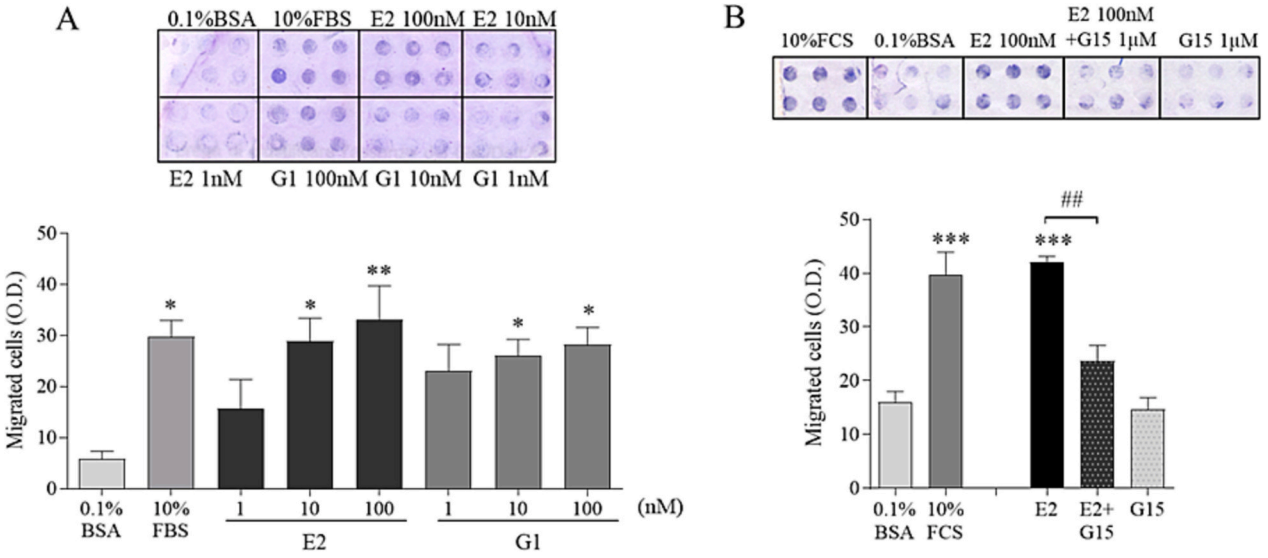

Fig. 1. Estrogenic agents promoted SKOV3 migration via GPER. (A–B) *Upper panels*. Representative image of SKOV3 migration in response to: (A) increasing concentrations of 17β-estradiol (E2) or selective GPER agonist G1 (1–100 nM); (B) the selective GPER antagonist G15 (1 μM) in presence or absence of E2 (100 nM). Cell migration was measured in a modified 48-well Boyden chamber after 6 h incubation at 37 °C. Basal migration (without chemoattractant stimulus): 0.1 % BSA. Positive control: 10 % FCS. Lower panels. Cell migration is shown as optical density values (O.D., arbitrary units). Each independent experiment was performed in sextuplicate. Data are expressed as mean ± SEM of 3–5 independent experiments; One-way ANOVA, Tukey's *post-hoc* test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. 0.1 % BSA; $^{\#\#}p < 0.01$.

agonist activity (Supplementary Fig. S2).

Overall, these data suggest that the pro-migratory effect of estrogens in the SKOV3 cell line is at least in part mediated by the membrane receptor GPER.

We then evaluated the cell proliferation rate in response to estrogenic agents. Results on the effect of ER agonists on SKOV3 proliferation are conflicting [30,31], and the role of GPER in the setting of ovarian cancer growth has not been fully elucidated in earlier studies [28,32]. As shown in Fig. 2, SKOV3 cell proliferation, as evaluated by MTT assay, did not change in cells challenged with E2 (1–100 nM) for 72 h (Fig. 2A), or with 100 nM E2 over time (24–72 h, Fig. 2B). Similarly, treatment with G1 (100 nM) did not influence cell proliferation, as measured after 72 h (Fig. 2C).

Thus, under our experimental conditions, SKOV3 cells did not exhibit proliferative response to E2 and G1, suggesting that SKOV3 could be growth resistant to estrogens.

3.2. E2 treatment increased PFKFB3 levels via GPER

Enhanced glycolytic metabolism is a key feature of cancer, and overexpression of the glycolytic enzyme PFKFB3 correlates with an invasive tumor phenotype [33]. Of note, Imbert-Fernandez and colleagues [20] reported that E2 treatment increases PFKFB3 mRNA and protein levels at early time points (3–6 h) in MCF-7 cells.

We assessed the estrogenic regulation of PFKFB3 in SKOV3 cells, and found that E2 treatment increased PFKFB3 protein levels after 6 h, already at the lowest concentration tested (1 nM; Fig. 3A). In time-course experiments, PFKFB3 protein levels significantly increased in response to 10 nM E2 treatment after 3 h but returned to baseline after 24 h (Fig. 3B). No changes in *PFKFB3* mRNA levels were observed after treatment for 1–6 h (Fig. 3C), suggesting that E2 increased PFKFB3 levels in a transcription-independent manner.

To investigate the role of GPER in this setting, cells were treated with GPER ligands in the presence or absence of E2. Treatment with G15 (1 μM) significantly prevented E2-mediated upregulation of PFKFB3 expression (Fig. 4A). In addition, similarly to E2, treatment with G1 (1–100 nM) concentration-dependently increased PFKFB3 levels (Fig. 4B), reaching statistical significance at 10 nM. These data further support a role for GPER in ER signaling in SKOV3 cells.

3.3. E2 and G1 induced FAK phosphorylation via GPER

There is evidence that PFKFB3 is functionally linked to FAK phosphorylation and activation; indeed, pharmacological inhibition of PFKFB3 causes a decrease in phosphorylated FAK (Tyr397) and cancer cell invasion [17]. Therefore, we explored a possible role for FAK in the regulation of SKOV3 migration by estrogenic agents.

In time-course experiments (15 min-3 h), treatment with 10 nM E2 significantly increased FAK phosphorylation at Tyr397 after 15–30 min (Fig. 5). A similar effect on FAK phosphorylation was observed after 15-min treatment with 100 nM G1 (Fig. 6).

3.4. miR-206 inhibited E2- and G1-mediated cell migration without affecting GPER

Based on previously published data by our group showing that miR-206 downregulates PFKFB3 and total FAK, resulting in impaired SKOV3 migration [25], we tested the role of miR-206 in estrogen-mediated SKOV3 invasiveness and PFKFB3 regulation.

As reported in Fig. 7A, SKOV3 cells transfected with miR-206 (37 nM) were no longer able to migrate more efficiently in response to either E2 or G1 when compared to cells transfected with a negative control (miR-NC). Moreover, in line with the relevant role of PFKFB3 in this process, miR-206 prevented E2- and G1-mediated increase in PFKFB3 protein amount (Fig. 7B).

Finally, based on published data showing that miR-206 down-regulates ERα expression in estrogen-responsive breast cancer cells [34], we investigated the effect of miR-206 on ERα and GPER protein levels in SKOV3. In cells transfected with miR-206 (0.3–37 nM) for 72 h, ERα abundance decreased, whereas GPER amount remained unchanged at all concentrations tested (Fig. 8A, B).

Overall, these results suggest that miR-206 blocks G1 signaling and SKOV3 invasiveness without affecting GPER levels.

3.5. miR-206 inhibited estrogen signaling and OVCAR5 but not CAOV3 cell migration

To validate our findings, we carried out experiments in 2 additional EOC lines, namely OVCAR5 and CAOV3.

Similar to what observed in SKOV3 cells, treatment with the selective GPER agonist G1 or E2 increased PFKFB3 protein levels and cell migration in OVCAR5 but not in CAOV3, which apparently express neither ERα nor GPER (Fig. 9A, D, E and F). Moreover, miR-206 overexpression decreased estrogen-induced OVCAR5 cell migration and, in line with an important role for PFKFB3 in this process, prevented G1-mediated increase in PFKFB3 protein levels (Fig. 9B and C). These results further highlight the relevance of GPER in EOC aggressiveness, and suggest that the functional role of miR-206 is essentially consistent across estrogen-responsive epithelial ovarian cancer cells.

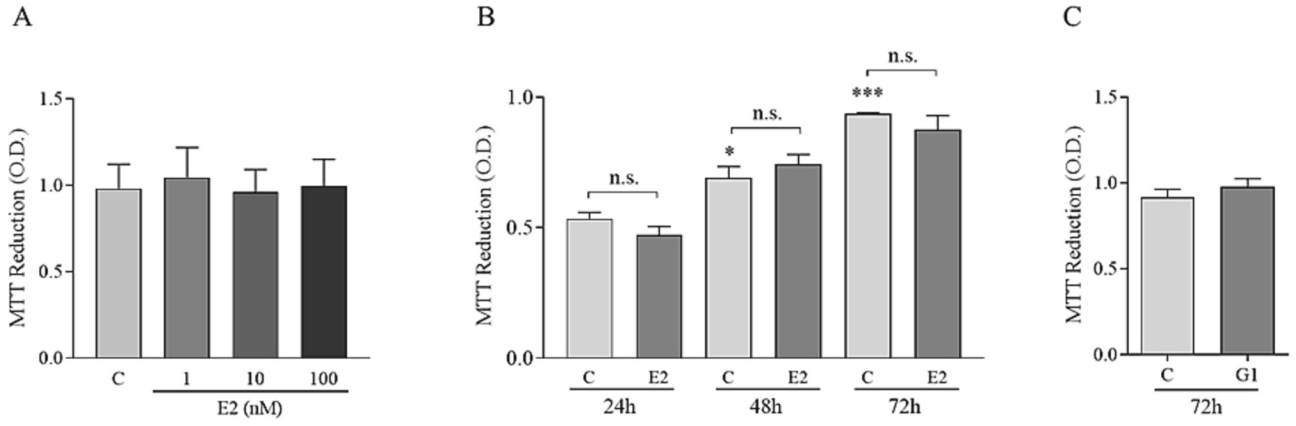

Fig. 2. Estrogen receptor ligands did not affect SKOV3 proliferation. SKOV3 (2.5×10^3 cells/well) were seeded in 96-well plates in complete culture medium. After 24 h, medium was replaced with phenol red–free medium with 2 % FCS, and cells were treated with: (A) 1–100 nM E2 for 72 h, (B) 100 nM E2 for 24–72 h, (C) 100 nM G1 for 72 h. Cell proliferation was measured by MTT assay. Control (C): 2 % FCS. Data are expressed as mean ± SEM of 3–4 independent experiments performed in quintuplicate. A-C, one-way ANOVA, *ns*. B, one-way ANOVA, Dunnett's *post-hoc* test: $*p < 0.05$; $***p < 0.001$ vs. control 24 h.

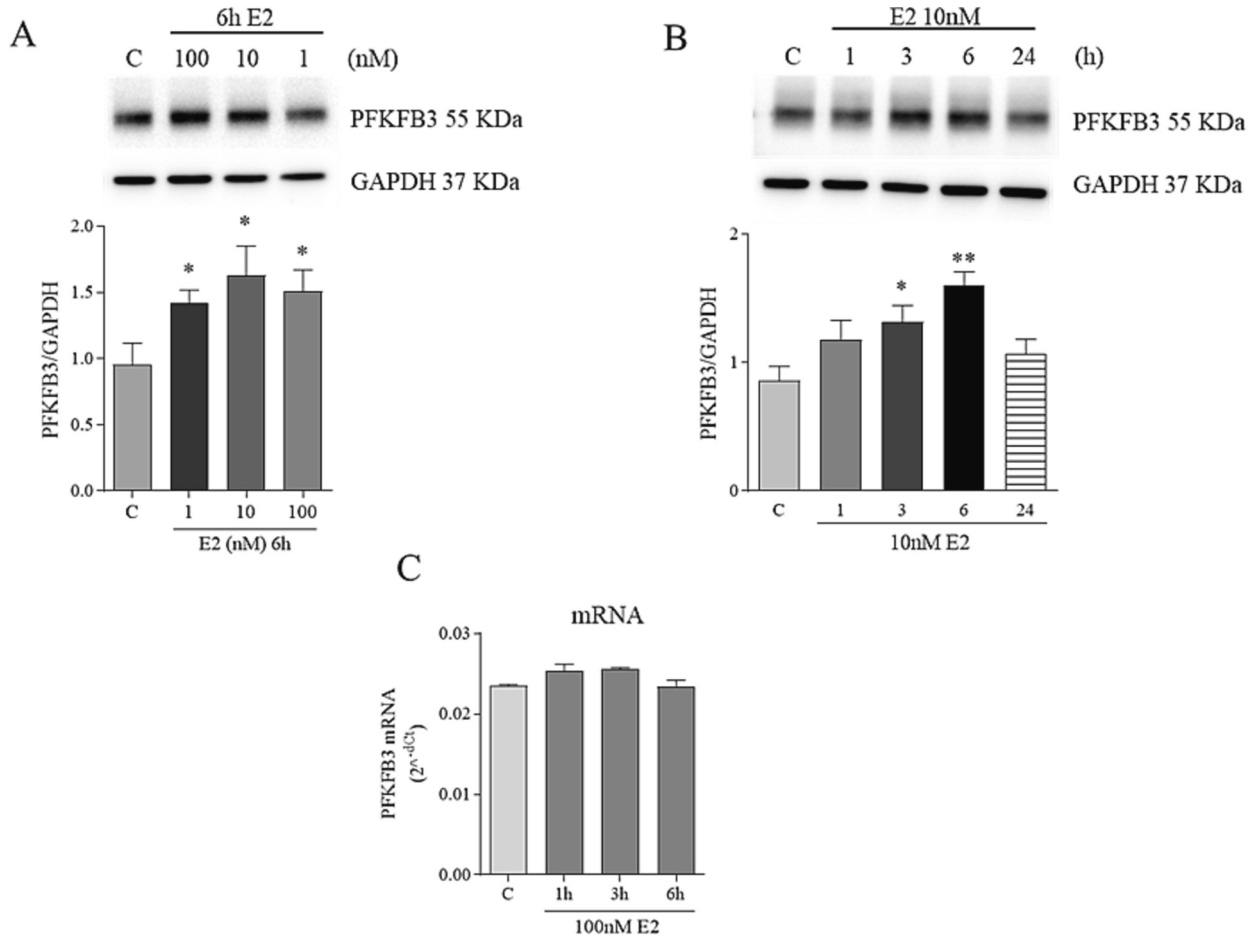

Fig. 3. E2 increased PFKFB3 protein but not mRNA levels in SKOV3 cells. SKOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes in complete culture medium. After 24 h, medium was replaced with phenol red–free medium with 5 % FCS, and cells were treated with E2 (1–100 nM) for 6 h (A), with 10 nM E2 for 1–24 h (B) or with 100 nM E2 for 1–6 h (C). A–B. *Upper panels*: Representative blot showing PFKFB3 immunodetection. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Data are expressed as mean ± S.E.M. of 4 independent experiments; *t*-test: $^*p < 0.05$, $^{**}p < 0.01$ vs. control. C. *PFKFB3* mRNA levels were measured by q-PCR and normalized to GAPDH levels. Data are expressed as mean ± SEM of 3 independent experiments run in triplicate. *t*-Test, *ns*.

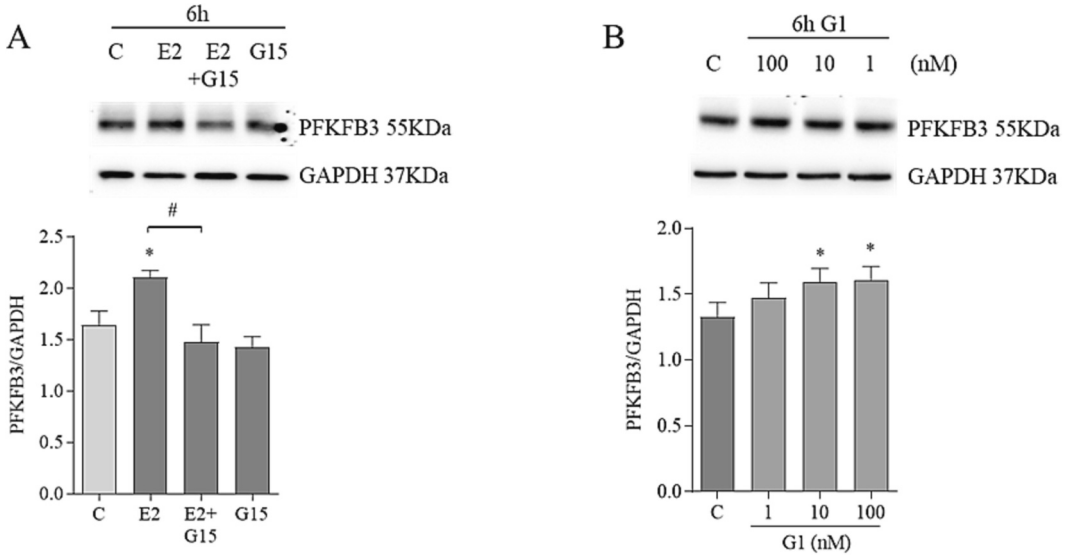

Fig. 4. The selective GPER agonist G1 increased PFKFB3 protein levels while the GPER antagonist G15 blocked E2-mediated PFKFB3 expression. SKOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes in complete culture medium. After 24 h, medium was replaced with phenol red–free medium with 5 % FCS and cells were treated with: (A) 100 nM E2 for 6 h in the presence or absence of 1 μM G15, which was added 30 min before E2, or (B) G1 (1–100 nM) for 6 h. *Upper panels*: Representative blot showing PFKFB3 immunodetection. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Data are expressed as mean ± S.E.M. of 3 independent experiments. (A) *t*-test: $^*p < 0.05$ vs. control; $^{\#}$E2 + G15 vs E2; (B) *t*-test: $^*p < 0.05$ vs. control.

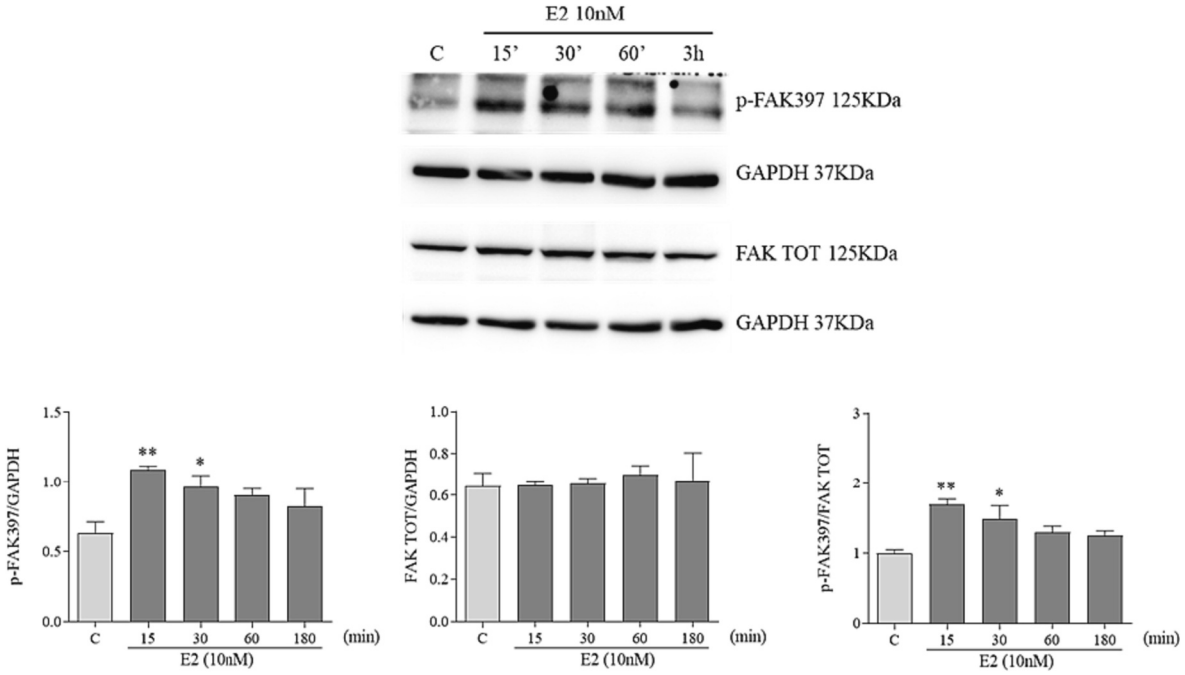

Fig. 5. E2 increased FAK phosphorylation at Tyr397. SKOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes in complete culture medium. After 24 h, medium was replaced with phenol red–free medium with 1 % FCS, and cells were treated with E2 (10 nM) for 15 min-3 h. *Upper panels*: Representative blot showing p-FAK397 and total FAK immunodetection. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels, and p-FAK/FAK ratio. 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.

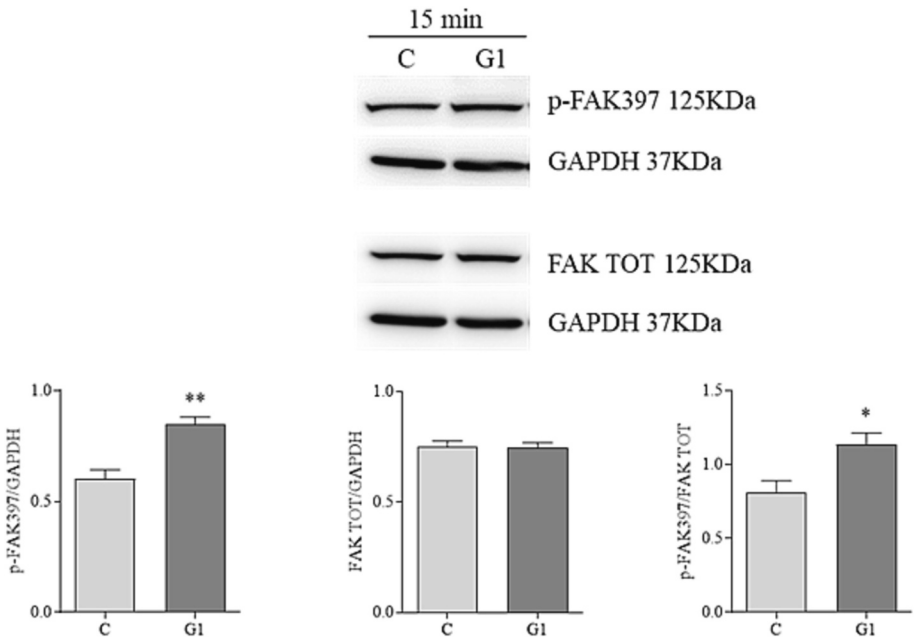

Fig. 6. G1 increased FAK phosphorylation at Tyr397. SKOV3 (2×10^5 cells/dish) were seeded in 35-mm dishes in complete culture medium. After 24 h, medium was replaced with phenol red–free medium with 1 % FCS, and cells were treated with G1 (100 nM) for 15 min. *Upper panels*: Representative blot showing p-FAK397 and total FAK immunodetection. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels, and p-FAK/FAK ratio. Data are expressed as mean ± S.E.M. of 3 independent experiments; *t*-test: $*p < 0.05$; $**p < 0.01$ vs. control.

4. Discussion

Activation of estrogen signaling pathways plays a crucial role in the etiology and progression of epithelial ovarian cancer, which can be halted by agents that inhibit estrogen production and/or activity via ER. However, the specific involvement of ERα and GPER in OC development as well as in the response to anti-estrogen treatments is unclear. miRNAs such as miR-206 act as post-transcriptional inhibitors of proteins involved in OC aggressiveness [35] and may represent a valuable strategy to control estrogen signaling irrespective from ER subtypes. In this study, we provide the first evidence that miR-206 mimics likely behave as pathway-preferential antagonists, by blocking estrogen signaling and G1-induced OC cell invasiveness without directly interfering with GPER.

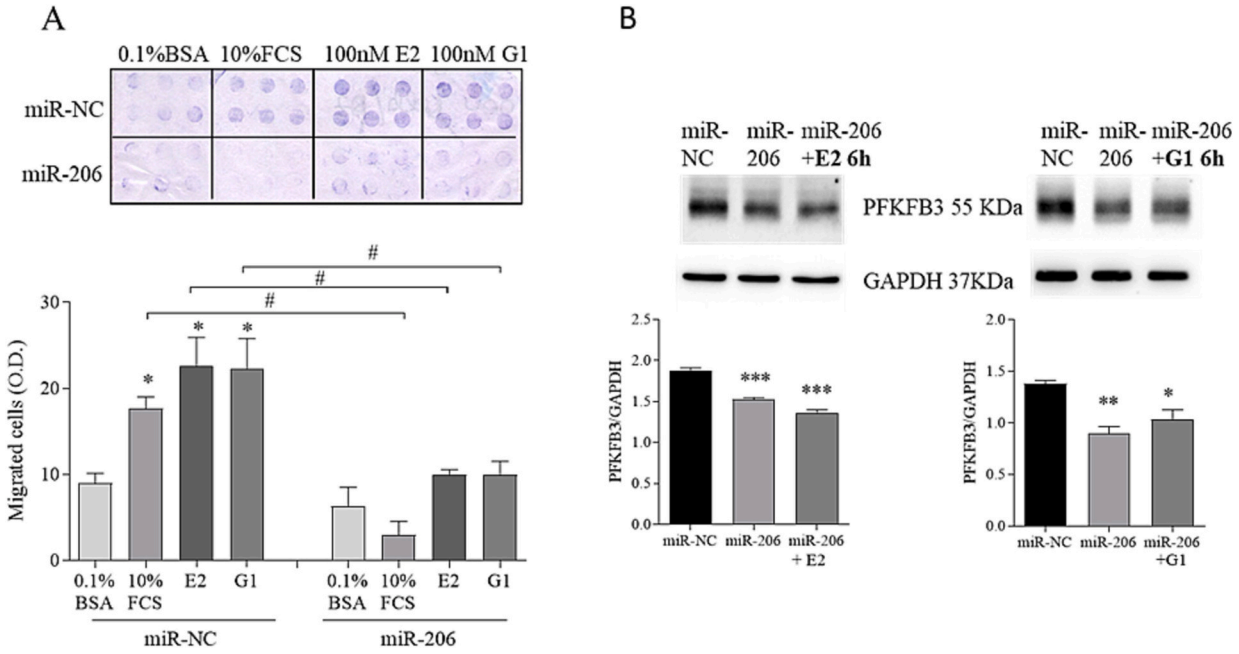

Fig. 7. miR-206 transfection blocked estrogen-induced SKOV3 cell migration, downregulating PFKFB3 protein levels. SKOV3 (1×10^5 cells/well) were seeded in 12-well plates in complete culture medium and, after 24 h, transfected with miRNA-206 (37 nM) for 72 h in phenol red–free medium with 5 % FCS. (A) *Upper panel*: Representative image of transfected SKOV3 migration in response to E2 or G1 (100 nM), measured in a modified 48-well Boyden chamber after 6 h incubation at 37 °C. miR-NC (37 nM) was used as a negative control. Basal migration (without chemoattractant stimulus): 0.1 % BSA. Positive control: 10 % FCS. *Lower panel*. Cell migration is shown as optical density values (O.D., arbitrary units). Each independent experiment was performed in sextuplicate. Data are expressed as mean ± SEM of 3 independent experiments; *t*-test: $^{*}p < 0.05$ vs. 0.1 % BSA; $^{\#}p < 0.05$ vs. miR-NC. (B) After transfection, fresh medium containing 5 % FCS was added and cells were treated with E2 or G1 (100 nM) for 6 h. MiR-NC (37 nM) was used as a negative control. *Upper panels*: Representative blot showing immunodetection of PFKFB3. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Data are the mean ± SEM of 3 independent experiments. One-way ANOVA, Dunnett's *post-hoc* test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. miR-NC.

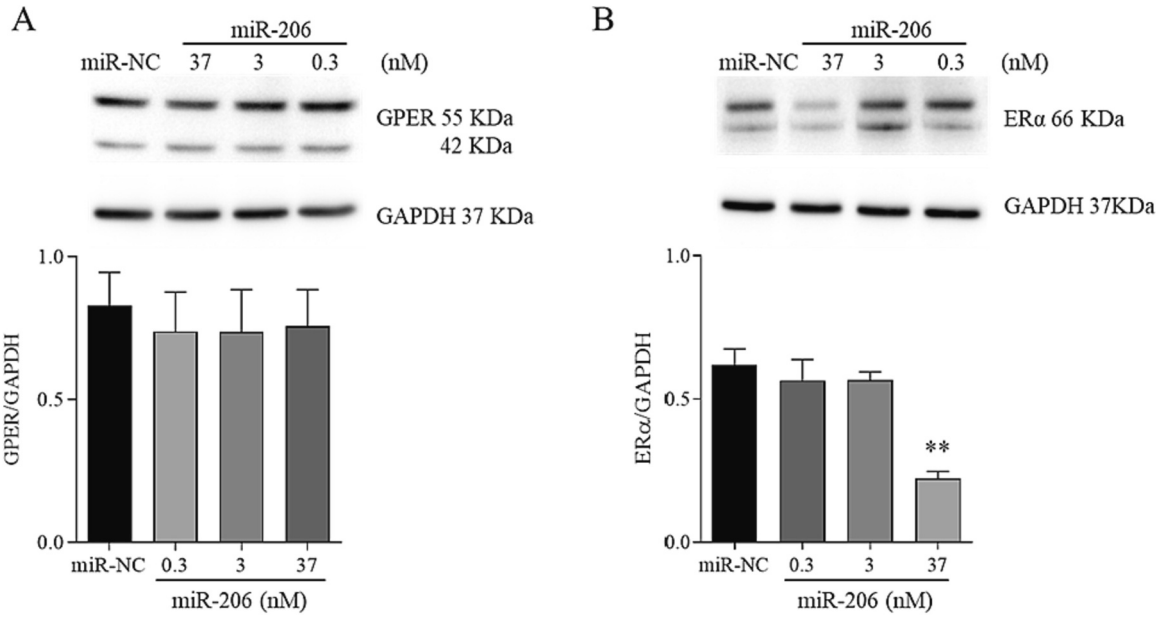

Fig. 8. miR-206 transfection did not affect GPER while negatively regulating ERα protein abundance. SKOV3 (1×10^5 cells/well) were seeded in 12-well plates in complete culture medium and, after 24 h, transfected with miRNA-206 (0.3–37 nM) in RPMI with 5 % FCS for 72 h. MiR-NC (37 nM) was used as a negative control. (A–B) *Upper panels*: Representative blot showing immunodetection of GPER and ERα respectively. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Data are the mean ± SEM of 3 to 4 independent experiments. (A) One-way ANOVA, *ns*. (B) One-way ANOVA, Dunnett's *post-hoc* test: $^{**}p < 0.01$ vs. miR-NC.

While protumor effects of ERα have been shown in several *in vitro* and *in vivo* OC models [1], conflicting results have been reported regarding involvement of GPER, which is highly expressed in a subset of aggressive and invasive tumors and correlates with disease progression [28]. However, Ignatov et al. [8] showed a tumor suppressor effect of GPER in OC cell lines including SKOV3, supporting the view that

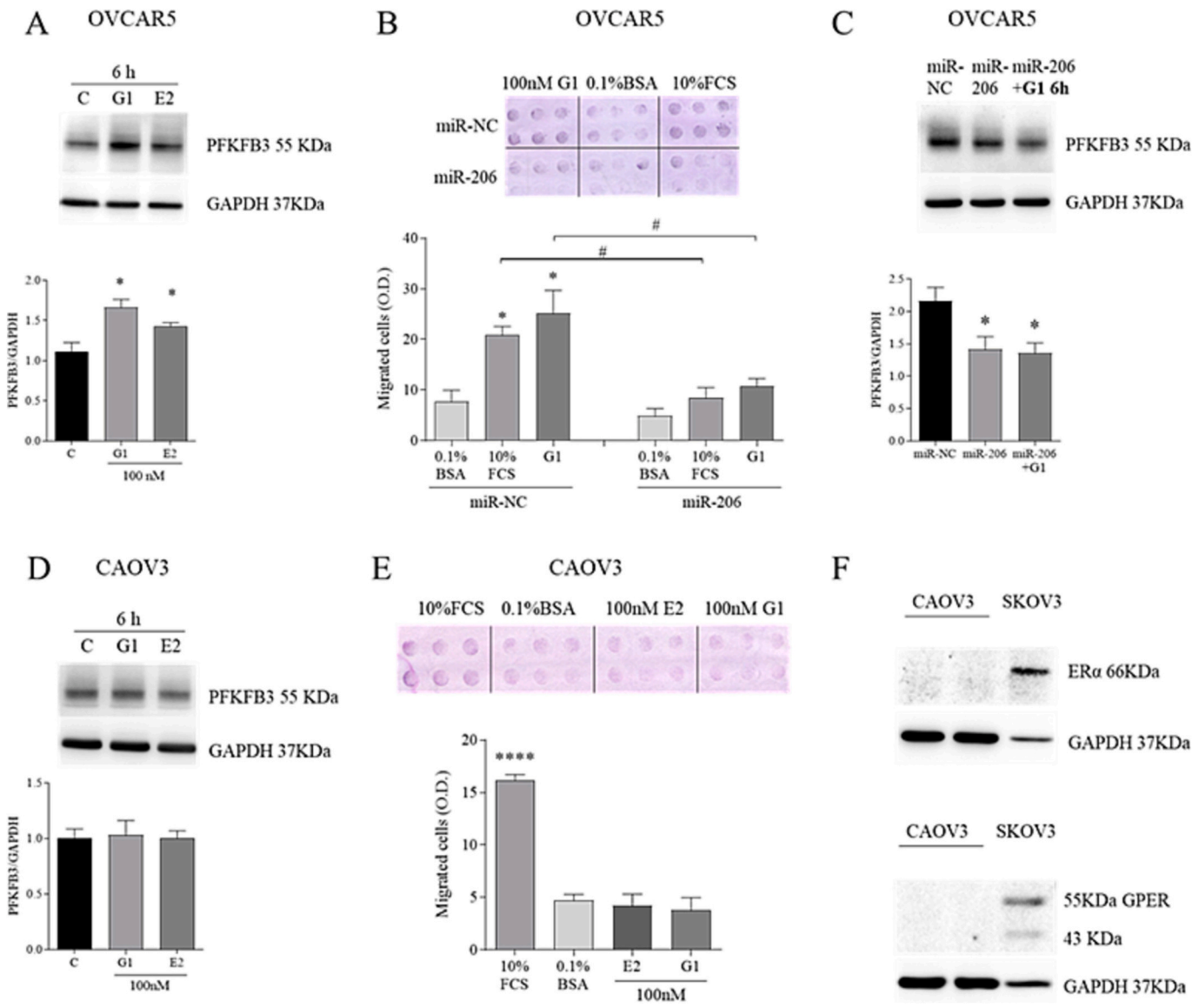

Fig. 9. Estrogenic agents increased PFKFB3 protein levels in OVCAR5 but not in CAOV3 cells, and miR-206 transfection blocked estrogen-induced OVCAR5 cell migration, downregulating PFKFB3 protein levels. OVCAR-5 and CAOV3 cells were treated with (A–D) E2 or G1 (100 nM) for 6 h, or (B–C) transfected with miR-206 (37 nM) for 72 h in phenol red–free medium with 5 % FCS. (C) After transfection, cells were treated with G1 for 6 h. (A–C–D–F) *Upper panels*: Representative blot showing immunodetection of PFKFB3, ERα and GPER. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Data are the mean ± SEM of 4 independent experiments. *t*-Test, $^{*}p < 0.05$ vs. C or miR-NC.

(B) *Upper panel*: Representative image of transfected OVCAR5 cell migration in response to G1 (100 nM), measured in a modified 48-well Boyden chamber after 6 h incubation at 37 °C. miR-NC (37 nM) was used as a negative control. Basal migration (without chemoattractant stimulus): 0.1 % BSA. Positive control: 10 % FCS. *Lower panel*. Cell migration is shown as optical density values (O.D., arbitrary units). Data are expressed as mean ± SEM of 3 independent experiments run in sextuplicate; *t*-test: $^{*}p < 0.05$ vs. 0.1 % BSA; $^{\#}p < 0.05$ vs. miR-NC. (E) *Upper panel*: Representative image of CAOV3 migration in response to E2 or G1 (100 nM). *Lower panel*. Cell migration is shown as optical density values (O.D., arbitrary units). Each independent experiment was performed in sextuplicate. Data are expressed as mean ± SEM of 4 independent experiments; *t*-test: $^{****}p < 0.0001$ vs. 0.1 % BSA.

targeting of GPER with selective agonists could represent a strategy to control advanced tumor growth [36].

We first tested the involvement of GPER in the proliferation of the highly invasive EOC cell line SKOV3 expressing both ERα and GPER. Under our experimental (low-serum) conditions, we were unable to detect a proliferative effect of E2 or the selective GPER agonist G1. This is in line with previous observations that OC cell lines including SKOV3 are growth resistant to E2 *in vitro*, while showing remarkable sensitivity to female hormones in xenograft models *in vivo* [30,37]. Although the reasons for the lack of response to estrogens *in vitro* remain unclear, we cannot exclude that antiestrogens inhibit OC growth when cell proliferation is stimulated by growth factors [38], consistent with the reported interplay between estrogen and growth factor signaling in estrogen-stimulated OC cell proliferation [10].

Looking at the invasive potential of EOC cells, we showed that G1 promoted, while G15 significantly inhibited SKOV3 migration induced by the endogenous ligand E2, which non-selectively binds all receptor subtypes. This was not the case in CAOV3 cells that apparently express neither ERα nor GPER. Of note, we also found that treatment with the SERM tamoxifen at a concentration (1 μM) close to the therapeutic peak plasma level (0.3 μM) was unable to prevent E2-induced OC cell migration and even induced it with respect to control, likely behaving as a GPER agonist [11]. These data are partially in line with previous findings reporting that G1 promotes cell invasion in GPER-positive, ERα-

negative cell lines [28,29] and overall support a central role for GPER in estrogen-induced OC cell migration, which is critical to cancer cell invasion and metastasis.

We next focused on the signaling proteins responsible for invasive potential of GPER in OC cells. Cell migration is achieved by the development of focal contacts involving the integrin-associated FAK phosphorylation and dephosphorylation according to cytoskeletal remodeling. In particular, cell adhesion to extracellular matrix causes the autophosphorylation of non-receptor tyrosine kinase FAK at Tyr397, thus creating a docking site for other signaling proteins involved in the regulation of cell motility and invasion [14]. Recent data support an interplay between PFKFB3 and FAK, suggesting that the functional activation of FAK is fostered by glycolysis in endothelial and cancer cells [18,19]. Indeed, the interrelated activation of these proteins is a common pro-migratory mechanism shared by several OC cell lines [25]. Remarkably, overexpression of PFKFB3 and FAK is an essential component of the malignant phenotype and invasive tumors such as OC. These proteins are therefore target of anticancer drugs [14,15]. We here show for the first time that G1 increased PFKFB3 levels as well as FAK phosphorylation at Tyr397 in OC cells. These data are consistent with the estrogen-mediated increase in PFKFB3 and FAK activity in endothelial cells of the tumor microenvironment promoting cell motility and angiogenesis [39,40], and highlight that estrogen signaling and OC aggressiveness are also related to GPER expression. These findings fill a gap in the literature and may be of relevance for the long-term response to antiestrogens, considering that GPER is activated by SERMs such as tamoxifen, which increases GPER expression in breast cancer cells [13]. Indeed, in the setting of breast cancer, GPER contributes to resistance to antiestrogenic agents [41], and patients with ERα-positive/GPER-positive tumors have lower survival rate than those who develop ERα-positive, GPER-negative tumors [13]. Although the functional and prognostic role of GPER in OC remains controversial [1,9], GPER expression may be considered in addition to ERα to predict response to endocrine therapy. GPER is also widely expressed in different tissues including heart and vessels, where its activation is linked to cardiovascular protection [42,43]. Hence, strategies to control estrogen signaling in a tissue-selective manner may represent a valuable alternative to non-selective antiestrogenic agents that inhibit all downstream signaling pathways.

miRNAs are small single-stranded non-coding RNA molecules that cause inhibition of protein translation or mRNA degradation, thereby acting as post-transcriptional regulators of protein abundance and functions. Since miRNAs have the potential to simultaneously reduce the expression of multiple signaling proteins [44], they could be useful to selectively target protein overexpressed in tumors including OC [45]. In this setting, recent findings from our group showed that miR-206 acts as an oncosuppressor in several OC cell lines including SKOV3 behaving as a dual anti-PFKFB3/FAK agent [25]. Accordingly, decreased miR-206 levels compared to controls have been found in OC tissues and EOC including SKOV3, which express the lowest miR-206 levels [46]. Moreover, several lines of evidence support a link between miR-206 and estrogen signaling. For example, E2 decreases miR-206 levels in breast cancer cells [47], while miR-206 decreases the expression of E2 target genes, thus contributing to estrogen signaling regulation [34]. In addition, Chen et al. [48] showed that E2 and the selective ERα agonist PPT decrease miR-206 expression in endometrial cancer cell lines, while miR-206 overexpression inhibits cell proliferation and invasion by regulating ERα expression.

In the present study, we report for the first time that miR-206 inhibited the increase in PFKFB3 levels induced by either E2 or G1 and, more important, prevented migration of SKOV3 and OVCAR5 cells induced by these estrogenic agents. In line with what observed in breast and endometrial cancer cells [34,48], we also found that transfecting SKOV3 cells with miR-206 significantly reduced ERα protein levels by about 60 % compared to miR-negative control, and provide the first evidence that miR-206 did not affect GPER levels. Hence, with respect to traditional SERMs including tamoxifen, miR-206 could be considered as a pathway-selective agent deprived of GPER agonist activity. Whether the reduction of ERα expression plays a role in the overall effect of miR-206 on GPER signaling remains to be clarified.

In summary, we here provide evidence that 1) GPER activation by estrogenic agents promotes EOC cell migration via PFKFB3 and FAK, key mediators of tumor progression and metastasis, and 2) miR-206 transfection reduces ERα levels and blocks the functional consequences of G1-mediated estrogen signaling in OC without targeting GPER, thus representing a potential strategy for treatment of OC characterized by GPER and ERα expression.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2023.122135.

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