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Cancer Research

GPER Mediates Activation of HIF1α/VEGF Signaling by Estrogens

Ernestina Marianna De Francesco[1], Michele Pellegrino[1], Maria Francesca Santolla[1], Rosamaria Lappano[1], Emilia Ricchio[1], Sergio Abonante[2], and Marcello Maggiolini[1]

Abstract

Biological responses to estrogens in normal and malignant tissues are mainly mediated by the estrogen receptors ERα and ERβ, which function as ligand-activated transcription factors. In addition, the G protein–coupled receptor GPR30 (GPER) mediates estrogenic signaling in breast cancer cells and cancer-associated fibroblasts (CAF) that contribute to cancer progression. In this study, we evaluated the role elicited by GPER in the estrogen-regulated expression and function of vascular endothelial growth factor (VEGF) in ER-negative breast cancer cells and CAF. We demonstrated that 17β-estradiol (E2) and the GPER-selective ligand G-1 triggered a GPER/EGFR/ERK/c-fos signaling pathway that leads to increased VEGF via upregulation of HIF1α. In further extending the mechanisms involved in E2-supported angiogenesis, we also showed that conditioned medium from CAF treated with E2 and G-1 promoted human endothelial tube formation in a GPER-dependent manner. *In vivo*, ligand-activated GPER was sufficient to enhance tumor growth and the expression of HIF1α, VEGF, and the endothelial marker CD34 in a mouse xenograft model of breast cancer. Our findings offer important new insights into the ability of estrogenic GPER signaling to trigger HIF1α-dependent VEGF expression that supports angiogenesis and progression in breast cancer. *Cancer Res; 74(15); 4053–64. ©2014 AACR.*

Introduction

Breast cancer is the most frequently diagnosed malignancy in women in the United States and the leading cause of cancer-related death in women worldwide (1). Although the molecular mechanisms involved in breast tumor development remain to be fully understood, it has been established that E2 triggers stimulatory effects by binding to the estrogen receptor (ER)α and ERβ that regulate the expression of genes involved in cell-cycle progression, cell migration, and survival (2, 3). In addition, the G protein–coupled receptor (GPR)30/GPER has been shown to mediate estrogenic signaling in different normal and malignant cell contexts, including breast cancer (3–7). In this regard, the identification of selective GPER agonists or antagonists (8–12) has allowed the evaluation of certain biological responses elicited through GPER. Actually, GPER activates a network of transduction pathways involving the epidermal growth factor receptor (EGFR), the intracellular cyclic AMP (cAMP), the mitogen-activated protein kinases (MAPK) cascade, and calcium mobilization (13–15). Moreover, the potential of GPER to mediate growth effects in diverse types of tumors has been evidenced (6, 7) together with its involvement in the estrogen responsiveness of ER-negative breast cancer cells (16). In accordance with these findings, the expression of GPER was associated with aggressive features and lower survival rates in patients with endometrial and ovarian cancer (17, 18), whereas GPER was negatively correlated with relapse-free survival and positively associated with the resistance to tamoxifen treatment in breast tumors (19). Therefore, GPER may be considered as a prognostic marker and a further player involved to some extent in the failure of endocrine therapy in estrogen-sensitive malignancies (20). In addition, the GPER/EGFR signaling mediates the expression of cell-cycle regulatory genes in cancer-associated fibroblasts (CAF) derived from patients with breast tumor, suggesting that the action of GPER may involve a functional interaction between these main components of the tumor microenvironment and cancer cells (21).

Tumor angiogenesis is a complex process initiated by paracrine signals occurring through tumor cells and the surrounding stroma (22). As the vascular endothelial growth factor-A (VEGF-A, also referred to as VEGF) mainly drives cancer progression upon hypoxic conditions (23), VEGF inhibitors are currently used in different chemotherapeutic strategies (24). Hence, great efforts are still addressed toward a deeper understanding of the transduction mechanisms involved in the expression and function of VEGF. In this regard, it should be mentioned that ERα mediates the upregulation of VEGF by 17β-estradiol (E2) in estrogen-sensitive tumors (25, 26). In addition, E2 induced through ERα the expression of hypoxia inducible factor-1α (HIF1α), which is an acknowledged VEGF

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regulator (27, 28). Nevertheless, it remains to be elucidated the potential of E2 in stimulating VEGF expression and angiogenesis in cell contexts lacking ER expression.

On the basis of the aforementioned data and our previous investigation showing that HIF1α/GPER signaling mediates the induction of VEGF by hypoxia in different model systems (29), in this study we have assessed the ability of ligand-activated GPER to regulate VEGF in ER-negative breast cancer cells as well as in CAFs and breast tumor xenografts. Our results provide novel insights into the ability of estrogens to induce VEGF expression and angiogenesis through GPER, hence extending the mechanisms through which estrogens trigger breast cancer progression.

Materials and Methods

Reagents

17β-Estradiol (E2), β-estradiol 6-(O-carboxy-methyl)oxime: BSA (E2-BSA), and 4-hydroxytamoxifen (OHT) were purchased from Sigma-Aldrich Srl. 1-[4-(-6-Bromobenzol[1,3]diodo-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c-] quinolin8yl] ethanone (G-1) and (3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G-15) were obtained from Tocris Bioscience. Tyrphostin AG1478 (AG) was purchased from Biomol Research Laboratories. PD98059 (PD) was obtained from Calbiochem. Human VEGF was purchased from Peprotech. All compounds were dissolved in dimethyl sulfoxide (DMSO) except E2 and OHT, which were solubilized in ethanol, and VEGF and E2-BSA, which were solubilized, respectively, in water and phosphate-buffered saline (PBS). Before use, E2-BSA stock solution was mixed with dextran (0.05 mg/mL) and charcoal (50 mg/mL) for 30 minutes, centrifuged at 3,000 × *g* for 10 minutes and passed through a 0.22-mm filter to remove the potential contamination of free E2.

Cell cultures

CAFs obtained from breast malignancies were characterized and maintained as we previously described (29). The SkBr3 breast cancer cells were obtained by ATCC, used less than 6 months after resuscitation, and maintained in RPMI-1640 without phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin/streptomycin (Life Technologies). Human umbilical vein endothelial cells (HUVEC), kindly provided by Dr. A. Caruso (University of Brescia, Brescia, Italy) and routinely tested and authenticated according to the ATCC suggestions, were seeded on collagen-coated flasks (Sigma-Aldrich Srl) and cultured in endothelial growth medium (EGM; Lonza), supplemented with 5% FBS (Lonza). Breast MCF-7 and prostate LNCaP cancer cells were obtained by ATCC, used less than 6 months after resuscitation, and maintained respectively in DMEM and RPMI-1640, both supplemented with 10% FBS and 100 μg/mL penicillin/streptomycin (Life Technologies). Cells were switched to medium without serum 24 hours before experiments.

Gene reporter assays

The 2.6-kb VEGF promoter-luciferase construct containing full-length VEGF promoter sequence (22,361 to +298 bp relative to the transcription start site) used in luciferase assays was a kind gift from Dr. P. Soumitro (Harvard Medical School, Boston, MA). The hypoxia responsive element (HRE)-mutated VEGF promoter (pVEGF-mut) reporter gene was kindly provided by Dr. J. Cheng (Shanghai Jiao Tong University School of Medicine, Shanghai, China). CAFs and SkBr3 cells (1×10^5) were plated into 24-well dishes with 500 μL/well culture medium containing 10% FBS and transfected for 24 hours with control shRNA, shHIF1α, and shGPER. A mixture containing 0.5 μg of reporter plasmid and 10 ng of pRL-TK was then transfected by using X-treme GENE 9 DNA Transfection Reagent, as recommended by the manufacturer (Roche Diagnostics). After 8 hours, cells were treated for 18 hours with E2 and G-1 in serum-free medium. Luciferase activity was measured with Dual Luciferase Kit (Promega) and normalized to the internal transfection control provided by *Renilla* luciferase. The normalized relative light unit values obtained from cells treated with vehicle were set as 1-fold induction, upon which the activity induced by treatments was calculated.

Gene expression studies

Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described (29). The expression of selected genes was quantified by real-time PCR using Step One sequence detection system (Applied Biosystems Inc.). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc.). For *ERα, ERβ, c-fos, HIF1α, VEGF*, and the ribosomal protein *18S*, the primers were: ERα Fwd: 5′-AGAGGGCATGGTGGAGATCTT-3′ and Rev: 5′-CAAACTCCTCTCCCTGCAGATT-3′; ERβ Fwd: 5′-GACCACAAGCCCAAATGTGTT-3′ and Rev: 5′-ACTGGCGATGGACCACTAAA-3′; c-fos: Fwd: 5′-GAGCCCTTTGATGACTTCCT-3′ and Rev: 5′-GAGCGGGCTGTCTCAGA-3′; HIF1α Fwd: 5′-TGCATCTCCATCTTCTACCCAAGT-3′ and Rev: 5′-CCGACTGTGAGTGCCACTGT-3′; VEGF: Fwd: 5′-TGCAGATTATGCGGATCAAACC-3′ and Rev: 5′-TGCATTCACATTTGTTGTGCTGTAG-3′; 18S Fwd: 5′-GGCGTCCCCCAACTTCTTA-3′ and Rev: 5′-GGGCATCACAGACCTGTTATT-3′. Assays were performed in triplicate and the RNA expression values were normalized using 18S expression and then calculated as fold induction.

Western blot analysis

SkBr3 cells, CAFs, and tumor homogenates obtained from nude mice were processed according to a previous described protocol (29). Protein lysates were electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel, electroblotted onto a nitrocellulose membrane and probed with primary antibodies against HIF1α (R&D Systems, Inc.), ERβ (Serotec), ERα (F-10), GPER (N-15), CD34 (ICO115), phosphorylated ERK1/2 (E-4), ERK2 (C-14), phosphorylated EGFR (Tyr 1173), EGFR (1005), and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA). Proteins were detected by horseradish peroxidase–linked secondary antibodies (Santa Cruz Biotechnology, DBA) and revealed using the ECL System (GE Healthcare).

Gene silencing experiments and plasmids

Cells were plated onto 10-cm dishes and transfected by X-treme GENE 9 DNA Transfection Reagent (Roche Molecular

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Biochemicals) for 24 hours before treatments with a control vector, a specific shRNA sequence for each target gene, the plasmid DN/c-fos encoding a c-fos mutant that heterodimerizes with c-fos dimerization partners but not allowing DNA binding (kindly obtained from Dr. C. Vinson, NIH, Bethesda, MD; ref. 30). The HIF1α shRNA and the respective control plasmid were purchased from SABioscience Corporation. The silencing of GPER expression was obtained by a construct previously described (31).

Immunofluorescence microscopy

SkBr3 cells and CAFs were grown on coverslips, then serum deprived and transfected for 24 hours with shHIF1α or shGPER and the respective control plasmids. Thereafter, cells were treated for 18 hours with E2 and G-1, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed 3 times with PBS, and incubated overnight with a mouse primary antibody against VEGF (C-1; Santa Cruz Biotechnology, DBA). After incubation, the slides were extensively washed with PBS and incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), (1:1,000; Sigma-Aldrich) and donkey anti-mouse IgG-FITC (1:300; purchased from Alexa Fluor; Life Technologies). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS) were used for the microscopy evaluation.

Conditioned medium

CAFs were cultured in regular growth medium, then cells were washed twice with PBS and transfected for 24 hours in serum-free RPMI-1640 with shGPER or control shRNA using X-treme GENE 9 DNA Transfection Reagent as recommended by the manufacturer (Roche Molecular Biochemicals). Cells were treated for 18 hours with E2 and G-1, the culture medium was then replaced for additional 18 hours with medium without serum and treatments. Thereafter, the supernatants were collected, centrifuged at 3,500 rpm for 5 minutes to remove cell debris, and used as conditioned medium in HUVECs.

Evaluation of E2 production

SkBr3 and CAFs cultured in regular growth medium were rinsed twice with PBS and incubated with serum-free medium for 24 hours. Culture supernatants were collected and centrifuged at 3,500 rpm for 5 minutes to remove cell debris. Three hundred pg/mL of E2 were added to collected supernatants in order to obtain a positive control. E2 production was measured by ELISA (Enzo Life Sciences) in three independent experiments performed in triplicate.

Tube formation assay

The day before the experiment, HUVECs were cultured in serum-free medium (EBM; Lonza). Growth factor–reduced Matrigel (Cultrex; Trevigen Inc.) was thawed overnight at 4°C on ice, plated on the bottom of prechilled 96-well plates, and left at 37°C for 1 hour for gelification. Starved HUVECs were collected by enzymatic detachment (0.25% trypsin-EDTA solution; Life Technologies), counted, and resuspended in conditioned medium from CAFs. Then, 10,000 cells/well were seeded on Matrigel and incubated at 37°C. Cord formation was observed 2 hours after cell seeding. Tube formation was quantified by using the software NIH ImageJ.

Proliferation assay

For quantitative proliferation assay, SkBr3 cells (1×10^5) were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped FBS with the indicated treatments; medium was renewed every 2 days (with treatments) and cells were counted using the Countess Automated Cell Counter, as recommended by the manufacturer's protocol (Life Technologies).

In vivo studies

Female 45-day-old athymic nude mice (nu/nu Swiss; Harlan Laboratories) were maintained in a sterile environment. At day 0, exponentially growing SkBr3 cells (8.0×10^6 per mouse) were inoculated subcutaneously in 0.1 mL of Matrigel (Cultrex; Trevigen Inc.). When the tumors reached average ~0.15 cm^3 (i.e., in about 1 week), mice were randomized and divided into four groups, according to treatments administered by intramuscular injection for 40 days. The first group of mice ($n = 7$) was treated daily with 100 μL of vehicle (0.9% NaCl with 0.1% albumin and 0.1% Tween-20; Sigma-Aldrich), the second group of mice ($n = 7$) was treated daily with 100 μL G-1 (0.5 mg/kg/die), the third group of mice ($n = 7$) was treated daily with 100 μL G-15 (3.5 mg/kg/die), and the fourth group of mice ($n = 7$) was treated daily with 100 μL G-1 in combination with G-15 (at the concentrations described above). G-1 and G-15 were dissolved in DMSO at 1 mg/mL. For treatments, 6.2 μL of G-1 were added to 93.8 μL of vehicle and 44 μL G-15 were added to 56 μL of vehicle. The doses of G-15 were chosen to represent an approximately 1:7-fold μg excess with respect to G-1. SkBr3 xenograft tumor growth was evaluated by caliper measurements, along two orthogonal axes: length (L) and width (W). Tumor volumes (in cm^3) were estimated by the following formula: $TV = L \times (W^2)/2$. At day 40, the animals were sacrificed following the standard protocols and tumors were dissected from the neighboring connective tissue. Specimens of tumors were frozen in nitrogen and stored at −80°C, the remaining tumor tissues of each sample were fixed in 4% paraformaldehyde and embedded in paraffin for the histologic analyses. Animal care, death, and experiments were done in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and in accordance with the Italian law (DL 116, January 27, 1992). The project was approved by the local ethical committee.

Histologic analysis

Formalin-fixed, paraffin-embedded sections of tumor xenografts were cut at 5 μm and allowed to air dry. Deparaffinized, rehydrated sections were stained for 6 minutes with hematoxylin (Bio-Optica), washed in running tap water,

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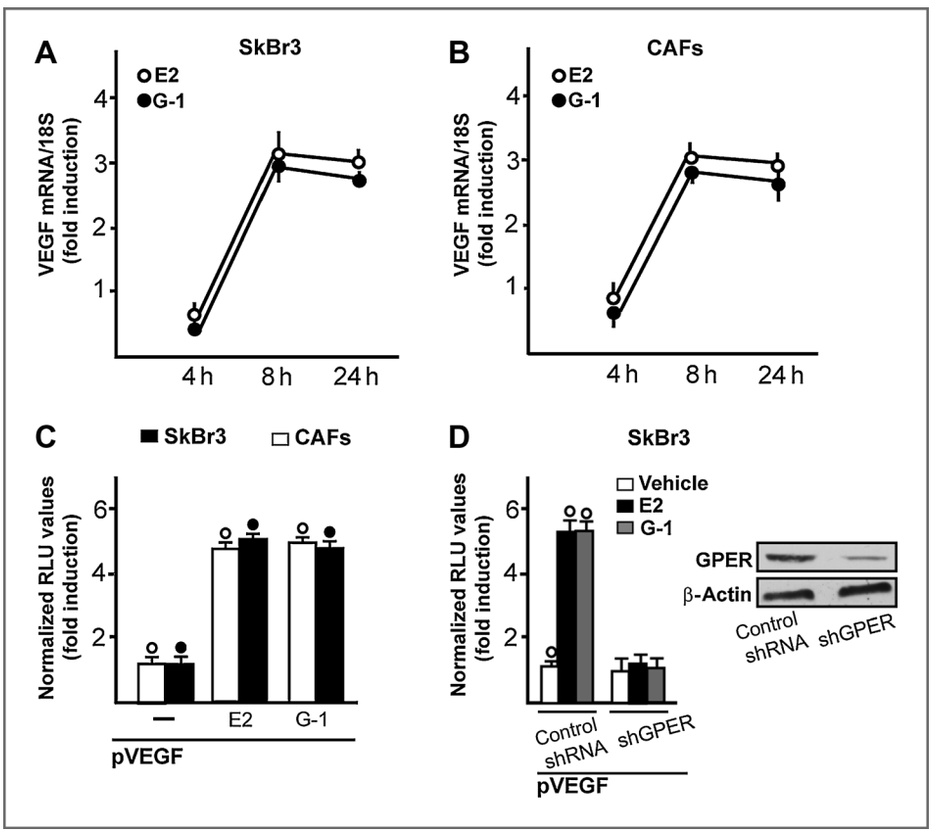

Figure 1. E2 and G-1 induce the expression of VEGF. In SkBr3 cells (A) and CAFs (B), 1 nmol/L E2 and 1 μmol/L G-1 upregulate the mRNA expression of *VEGF*, as evaluated by real-time PCR. Gene expression was normalized to 18S expression and results are shown as fold changes of mRNA expression compared with cells treated with vehicle. C, an expression vector encoding the VEGF promoter sequence (pVEGF) is transactivated in SkBr3 cells and CAFs treated for 18 hours with 1 nmol/L E2 and 1 μmol/L G-1. D, in SkBr3 cells, the transactivation of the pVEGF induced by a 18-hour treatment with 1 nmol/L E2 and 1 μmol/L G-1 is abrogated, silencing the expression of GPER. Side panel shows the efficacy of GPER silencing, as evaluated by immunoblotting. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction, upon which the activities induced by treatments were calculated. Each data point represents the mean ± SD of three independent experiments performed in triplicate. ○ and ●, $P < 0.05$ for cells receiving vehicle (−) versus treatments.

and counterstained with eosin Y (Bio-Optica). Sections were then dehydrated, cleared with xylene, and mounted with resinous mounting medium. Morphologic analyses were carried out on paraffin-embedded tumor sections, stained with hematoxylin and eosin (H&E), or immunolabeled with human cytocheratin 18 (Santa Cruz Biotechnology, DBA). Tumor sections were immunolabeled with CD34, which served as a vessel marker (32, 33).

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls' testing to determine differences in means. Statistical comparisons for *in vivo* studies were made using the Wilcoxon–Mann–Whitney test. $P < 0.05$ was considered statistically significant.

Results

E2 and G-1 induce VEGF expression through GPER

Considering the ability of estrogens to stimulate the expression of the proangiogenic mediator VEGF (25, 26), we aimed to evaluate whether GPER may be involved in this response to estrogens. To this end, we used as a model system the SkBr3 breast cancer cells and CAFs that do not express the classical ERs (Supplementary Fig. S1) and are not able to produce E2 following our experimental conditions (Supplementary Fig. S1). As determined by real-time PCR, the selective GPER agonist G-1 and E2 induced the mRNA expression of *VEGF* (Fig. 1A and B). Accordingly, G-1 and E2 transactivated a VEGF promoter construct (Fig. 1C) through GPER, as the luciferase activity was repressed knocking down the expression of GPER in SkBr3 cells (Fig. 1D) and CAFs (data not shown). The aforementioned findings were then confirmed by immunofluorescence experiments performed in both SkBr3 cells (Fig. 2) and CAFs (Supplementary Fig. S2), further corroborating the involvement of GPER in the upregulation of VEGF protein expression induced by estrogens in these cells.

HIF1α expression is regulated by estrogens through GPER along with the EGFR/ERK/c-FOS transduction pathway

On the basis of previous data showing that estrogens induce the expression of HIF1α, which has been largely involved in the transcriptional regulation of VEGF (27, 28), we asked whether this action of estrogens may occur through GPER. Notably, E2 and G-1 upregulated the mRNA expression (Fig. 3A and B) and the protein levels of HIF1α (Fig. 3C–F) in a GPER-dependent manner as the GPER silencing abrogated these responses (Fig. 3G and H and Supplementary Fig. S3). Next, the HIF1α protein increase by E2 and G-1 was abrogated in presence of the EGFR inhibitor AG1478 (AG), the MEK inhibitor PD, or using a plasmid encoding a c-fos mutant named dominant/negative c-fos (DN/c-fos) in both SkBr3 cells (Fig. 4A and B) and CAFs (Fig. 4C and D). Taken together, these data indicate that

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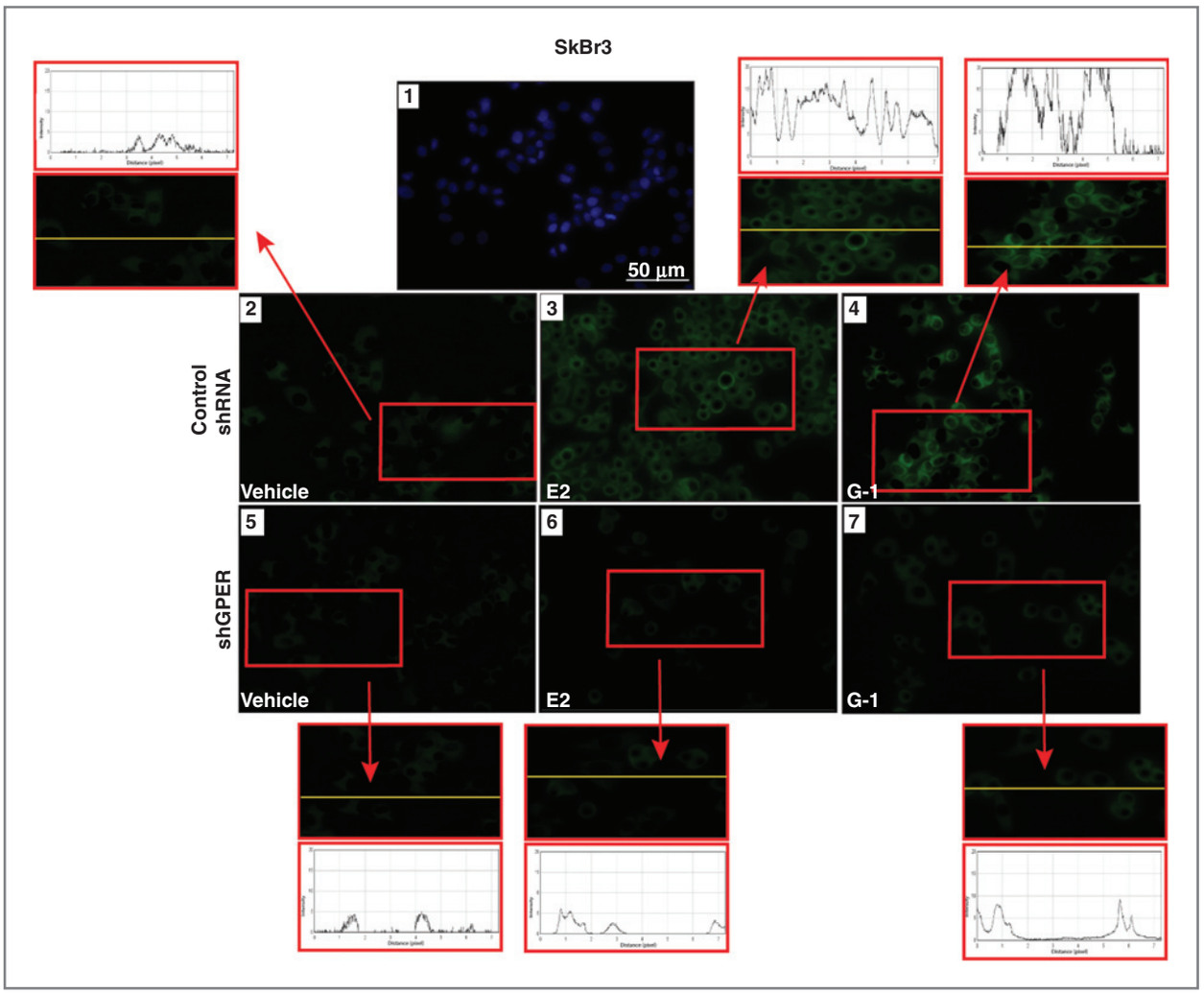

Figure 2. GPER mediates the upregulation of VEGF protein expression induced by E2 and G-1. Evaluation of VEGF protein expression by immunofluorescent microscopy in SkBr3 cells transfected for 24 hours with control shRNA (panels 2–4) or shGPER (panels 5–7). Cells were treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1, as indicated; the accumulation of VEGF is evidenced by the green signal. Nuclei were stained by DAPI (blue signal in panel 1). Using the program WCIF Image J for Windows for expressive analysis, the plot profiles obtained at the level of the yellow line of the corresponding inset are shown in each side panel. Note the high values indicating zones of intense labeling. Images shown are representative of 20 random fields of three independent experiments.

GPER along with the EGFR/ERK/c-fos transduction pathway mediate the HIF1α expression induced by E2 and G-1.

HIF1α is involved in the upregulation of VEGF induced by E2 and G-1

Considering that HIF1α exerts a crucial role in the regulation of VEGF in diverse pathophysiologic conditions, we aimed to evaluate whether the VEGF protein induction by E2 and G-1 is mediated by HIF1α. Performing immunofluorescence assays we ascertained that the upregulation of VEGF induced by these ligands is abrogated knocking down HIF1α expression in both SkBr3 cells (Fig. 5 and Supplementary Fig. S3) and CAFs (Supplementary Figs. S3 and S4). Accordingly, the transactivation of a VEGF promoter construct triggered by E2 and G-1 was prevented silencing HIF1α expression in SkBr3 cells (Supplementary Fig. S5) and CAFs (data not shown). Likewise, the luciferase activity induced by E2 and G-1 was prevented transfecting cells with a HRE-mutated VEGF promoter construct (Supplementary Fig. S5). It is worth noting that E2 and G-1 were also not able to stimulate VEGF expression transfecting both SkBr3 cells and CAFs with the DN/c-fos plasmid (data not shown).

Further supporting the aforementioned findings, in SkBr3 cells the membrane impermeable E2-BSA induced the EGFR and ERK1/2 phosphorylation (Supplementary Fig. S6) as well as the upregulation of c-fos, HIF1α, and VEGF mRNA expression (Supplementary Fig. S6). In addition, E2 and G-1 alone and in the presence of the ER antagonist OHT promoted the activation of EGFR and ERK1/2 (Supplementary Fig. S6) as well as the upregulation of *c-fos*, *HIF1α*, and *VEGF* mRNA

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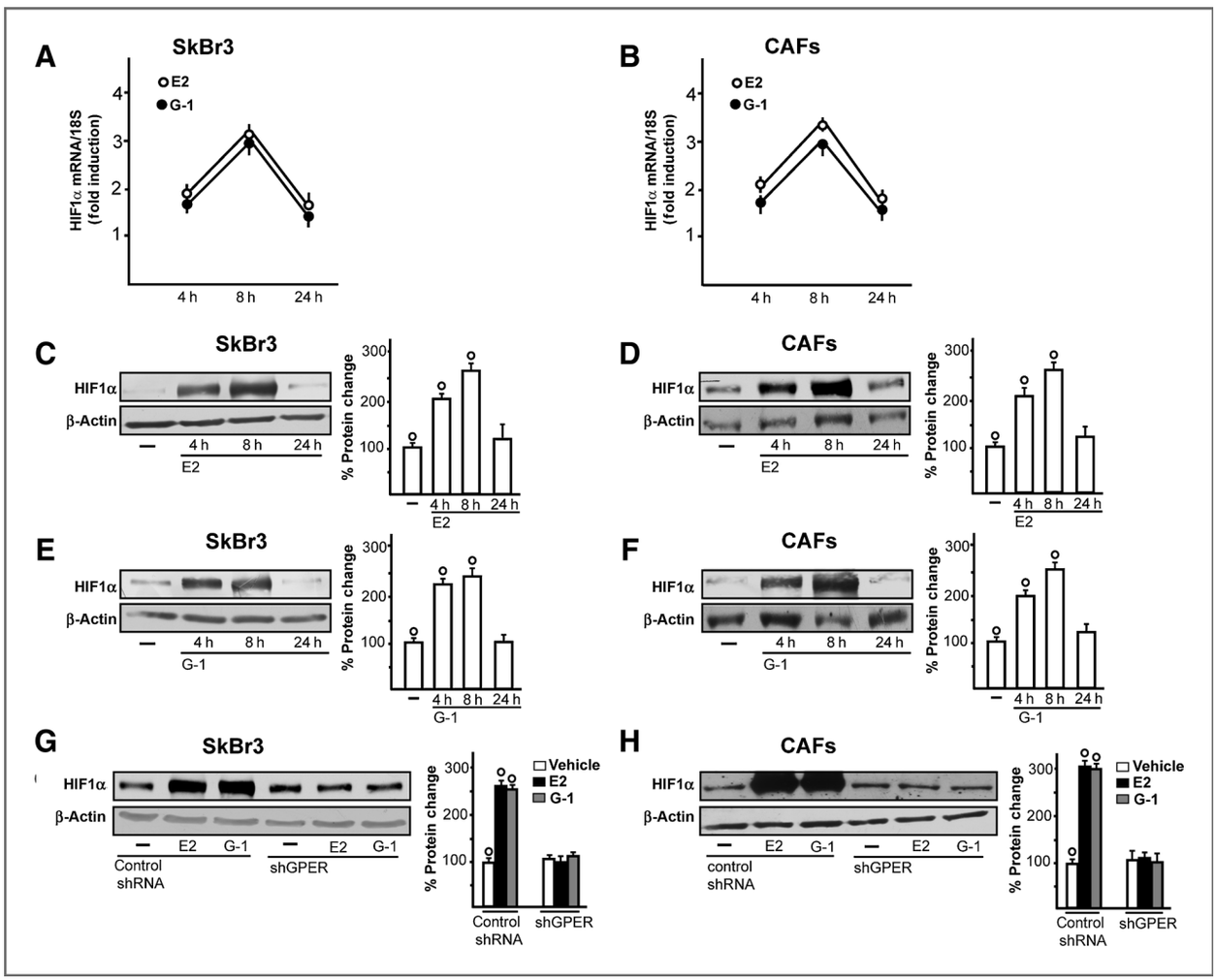

Figure 3. The HIF1α expression induced by E2 and G-1 occurs through GPER. In SkBr3 cells and CAFs, 1 nmol/L E2 and 1 μmol/L G-1 upregulate HIF1α expression at both mRNA (A and B) and protein levels (C–F), as indicated. SkBr3 (G) cells and CAFs (H) were transfected with control shRNA or shGPER for 24 hours and then treated for 12 hours with vehicle (–), 1 nmol/L E2, and 1 μmol/L G-1. Gene expression was evaluated by real-time PCR and normalized to 18S expression. Results are shown as fold changes of mRNA expression compared with cells treated with vehicle. Protein expression was evaluated by immunoblotting experiments and side panels show densitometric analysis of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. ○, $P < 0.05$ for cells receiving vehicle (–) versus treatments.

expression (Supplementary Fig. S6). It is worth noting that OHT stimulated the aforementioned responses (Supplementary Fig. S6) acting as a GPER agonist, according to previous studies (4, 7). Altogether, these data may suggest that GPER mediates the estrogen-induced expression of VEGF through the EGFR/ERK/c-fos transduction pathway and the involvement of HIF1α.

GPER is involved in VEGF-mediated tube formation induced by E2 and G-1

Having established that GPER mediates the estrogen-induced upregulation of VEGF, we analyzed the potential role of GPER in endothelial tubulogenesis. HUVECs that represent a useful model system for *in vitro* evaluation of neoangiogenesis (34) were cultured using conditioned medium from CAFs previously treated with E2 and G-1. Interestingly, a ramified network of tubules was generated in HUVECs grown in medium from CAFs treated with E2 and G-1 (Fig. 6A), whereas these ligands had no effects knocking down the expression of GPER in CAFs (Fig. 6B). Further supporting these data, the addition of VEGF to medium collected from GPER-silenced CAFs rescued the generation of tubule structures in HUVECs (Fig. 6C). These results, recapitulated in Fig. 6D–F, indicate that VEGF may be considered as a target of the estrogenic GPER signaling toward new blood vessels formation.

GPER mediates HIF1α and VEGF expression along with growth effects in breast cancer xenografts

Then, we turned to an *in vivo* model system. Female nude mice bearing into the intrascapular region SkBr3 cell tumor xenografts were treated with vehicle, G-1 alone, and in combination with the GPER antagonist G-15 (10). These

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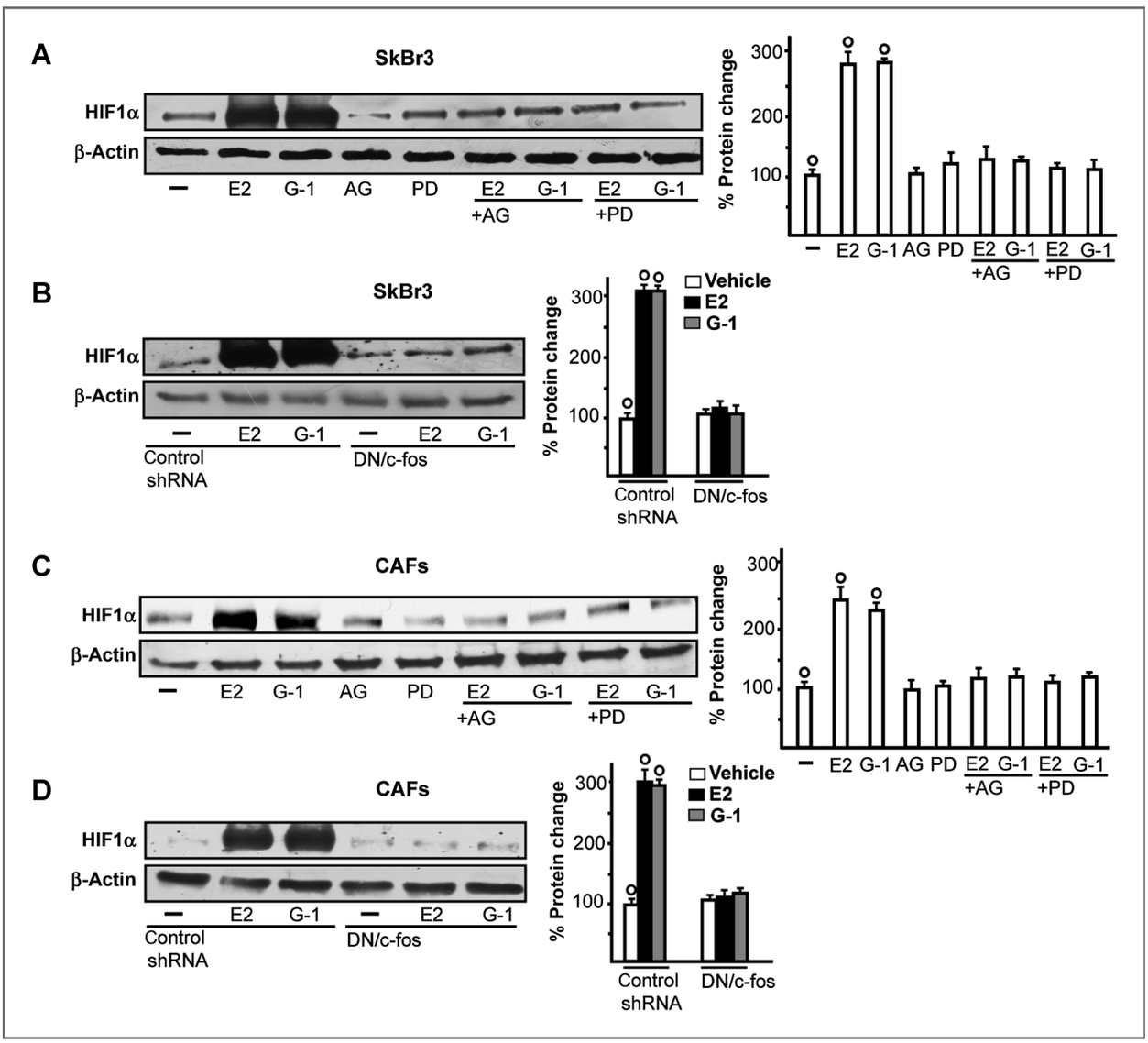

Figure 4. The EGFR/ERK/c-FOS transduction pathway mediates the HIF1α expression induced by E2 and G-1. Immunoblots showing HIF1α protein expression in SkBr3 cells (A) and CAFs (C) treated for 12 hours with vehicle (–), 1 nmol/L E2, and 1 μmol/L G-1 alone or in combination with 10 μmol/L EGFR inhibitor AG1478 (AG), 10 μmol/L MEK inhibitor PD98089 (PD). Evaluation of HIF1α protein expression in SkBr3 cells (B) and CAFs (D), which were transfected for 24 hours with control shRNA or a plasmid encoding for a dominant negative form of c-fos (DN/c-fos) and treated for 12 hours with vehicle (–), 1 nmol/L E2 and 1 μmol/L G-1. Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. ○, $P < 0.05$ for cells receiving vehicle (–) versus treatments.

administrations were well tolerated as no change in body weight or in food and water consumption was observed together with no evidence of reduced motor function. In addition, no significant difference in the mean weights or histologic features of the major organs (liver, lung, spleen, and kidney) was observed after sacrifice among vehicle and ligands-treated mice, thus indicating a lack of toxic effects. Histologic examination of SkBr3 xenografts revealed that tumors were primarily composed of tumor epithelial cells (Supplementary Fig. S7). After 40 days of treatment, the tumor growth induced by G-1 was prevented by G-15 (Fig. 7A). Representative tumor images are shown in Fig. 7B. As evidenced in Supplementary Fig. S6, E2 and G-1 stimulated the proliferation of SkBr3 cells also in growth assays performed *in vitro*. Next, we found increased HIF1α and VEGF protein levels in tumor homogenates obtained from G-1–stimulated mice with respect to vehicle-treated mice; however, these stimulatory effects were abrogated in the animal group receiving G-15 in addition to G-1 (Fig. 7C). The acknowledged marker of endothelial cell proliferation CD34 (32, 33) paralleled the increased expression of HIF1α and VEGF upon treatments (Fig. 7C and Supplementary Fig. S8), suggesting that ligand-activated GPER may stimulate tumor growth and angiogenesis.

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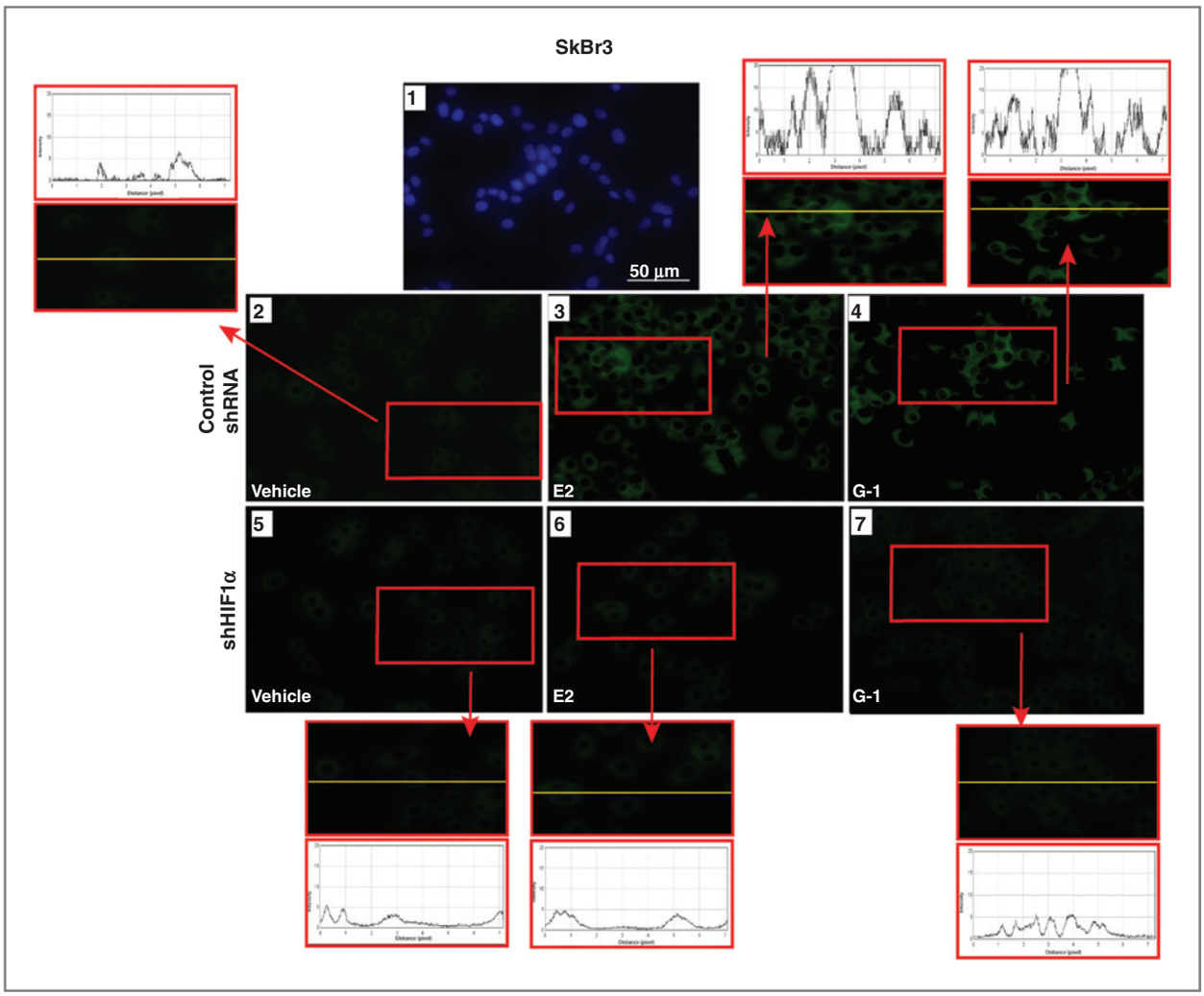

Figure 5. VEGF expression induced by E2 and G-1 occurs through HIF1α. Evaluation of VEGF expression by immunofluorescent microscopy in SkBr3 cells transfected for 24 hours with control shRNA (panels 2–4) or shHIF1α (panels 5–7). Cells were treated with vehicle, 1 nmol/L E2, and 1 μmol/L G-1 for 18 hours, as indicated. VEGF accumulation is evidenced by the green signal. Nuclei were stained by DAPI (blue signal in panel 1). Using the program WCIF Image J for Windows for expressive analysis, the plot profiles obtained at the level of the yellow line of the corresponding inset are shown in each side panel. Note the high values, indicating zones of intense labeling. Images shown are representative of 20 random fields of three independent experiments.

Discussion

In this study, we have demonstrated that GPER mediates the upregulation of VEGF expression induced by E2 and G-1 in breast cancer cells and CAFs. In particular, we have ascertained that GPER activation by both ligands engages the EGFR/ERK/c-fos transduction signaling toward the induction of HIF1α and VEGF transcription. As a biological counterpart, we have evidenced that GPER mediates endothelial tube formation in HUVECs cultured in medium from CAFs, which were previously treated with E2 and G-1. In addition, we have determined that G-1 induces growth effects in SkBr3 tumor xenografts and increases the expression of HIF1α, VEGF, and CD34 in tumor homogenates. Therefore, the present findings provide novel insights into the potential of GPER to mediate estrogen-dependent regulation and function of VEGF, which plays a main role in tumor angiogenesis and progression (23, 24).

Breast cancer is stimulated by estrogens that activating the classical ERs modulate cell proliferation, adhesion, migration, and invasion in estrogen-sensitive tumors (2, 3). In addition, estrogens promote the formation of new blood vessels within the tumor mass (35), hence suggesting that these steroids elicit a stimulatory role not only in cancer cells but also in components of the surrounding stroma in accordance with the results obtained in this study. As the complex process of angiogenesis is required for tumor progression, it represents a central biological target in cancer (36). VEGF is one of the most potent proangiogenic factor playing a paramount role in the formation of blood vessels in the development of different types of tumors, including breast cancer (23). Accordingly, VEGF is highly expressed in breast cancer specimens compared with normal breast tissue (37) and its suppression leads to the inhibition of breast tumor development (38). Diverse factors

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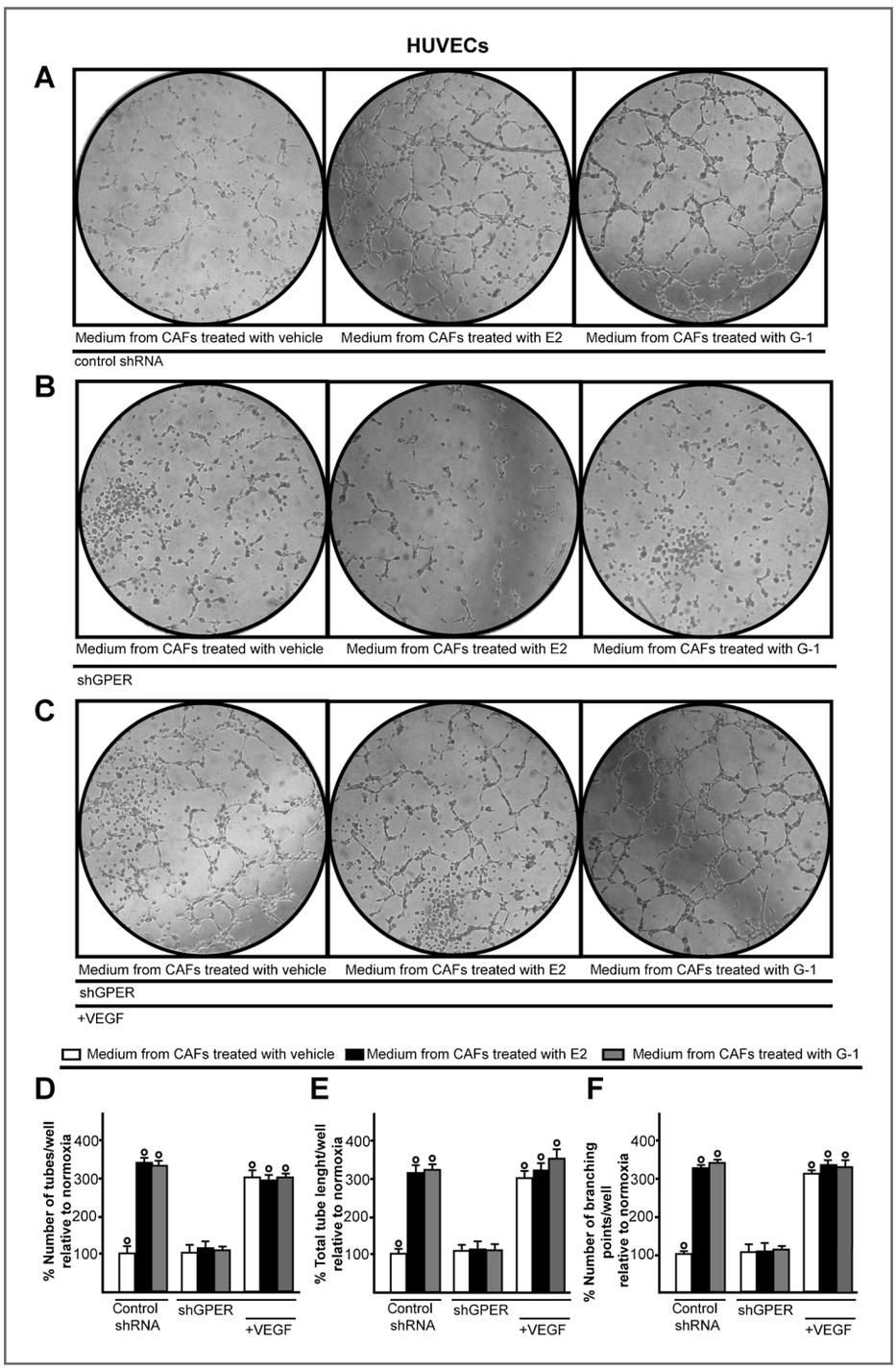

Figure 6. GPER mediates the endothelial tube formation triggered by E2 and G-1. Tube formation was evaluated in HUVECs cultured for 2 hours in medium collected from CAFs transfected for 24 hours with control shRNA (A) or shGPER (B) and then treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1, as indicated. C, tube formation is rescued using 10 ng/mL VEGF in HUVECs cultured in medium from CAFs, which were transfected for 24 hours with shGPER and then treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1. Quantification of the number of tubes (D), total tube length (E), and number of branching points (F) observed in HUVECs. Data are representative of three independent experiments performed in triplicate. ○, $P < 0.05$ for cells receiving medium of CAFs treated with vehicle versus medium of CAFs treated with ligands.

regulate VEGF expression like hypoxia, cytokines, growth factors, and hormones (23). In this regard, previous studies have demonstrated the ability of estrogens to upregulate VEGF levels activating ERα, which binds to the estrogen response elements (ERE) located within the VEGF promoter region (25, 26, 39). As these studies evaluated the mechanisms involved in the estrogen-regulated VEGF expression in ER-positive cells, in this study we evaluated the potential of estrogens to trigger the transcription of VEGF in ER-negative cells. In particular, using both *in vitro* and *in vivo* model systems we have provided novel evidence about the GPER-mediated stimulation of VEGF, although further investigations are needed to better clarify the role played by GPER in the modulation of VEGF in diverse pathophysiologic conditions.

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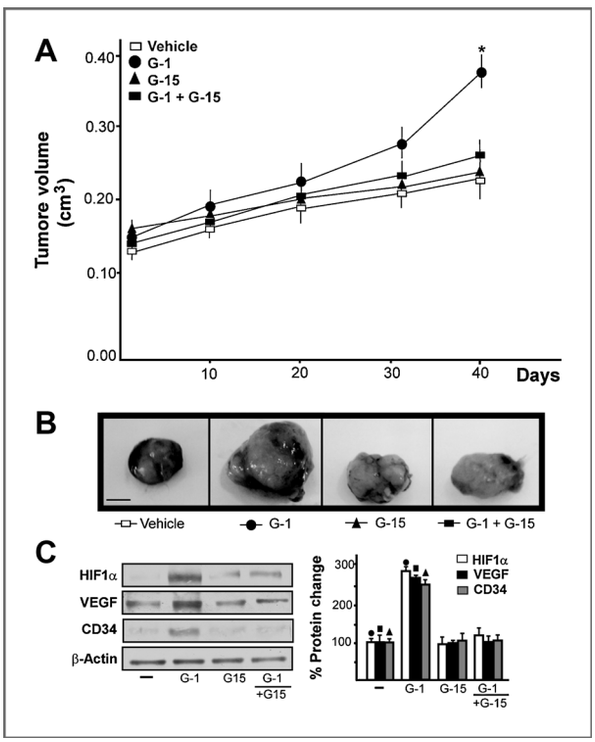

Figure 7. GPER is involved in SkBr3 cells xenograft growth. A, tumor volume from SkBr3 xenografts implanted in female athymic nude mice treated for 40 days with vehicle, G-1 (0.25 mg/kg/die), and G-15 (3.5 mg/kg/die) alone or in combination, as indicated. *, $P < 0.05$ for G-1–treated animals versus vehicle-treated animals. B, representative images of experimental tumors at day 40; scale bar, 0.3 cm. HIF1α, VEGF, and CD34 protein expression in tumor homogenates from SkBr3 xenografts treated as reported above (C). Side panels show densitometric analysis of the blots normalized to β-actin. ●, ■, ▲, $P < 0.05$ for G-1–treated animals versus vehicle-treated animals.

Interestingly, our data may recall previous findings showing that the ER antagonist and GPER agonist OHT (4, 7) is able to upregulate VEGF in different tumor types, including breast cancer (40, 41). In addition, it has been recently reported that low survival rates in patients with endometrial cancer are associated with both elevated GPER expression and VEGF levels (42), further providing a relationship between these 2 main players of tumor cells and the surrounding stroma.

HIF1α constitutes with HIF1β the active transcriptional complex HIF1 that regulates many genes involved in important biological functions in cancer cells like energy metabolism, survival, cell migration, and neovascularization (43). Cytokines, growth factors, and hormones beyond hypoxia were shown to upregulate the expression of HIF1α (44). For instance, in normoxic conditions activated EGFR increased HIF1α expression through the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and MAPK transduction pathways (45). Further extending these data, our present results indicate that the GPER/EGFR/ERK/c-fos transduction signaling is involved in the upregulation of HIF1α by estrogens, thus providing novel insights into the mechanisms mediating the HIF1α-dependent stimulation of VEGF. Moreover, the current results corroborate our previous studies showing that a cross-talk between HIF1α and GPER regulates the expression and function of VEGF upon hypoxic conditions (29).

The stromal contribution to the development of a wide variety of tumors has been extensively assessed using both *in vitro* and *in vivo* model systems (46, 47). For instance, it has been shown that malignant cells may recruit into the tumor mass diverse components of the microenvironment like CAFs, inflammatory and vascular cells that actively cooperate toward cancer progression (46, 47). In particular, increasing evidence has suggested that CAFs contribute to cancer aggressiveness through the production of secreted factors that target numerous stromal components and cancer cell types (46–48). In breast carcinomas, CAFs have been shown to elicit relevant biological activities including the stimulation of new blood vessels formation, which closely correlates with cancer growth, metastasis, and poor prognosis (29, 46–48). Our results further extend these findings as the medium collected from CAFs, which were stimulated by estrogens, induced tube formation in HUVECs. This response occurred through the GPER-mediated release of VEGF, suggesting that the paracrine signaling between CAFs and endothelial cells may trigger angiogenic processes toward tumor progression. Likewise, the growth effects observed in breast cancer xenografts upon ligand-activated GPER were paralleled in tumor homogenates by an increased expression of HIF1α, VEGF, and the vessel marker CD34. In line with these data, an enhanced tumor growth occurred *in vivo* using breast cancer cells engineered to express elevated levels of VEGF (49), on the contrary the inhibition of VEGF led to the growth arrest of breast carcinomas in nude mice (38). In addition, an increased VEGF expression upon E2 exposure was found in rat mammary cancer (50), further highlighting the main role played by VEGF in breast tumorigenesis.

The present data provide novel insights into the potential of estrogenic GPER signaling to trigger the HIF1-mediated increase of VEGF toward angiogenesis and cancer progression. Furthermore, the paracrine responses mediated by GPER extend the current knowledge on the critical interaction between cancer cells and the surrounding stroma, which plays a pivotal role in tumor development and metastasis. Altogether, these findings may be taken into account in setting novel therapeutic strategies targeting the stimulatory action of estrogens in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E.M. De Francesco, M. Maggiolini
Development of methodology: E.M. De Francesco, M.F. Santolla, R. Lappano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.M. De Francesco, M. Pellegrino, M.F. Santolla, E. Ricchio, S. Abonante
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.M. De Francesco, M. Pellegrino, E. Ricchio, M. Maggiolini
Writing, review, and or revision of the manuscript: E.M. De Francesco, M. Maggiolini
Study supervision: M. Maggiolini

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GPER Mediates Activation of HIF1α/VEGF Signaling by Estrogens

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