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GPER1 is regulated by insulin in cancer cells and cancer-associated fibroblasts

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Abstract

Elevated insulin levels have been associated with an increased cancer risk as well as with aggressive and metastatic cancer phenotypes characterized by a poor prognosis. Insulin stimulates the proliferation, migration, and invasiveness of cancer cells through diverse transduction pathways, including estrogen signaling. As G protein estrogen receptor 1 (GPER1) mediates rapid cell responses to estrogens, we evaluated the potential of insulin to regulate GPER1 expression and function in leiomyosarcoma cancer cells (SKUT-1) and breast cancer-associated fibroblasts (CAFs), which were used as a model system. We found that insulin transactivates the *GPER1* promoter sequence and increases the mRNA and protein expression of GPER1 through the activation of the PRKCD/MAPK1/c-Fos/AP1 transduction pathway, as ascertained by means of specific pharmacological inhibitors and gene-silencing experiments. Moreover, cell migration triggered by insulin occurred through GPER1 and its main target gene *CTGF*, whereas the insulin-induced expression of GPER1 boosted cell-cycle progression and the glucose uptake stimulated by estrogens. Notably, a positive correlation between insulin serum levels and GPER1 expression was found in cancer fibroblasts obtained from breast cancer patients. Altogether, our data indicate that GPER1 may be included among the complex network of transduction signaling triggered by insulin that drives cells toward cancer progression.

Key Words

- ▶ insulin
- ▶ GPER1
- ▶ estrogen
- ▶ cancer cells
- ▶ cancer-associated fibroblasts (CAFs)

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Introduction

Insulin, a key mediator of important metabolic functions, coordinates and regulates the storage and release of the body's fuel (Belfiore & Malaguarnera 2011). Insulin, which is secreted by β -cells of the pancreas in response to increasing blood glucose levels, binds to the cognate receptor (INSR) mainly expressed by hepatocytes, adipocytes, and muscle cells as well as prostate and breast tissues

(Belfiore & Malaguarnera 2011). So far, two INSR isoforms have been identified, isoform A (INSR-A) and isoform B (INSR-B), which are usually co-expressed and regulated by several factors (Belfiore 2007). Expression of INSR-A has been predominantly detected in fetal tissues and tumors as found in leiomyosarcoma cells (Vigneri *et al.* 2009, Morcavallo *et al.* 2011). Aside from its important

contribution to maintenance of metabolic activity and glucose homeostasis, insulin shows mitogenic potential which can lead to an increased risk of certain types of cancer (Belfiore & Malaguarnera 2011). Accordingly, it has been suggested that direct INSR stimulation activates diverse transduction mechanisms involved in tumor development (Belfiore & Malaguarnera 2011). Moreover, in cancer patients affected by insulin resistance, increased insulin levels combine with frequent INSR overexpression in tumor cells, leading to abnormal stimulation of nonmetabolic effects mediated by INSR, such as cell survival, proliferation, and migration (Belfiore & Malaguarnera 2011). In particular, high insulin levels are associated with an augmented risk of breast cancer and breast cancer relapses in diabetic and nondiabetic women (Duggan et al. 2011, Cohen & Le Roith 2012, Sieri et al. 2012). It has been well established that a cooperative crosstalk between insulin and estrogen signaling pathways triggers multiple biological events in breast carcinogenesis (Rose & Vona-Davis 2012, Catsburg et al. 2014). Estrogens mainly act through the classical estrogen receptor α (ESR1) and β (ESR2) (Hall et al. 2001), however many effects induced by these steroids are mediated by the G protein estrogen receptor 1 (GPER1, formerly known as GPR30) in several types of tumor cells and cancer-associated fibroblasts (CAFs), major players in the tumor microenvironment driving tumor progression (Madeo & Maggiolini 2010, Pupo et al. 2012, Lappano et al. 2013). In this respect, the recent identification of molecules acting as selective agonist or antagonist ligands of GPER1 has allowed the evaluation of the transduction mechanisms involved in the estrogenic GPER1 signaling in numerous pathophysiological conditions (Dennis et al. 2011, Lappano et al. 2012, Rosano et al. 2012). Indeed, GPER1 has been implicated not only in cancer, but also in cardiovascular, immunological, and neurological functions as well as diabetes (Mizukami 2010). GPER1 has been proposed as an interesting therapeutic target in diabetes and pancreatic islet transplantation (Mårtensson et al. 2009, Liu et al. 2013). Accordingly, GPER1 has been detected in pancreatic β -cells and GPER1 ligands have shown insulinotropic effects by mediating pancreatic β -cell survival and stimulating insulin release (Liu et al. 2009, Balhuizen et al. 2010). Pharmacological manipulations and gene deletion of *Gper1* in mice (*Gpr30*^{-/-}) were associated with an altered insulin release upon estrogen exposure (Mårtensson et al. 2009, Balhuizen et al. 2010). GPER1 deficiency resulted also in insulin resistance, dyslipidemia, obesity, and increased circulating pro-inflammatory cytokines, indicating a role for GPER1 in the regulation of metabolism and the inflammatory state (Sharma et al. 2013).

As regards the regulation of GPER1, our previous studies have shown that some important growth-factor-mediated transduction pathways such as EGFR (Albanito et al. 2008, Vivacqua et al. 2009) and IGF1 (Bartella et al. 2012, De Marco et al. 2013) are involved in the expression and function of GPER1 in cancer cells. Interestingly, high levels of expression of GPER1 in breast, endometrial, and ovarian tumors have been associated with a higher risk of developing metastatic disease and poor survival (Prossnitz & Barton 2011). Moreover, high levels of GPER1 were identified in inflammatory breast cancer (IBC), an aggressive and commonly hormone-independent form of breast cancer (Arias-Pulido et al. 2010). Recently, the overexpression of GPER1 and its plasma membrane localization have been suggested to be critical events in breast cancer progression, whereas the lack of GPER1 in the plasma membrane was associated with excellent long-term prognosis in ESR1-positive (Sjöström et al. 2014) tamoxifen-treated breast cancer.

In this study, we ascertained the capability of insulin in regulating GPER1 expression and activity in INSR-positive CAFs and SKUT-1 leiomyosarcoma cells, which were used as model systems. In particular, our results reveal that insulin up-regulates GPER1 by the activation of the PRKCD/MAPK1/c-Fos/AP1 transduction pathway. In addition, GPER1 and its target gene *CTGF* were required for insulin-induced cell migration. Also, the up-regulation of GPER1 by insulin boosted the glucose uptake and cell-cycle progression upon estrogen exposure. Interestingly, a significant positive correlation between GPER1 expression and serum insulin levels have been found in CAFs obtained from breast cancer patients. Our data provide novel insights into the functional crosstalk between GPER1 signaling and the insulin-mediated transduction pathway, highlighting the potential of GPER1 to contribute to cancer progression in patients with elevated insulin levels.

Materials and methods

Reagents

Bovine insulin and 17 β -estradiol (E₂) were purchased from Sigma-Aldrich Corp. Bisindolylmaleimide I (GF109203X), PD98059 (PD), Rottlerin, and Tyrphostin AG1478 were bought from Calbiochem (Milan, Italy). ICI 182 780 (ICI) and (3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G15) were obtained from Tocris Bioscience (Bristol, UK). Insulin was solubilized in HEPES 25 mM. PD and E₂ were dissolved

in ethanol, while GFX, Rottlerin, and AG were solubilized in dimethylsulfoxide.

Cell culture

R⁻ mouse fibroblasts (kindly provided by Renato Baserga, Philadelphia, PA, USA) are mouse 3T3-like cells derived from animals with a targeted disruption of the *Igflr* gene. R⁻ cells, which express low levels of endogenous INSR (approximately 5×10^3 receptors per cell) (Sell et al. 1994), were cotransfected with the pNTK2 expression vector containing the cDNA for the human INSR-A (Ex11⁻) or INSR-B (EX 11⁺) and with the pPDV61 plasmid encoding the puromycin resistance gene, by using the Lipofectamine reagent (Life Technologies, Inc./BRL, Bethesda, MD, USA), as described previously (Yamada et al. 2007). The cell clones obtained (R⁻/INSR-A and R⁻/INSR-B cells) express approximately 3×10^5 to 5×10^5 receptors per cell (Frasca et al. 1999). All cell types were grown in DMEM (4.5 g/l glucose) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 2 µg/ml of puromycin. Leiomyosarcoma SKUT-1 cells were kindly provided by Dr Colombatti (Aviano, Italy). The cells were grown in DMEM supplemented with 10% FBS and 1 mM Na pyruvate. CAFs were obtained from surgical specimens of breast cancer tissues from 47 patients who underwent mastectomy at the Regional Hospital in Cosenza (Italy). The samples were immediately incised in 5 ml of medium and incubated overnight in digestion solution (400 IU collagenase, 100 IU hyaluronidase, and 10% FBS, containing antibiotic and antimycotic solutions). The cells were then separated by differential centrifugation at 90 g for 2 min. The supernatants containing fibroblasts were centrifuged at 485 g for 8 min, the pellets obtained were suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1 and supplemented with 10% FBS and 1% penicillin) and cultured at 37 °C and 5% CO₂. At 80% of confluence, the fibroblasts were stored at -80 °C for the next isolation of RNA. Primary cell cultures of breast fibroblasts were characterized by immunofluorescence. Briefly, cells were incubated with human anti-vimentin (V9) and human anti-cytokeratin 14 (LL001) (Santa Cruz Biotechnology, DBA). In order to assess fibroblast activation, anti-fibroblast-activated protein α (FAPα) antibody (H-56, Santa Cruz Biotechnology, DBA) was used. All experiments were carried out in a mixed population of CAFs obtained from five patients with low serum insulin levels. Signed informed consent from all the patients was obtained and all samples were collected, identified, and used in accordance with the approval by the Institutional Ethical Committee Board (Regional Hospital of Cosenza, Italy).

Plasmids

The plasmid DN/c-Fos, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding, was a kind gift from Dr C Vinson (NIH, Bethesda, MD, USA). Short hairpin constructs against human GPER1 (shGPER1) and CTGF (shCTGF) were generated and used as described previously (Albanito et al. 2008). In brief, they were generated in the lentiviral expression vector pLKO.1 purchased from Euroclone, Milan, Italy. The targeting strand generated from the shGPER1 construct is 5'-CGCTCCCTGCAAGCAGTCTTT-3'. The targeting strand generated from the shCTGF construct is 5'-TAGTACAGCGATTCAAAGATG-3'. The shINSR was purchased from SABiosciences (Qiagen).

Transfections and luciferase assays

The cells (1×10^5) were plated into 24-well dishes with 500 µl/well of regular growth medium the day before transfection. The medium was replaced with DMEM, lacking serum and phenol red on the day of transfection, which was performed using X-tremeGene9 reagent, as recommended by the manufacturer (Roche Molecular Biochemical), with a mixture containing 0.5 mg of reporter plasmid and 2 ng of pRL-TK. After 6 h, the medium was replaced again with DMEM, lacking serum and phenol red, treatments were added and the cells were incubated for an additional 24 h. Luciferase activity was then measured with the Dual Luciferase Kit (Promega Italia) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity. The normalized relative light unit values obtained from cells treated with vehicle were defined as onefold induction, relative to which the activity induced by treatments was calculated.

RT and real-time PCR

Total RNA was extracted using the Trizol Commercial Kit (Invitrogen) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Total cDNA was synthesized from RNA by RT using the murine leukemia virus reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer. The expression of selected genes was quantified by real-time PCR using the Step One (TM) sequence detection system (Applied

Biosystems, Inc.), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems, Inc.). The assays were carried out in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. For GPER1 (mouse) the primers used were: 5'-TGGTGGTGAACATCAGTCTC-3' (GPER1 forward); 5'-AAGCTCATCCAGCTGAGGAA-3'. For GPER1 (human) the primers used were: 5'-ACACACCTGGGTGGACACAA-3' (GPER1 forward); 5'-GGAGCCAGAAGCCACATCTG-3' (GPER1 reverse). For the ribosomal protein 18S (human and mouse), which was used as a control gene to obtain normalized values, the primers used were: 5'-GGCGTCCCCAACTTCTTA-3' (18S forward) and 5'-GGGCATCACAGACCTGTTATT-3' (18S reverse). The assays were carried out in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression. To quantify the GPER1 expression in CAFs obtained from breast cancer patients, standard curve was generated using serially diluted solutions of cDNA from a mixture of all samples. 5 µl cDNA of each sample were mixed to obtain the solution of the standard stock (tube 1, first dilution point), which was used to prepare the other four dilution points. Each dilution point (in triplicate) was added into well plates containing the Master Mix solution and, according to the protocol of the real-time software, the concentration of each solution (ng/ml) was recorded. The absolute quantification of unknown values was obtained by interpolating the PCR signals into the standard curve provided by the serially diluted solutions. PCR amplification was carried out in duplicate for each sample and the results are expressed as mean values. The content of *GPER1* transcript was normalized to the content of the housekeeping gene *18S*.

Western blotting

The cells were grown in 10-cm dishes and exposed to ligands before lysis in 500 µl of lysis buffer containing the following: 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, and a mixture of protease inhibitors (Aprotinin, PMSF, and Na-orthovanadate). Protein concentrations were determined according to the Bradford method (Sigma–Aldrich). Equal amount of whole protein extracts were electrophoresed through a reducing SDS/10% (w/v) polyacrilamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membranes were blocked and probed with primary antibodies against

GPER1 (N-15), CTGF (L-20), c-Fos (H-125), phosphorylated MAPK1/2 (E-4), MAPK2 (C-14), phosphorylated PRKCD (Thr 507), PRKCD (C-20), β-actin (C2), and β-tubulin (sc-9104) purchased from Santa Cruz Biotechnology (DBA); insulin receptor α (INSRα) from BD Bioscience (Milan, Italy). The levels of protein and phosphoproteins were detected with appropriate secondary HRP-conjugated antibodies and the ECL System (GE Healthcare, Milan, Italy). All experiments were carried out in triplicate and blots shown are representative.

Chromatin immunoprecipitation

The cells grown on 10-cm plates were shifted and treated for 24 h in a medium lacking serum and then with vehicle or insulin (10 nM). Chromatin immunoprecipitation (ChIP) assay was carried out as described previously (De Marco et al. 2013). The immune-cleared chromatin was immunoprecipitated with anti c-Fos (H-125) or nonspecific IgG (Santa Cruz Biotechnology, DBA). A 4 µl volume of each immunoprecipitated DNA sample and input were used as a template to amplify by PCR the region containing an AP-1 site located in the *GPER1* promoter region. The primers used to amplify this fragment were as follows: 5'-CGTGCCCATACCTTCAT-TGCTTCC-3' (forward) and 5'-CCTGGCCGGGTGT-CTGTG-3' (reverse).

Immunostaining assay

Fifty percent confluent, cultured, CAFs and leiomyosarcoma SKUT-1 cells grown on coverslips were serum deprived and transfected for 12 h with a control shRNA or a shRNA specific for GPER1 (shGPER1), using X-tremeGene9 reagent (Roche Molecular Biochemical), as recommended by the manufacturer, and then treated for 24 h with vehicle or 10 nM insulin. Thereafter, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% TritonX-100, washed three times with PBS and blocked and incubated overnight with primary antibody human GPER1 (N-15). After incubation, the slides were extensively washed with PBS and incubated with donkey anti-rabbit IgG-FITC (1:500, from Santa Cruz Biotechnology) and propidium iodide (1:1000, Sigma–Aldrich). A Leica AF6000 Advanced Fluorescence Imaging System supported by the quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS, GbH Mannheim, Germany) were used for evaluation of experiments.

Migration assay

Migration assays were carried out using Boyden Chambers (Costar Transwell, 8 mm polycarbonate membrane). For knockdown experiments, the cells were transfected with shRNA constructs directed against GPER1 or CTGF and with an unrelated shRNA construct (500 ng DNA/well transfected with X-tremeGene9 reagent in the medium without serum). After 24 h, the cells were seeded into the upper chambers.

Glucose uptake assay

The fluorescent analog of glucose 2-(*N*-(7 nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-D-glucose (2-NBDG; Life Technologies) was used to measure glucose uptake. Around 1×10^5 cells per well were seeded into 12-well plates and maintained in the medium for 24 h. For knockdown experiments, the cells were transfected for 48 h with shRNA constructs directed against GPER1 and with an unrelated shRNA construct (3 μ g DNA/well transfected with X-tremeGene9 reagent in medium without serum). Subsequently, the fluorescence was measured using an FLX-800 micro plate fluorimeter (Bio-Tek Instruments, Inc., Winooski, VT, USA) with an excitation wavelength of 465 nm and an emission wavelength of 540 nm (Yamada *et al.* 2007).

Cell-cycle analysis

Around 1×10^5 cells per well were seeded into 12-well plates and maintained in the medium for 24 h. For knockdown experiments, the cells were transfected for 48 h with shRNA constructs directed against GPER1 and with an unrelated shRNA construct (3 μ g DNA/well transfected with X-tremeGene9 reagent in medium without serum). The cells were then treated with 10 nM insulin for 8 h, thereafter the medium was removed and replaced by medium without serum, containing 10 nM E₂. After 8 h, the cells were pelleted, washed once with PBS, and resuspended in 0.5 ml of a 50 μ g/ml propidium iodide in $1 \times$ PBS (PI) solution containing 20 U/ml RNase-A and 0.1% triton and incubated for 1 h (Sigma–Aldrich). The cells were analyzed for DNA content by fluorescence-activated cell sorting (BD, FACS JAZZ). The cell phases were estimated as a percentage of a total of 10 000 events.

Immunohistochemistry

Immediately after excision, the tissue samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin wax blocks at 56 °C. GPER1 was

analyzed by immunohistochemical (IHC) staining using 3 μ m thick consecutive paraffin sections. The sections were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was achieved by boiling in 0.01 M citrate buffer of pH 6. Endogenous peroxidase was removed with 3% H₂O₂; nonspecific binding was blocked by incubating the slides for 30 min with 1.5% BSA in PBS. Next, the sections were incubated with the primary antibodies for 1 h at room temperature. GPER1 was detected using GPER1 mouse MAb (DakoCytomation, Glostrup, Denmark) at dilution 1:100. Ab–antigen reactions were revealed using a streptavidin–biotin–peroxidase complex (LSAB kit, DakoCytomation). All slides were counterstained with hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary antibodies were omitted. The expression of GPER1 was independently scored by two investigators by light microscopy of ten different section fields.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls' testing to determine differences in means. $P < 0.05$ was considered as statistically significant. Relationships between variables were assessed with the Spearman's correlation coefficient. Differences and relationships were considered statistically significant when $P < 0.05$.

Results

Insulin induces GPER1 expression

On the basis of previous data, showing that a functional cross-talk may occur between GPER1 and insulin-mediated action (Sharma *et al.* 2013), we aimed to evaluate whether insulin could regulate GPER1 expression and function. As shown in Fig. 1A, we first ascertained that insulin transactivates a *GPER1* promoter construct, which was transiently transfected into mouse fibroblasts expressing only INSR-A or INSR-B but not IGF1R (R^- /INSR-A and R^- /INSR-B cells respectively) (Pandini *et al.* 2002), in breast CAFs that express both INSR isoforms (data not shown) and in leiomyosarcoma cells (SKUT-1) that express almost only INSR-A (Sciacca *et al.* 2002). In accordance with these findings, insulin upregulated the mRNA (Fig. 1B) and protein levels of GPER1 in all cells used (Fig. 2A, B, C, D, and E), except for the mouse fibroblasts that lack IGF1R (R^-) and express low endogenous INSR

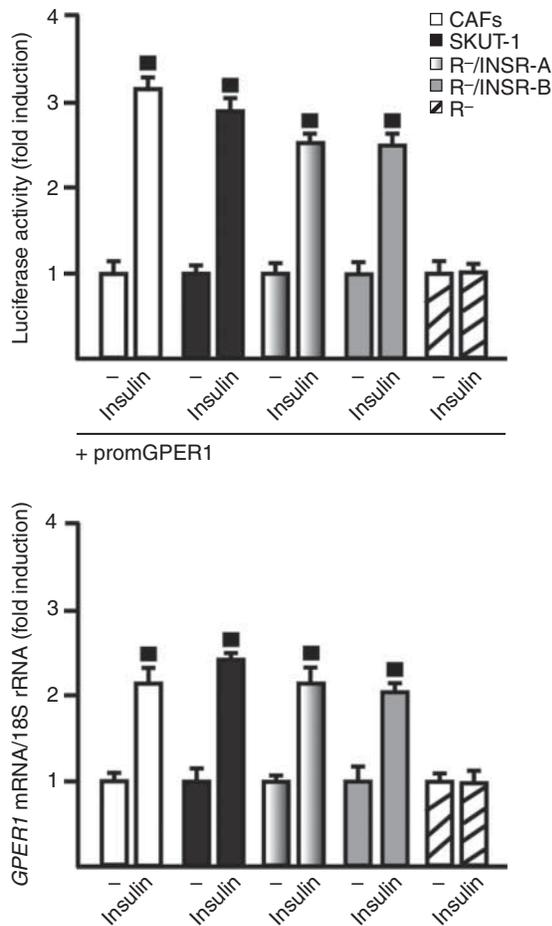


Figure 1

(A) 10 nM insulin induces the transactivation of the *GPER1* promoter construct only in mouse fibroblasts expressing insulin receptor isoform A (R⁻/INSR-A) or isoform B (R⁻/INSR-B), in cancer-associated fibroblasts (CAFs) and leiomyosarcoma SKUT-1 cells. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (–) were defined as onefold induction, relative to which the activities induced by insulin were calculated. (B) A 4 h treatment with 10 nM insulin upregulated *GPER1* mRNA expression only in R⁻/INSR-A, R⁻/INSR-B, CAFs, and SKUT-1 cells, as evaluated by real-time PCR. The mRNA expression of *GPER1* was normalized to (*18S*) expression. Each column represents the mean \pm s.d. of three independent experiments carried out in triplicate. (closed square) $P < 0.05$ for cells receiving vehicle (–) vs treatments.

levels (Sciacca *et al.* 2002). Further corroborating these results, the upregulation of GPER1 protein levels by insulin was no longer evident after silencing of INSR expression in CAFs and SKUT-1 cells (Fig. 2F and I). Results from immunofluorescence studies on CAFs and SKUT-1 cells transfected with a shGPER1 indicated that insulin-induced GPER1 expression was abolished, confirming the aforementioned observations (Fig. 3). Next, the induction of GPER1 protein by insulin was prevented by using the PRKC inhibitor GF109203X (GF), the MEK inhibitor

PD98059 (PD), and the PRKCD inhibitor Rottlerin (Rot), but still persisted in presence of the EGFR inhibitor AG1478 (AG) (Fig. 4A, B, C, and D). It is concluded that, insulin induced PRKCD and MAPK1 activation in R⁻/INSR-A, R⁻/INSR-B, CAFs, and SKUT-1 cells, but not in R⁻ cells (Supplementary Figure 1, see section on supplementary data given at the end of this article).

GPER1 upregulation by insulin involves activation of c-Fos/AP1 transcription

It has been previously shown that the activation of MAPK1 transduction pathway leads to a rapid induction of c-Fos (Maggiolini *et al.* 2004, Vivacqua *et al.* 2006a,b, Albanito *et al.* 2007), which has a growth stimulatory role in normal and cancer cells mainly activating the AP1 transcription complex together with Jun family members (Hess *et al.* 2004). In line with these data, in both CAFs and SKUT-1 cells, insulin induced the expression of c-Fos (Fig. 5A and B), which was recruited to the AP1 site located within the promoter sequence of GPER1, as ascertained by CHIP assay (Fig. 5C and D). Confirming these findings, insulin transactivated an *AP1* promoter construct transfected into CAFs and SKUT-1 cells; however, the luciferase activity was abrogated by co-transfecting an expression vector encoding a dominant-negative form of c-Fos (DN/c-Fos) (Fig. 5E and F). Likewise, the transactivation of the *GPER1* promoter construct (Fig. 5G and H) as well as the upregulation of GPER1 protein levels (Fig. 5I and J) were prevented in the presence of the DN/c-Fos. Taken together, our results indicate that insulin upregulates GPER1 expression through INSR-A and INSR-B along with the activation of PRKCD/MAPK1/c-Fos/AP1 transduction pathway.

GPER1 mediates CTGF expression and cell migration induced by insulin

Next, we sought to evaluate whether the insulin-induced GPER1 expression could be followed by the upregulation of a main GPER1 target gene, *CTGF* (Pandey *et al.* 2009). Noteworthy, insulin triggered CTGF protein induction in R⁻/INSR-A, R⁻/INSR-B, CAFs, and SKUT-1 cells (Fig. 6A, B, C, and D). In the last two cell types, the increase in CTGF protein levels was abolished by silencing INSR and GPER1 expression as well as transfecting cells with the DN/c-Fos plasmid (Fig. 7A, B, C, D, E, F, G, H, I, and J). As a biological counterpart, the migration stimulated by insulin in CAFs and SKUT-1 was prevented by treatment with the GPER1 antagonist G15 (Supplementary Figure 2, see section on supplementary data given at the end of this article), as well

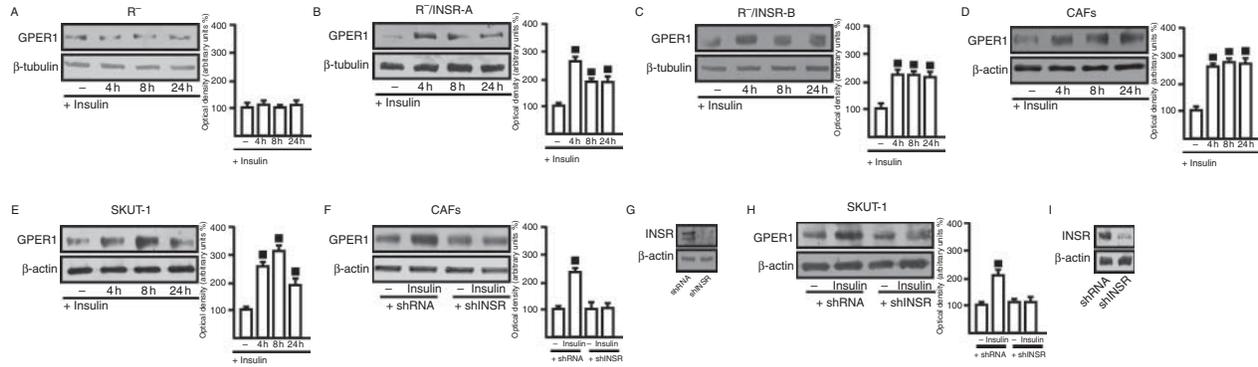


Figure 2

(A, B, C, D, and E) Treatment with 10 nM insulin induces increases in the GPER1 protein levels only in R⁻/INSR-A or R⁻/INSR-B, CAFs, and SKUT-1 cells. (F, G, H, and I) In CAFs and SKUT-1 cells transfected with a shINSR, treatment for 8 h with 10 nM insulin does not trigger an increase in GPER1

as transfecting cells with the shGPER1 or shCTGF constructs (Fig. 7K, L, M, N, O, and P), while the ER antagonist ICI did not have any inhibitory effect (Supplementary Figure 2). Taken together, these results indicate that GPER1 is involved in the upregulation of CTGF, and that both GPER1 and CTGF are required for the migratory effects stimulated by insulin.

protein levels. The charts show results of densitometric analysis of the blots normalized to β-tubulin or β-actin levels. Each column represents the mean ± S.D. of three independent experiments. (closed square) P < 0.05 for cells receiving vehicle (-) vs treatments.

GPER1 is involved in the glucose uptake and cell-cycle progression stimulated by insulin

Recently, estrogens have been reported to increase glucose uptake in breast cancer cells through a mechanism which involves ESR1 (Garrido *et al.* 2013). As GPER1 mediates estrogen signaling (Maggiolini & Picard 2010) and

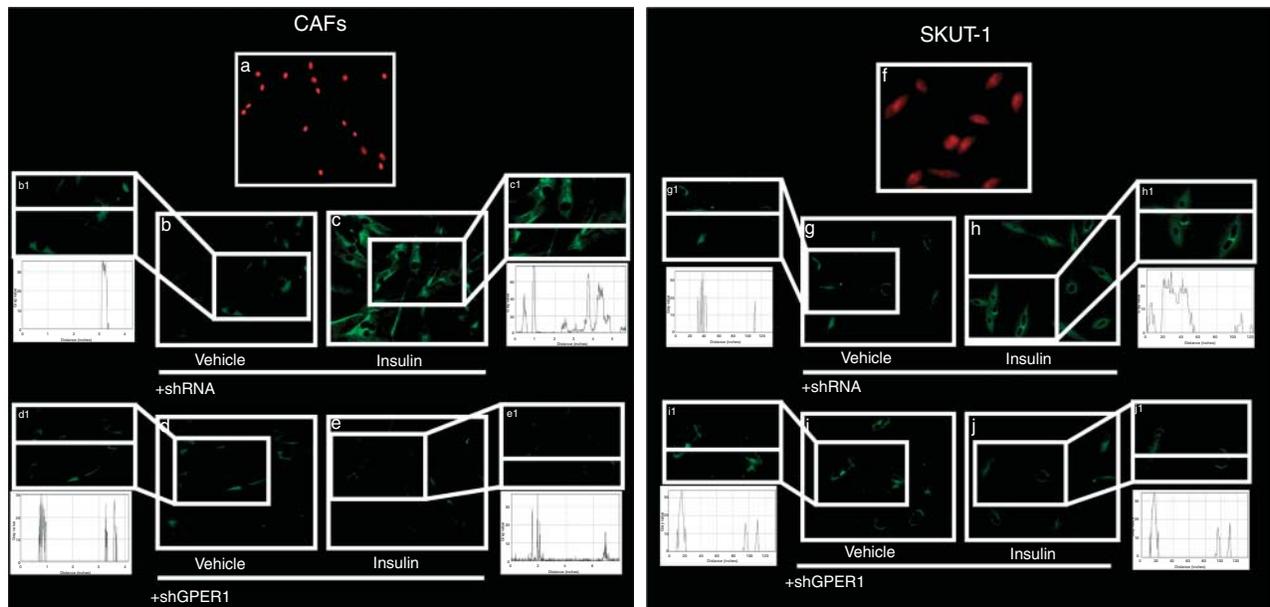


Figure 3

CAF and SKUT-1 cells were fixed, permeabilized, and stained with anti-GPER1 antibody. Nuclei (red) were stained with propidium iodide (a and f). The cells were transfected with a control shRNA (b, c and g, h) or with a shGPER1 (d, e and i, j) and treated for 8 h with vehicle (-) or 10 nM insulin and then stained with the GPER1 antibody. For descriptive purposes, panels b1, c1, d1, e1, g1, h1, i1 and j1 show the plot profiles

obtained at the level of white lines of the corresponding insets, as calculated by using the program WCIF Image J for Windows. Note the higher values indicating zones of intense labeling. Each experiment shown is representative of ten random fields. Data are representative of three independent experiments.

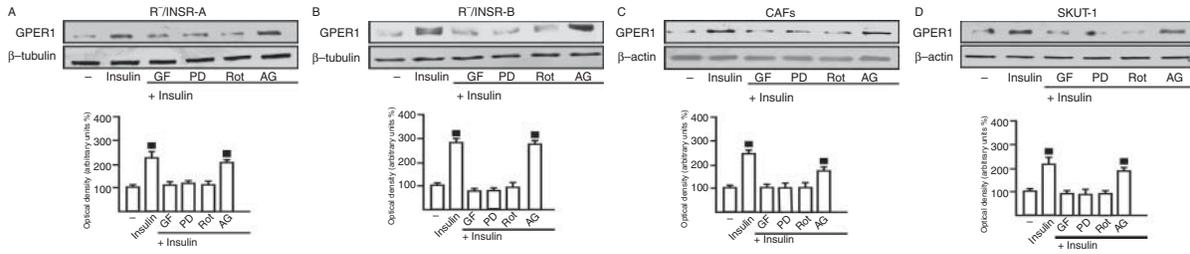


Figure 4

(A, B, C, and D) GPER1 protein expression evaluated in R^{-/-}INSR-A or R^{-/-}INSR-B, CAFs and SKUT-1 cells treated for 4 h with vehicle (–) or 10 nM insulin alone and in combination with 10 μM PRKC inhibitor GF109203X (GF), 10 μM MEK inhibitor PD98059 (PD), 10 μM PRKCD inhibitor Rottlerin (Rot), and

10 μM EGFR inhibitor AG1478 (AG). The charts show the results of densitometric analysis of the blots normalized to β-tubulin or β-actin. Each column represents the mean ± s.d. of three independent experiments. (closed square) Indicates P < 0.05 for cells receiving vehicle (–) vs treatments.

contributes to certain metabolic responses to insulin (Liu *et al.* 2009, Balhuizen *et al.* 2010, Sharma *et al.* 2013), we investigated whether glucose uptake could be stimulated by estrogens through GPER1. Moreover, before

exposure to estrogens, the cells were also treated with insulin, which upregulates GPER1 expression, as demonstrated above. Interestingly, the glucose uptake stimulated by E₂ was further boosted in CAFs and SKUT-1 cells

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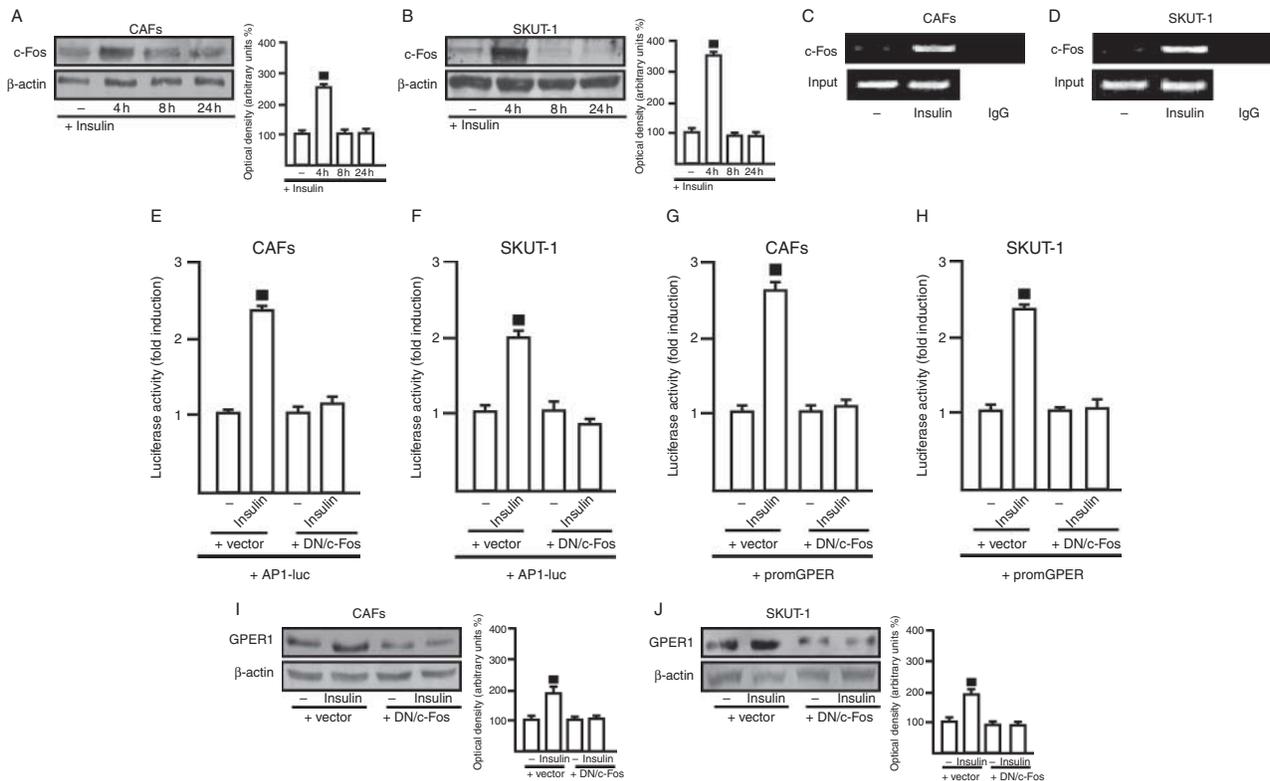


Figure 5

In CAFs and SKUT-1 cells, 10 nM insulin induced the expression of c-Fos (A and B), which is recruited to the AP1 site located within the GPER1 promoter sequence by a 4 h treatment with 10 nM insulin (C and D). The transactivation of an AP1-LUC reporter gene (E and F) and the GPER1 promoter construct (G and H) induced by a 18 h treatment with 10 nM insulin as well as the GPER1 protein increase induced by a 4 h treatment with 10 nM insulin were prevented in the presence of a dominant negative form of c-Fos construct (DN/c-Fos). (I and J) Each transfection experiment

was performed in triplicate, the luciferase activities from three independent experiments were normalized to the internal transfection control and values for cells receiving vehicle (–) were defined as onefold induction relative to which the activities induced by insulin were calculated. In immunoblotting, the charts show results of densitometric analysis of the blots normalized to β-tubulin or β-actin and each column represents the mean ± s.d. of three independent experiments. (closed square) P < 0.05 for cells receiving vehicle (–) vs treatments.

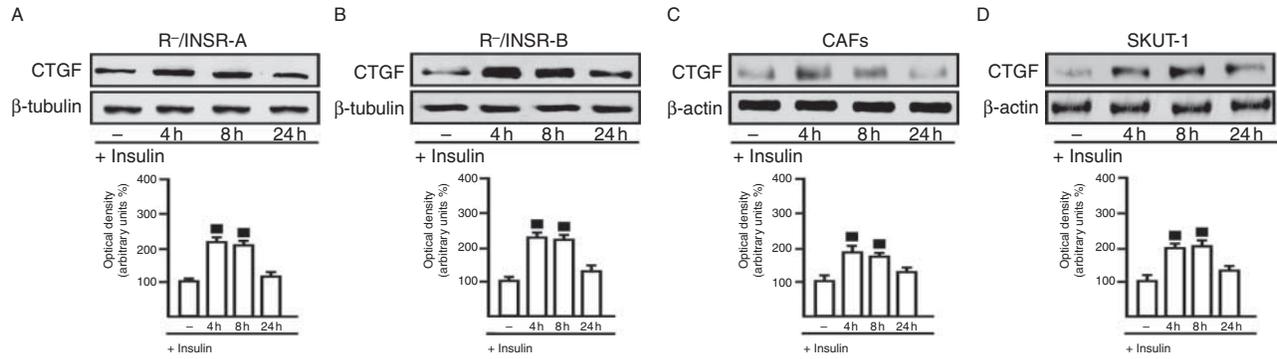


Figure 6

(A, B, C, and D) CTGF protein expression is upregulated by 10 nM insulin in R⁻¹/INSR-A and R⁻¹/INSR-B, CAFs, and SKUT-1 cells. The charts show the results from densitometric analysis of the blots normalized to β-tubulin or

β-actin. Each column represents the mean ± s.d. of three independent experiments. (closed square) Indicates *P* < 0.05 for cells receiving vehicle (–) vs treatments.

treated with insulin before the treatment with E₂, as assessed by fluorescence assays (Fig. 8A and B). The glucose uptake induced by E₂ alone and the additional stimulation obtained by insulin were prevented by silencing of GPER1 expression (Fig. 8A and B) and by use of the GPER1 antagonist G15, but not in the presence of the ER

inhibitor ICI (Supplementary Figure 3, see section on supplementary data given at the end of this article). By silencing GPER1 expression, we then ascertained that GPER1 mediates both the E₂-induced increase in the percentage of CAFs and SKUT-1 cells in the G2/M phase and the further boost observed from treating cells with

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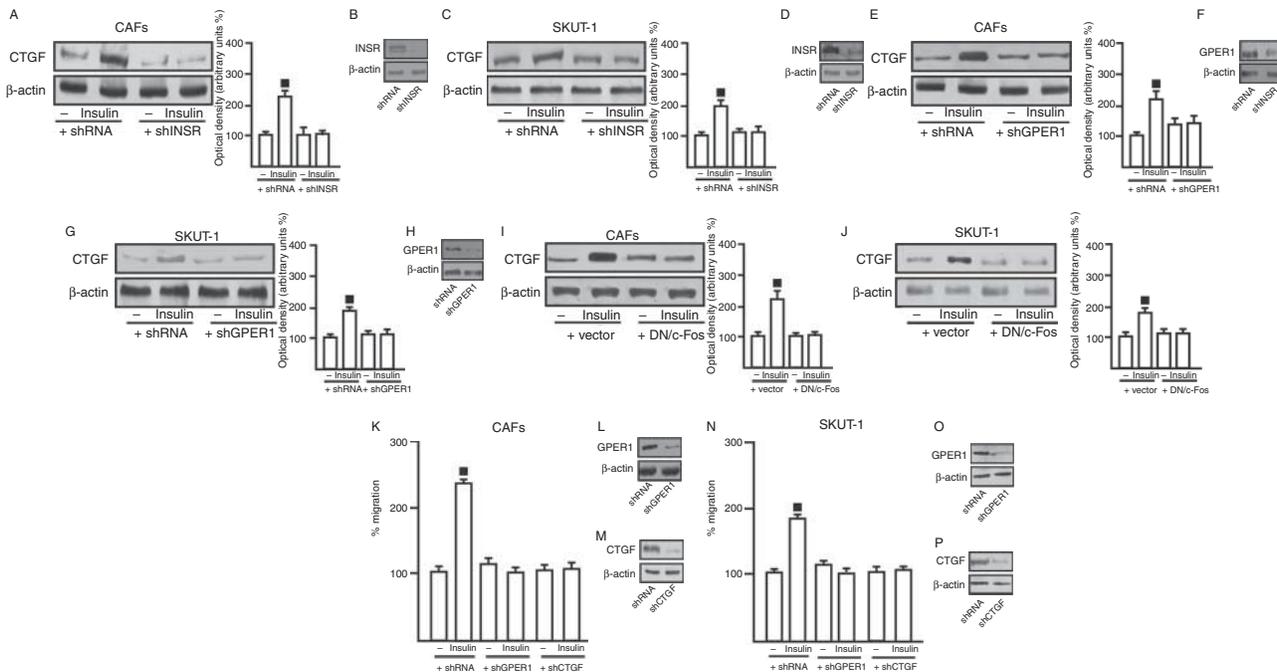


Figure 7

The upregulation of CTGF protein levels induced by a 4 h treatment with 10 nM insulin was abolished by transfecting CAFs and SKUT-1 cells with shINSR (A, B, C, and D), shGPER1 (E, F, G, and H), or a dominant negative form of c-Fos (DN/c-Fos) (I and J). The migration of CAFs and SKUT-1 cells after a 6 h treatment with 10 nM insulin was prevented by silencing GPER1

and CTGF expression (K, L, M, N, O, and P). Results shown are representative of three independent experiments. The charts show the results from densitometric analysis of the blots normalized to β-actin. (closed square) *P* < 0.05 for cells receiving vehicle (–) vs treatments.

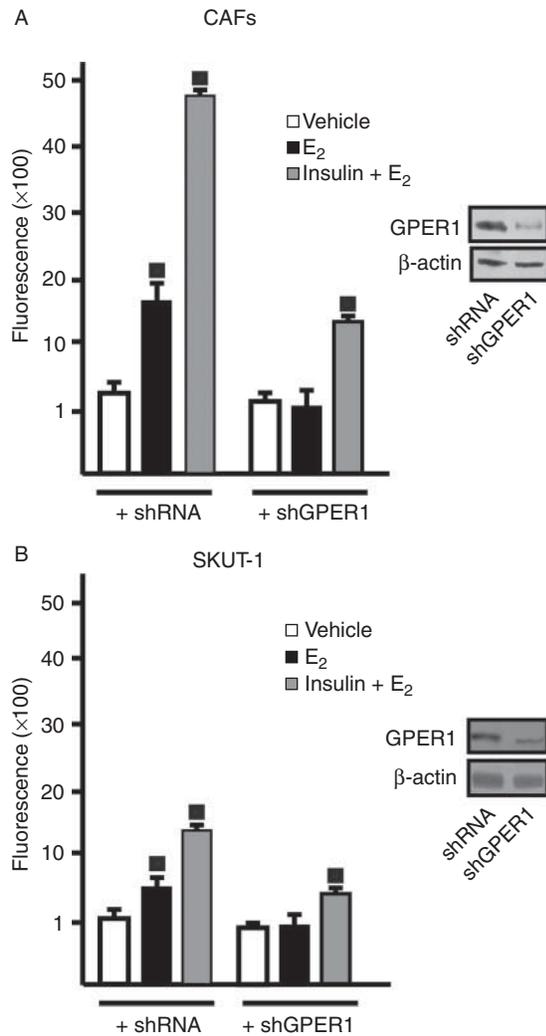


Figure 8

(A and B) CAFs and SKUT-1 cells were transfected with shRNA or shGPER1 and then treated with 10 nM E₂ for 30 min. Where indicated, cells were treated with 10 nM insulin for 8 h, thereafter the medium was removed and replaced before the treatment for 30 min with 10 nM E₂. Each column shows the fluorescence obtained by the measurement of the 2-NBDG uptake and represents the mean ± s.d. of three independent experiments performed in triplicate. (closed square) $P < 0.05$ for cells receiving vehicle vs treatments.

insulin before E₂ (Fig. 9). Collectively, these results indicate that insulin potentiates the action of estrogens elicited through GPER1 on glucose uptake and cell-cycle progression.

GPER1 expression in CAFs is positively associated with serum insulin levels

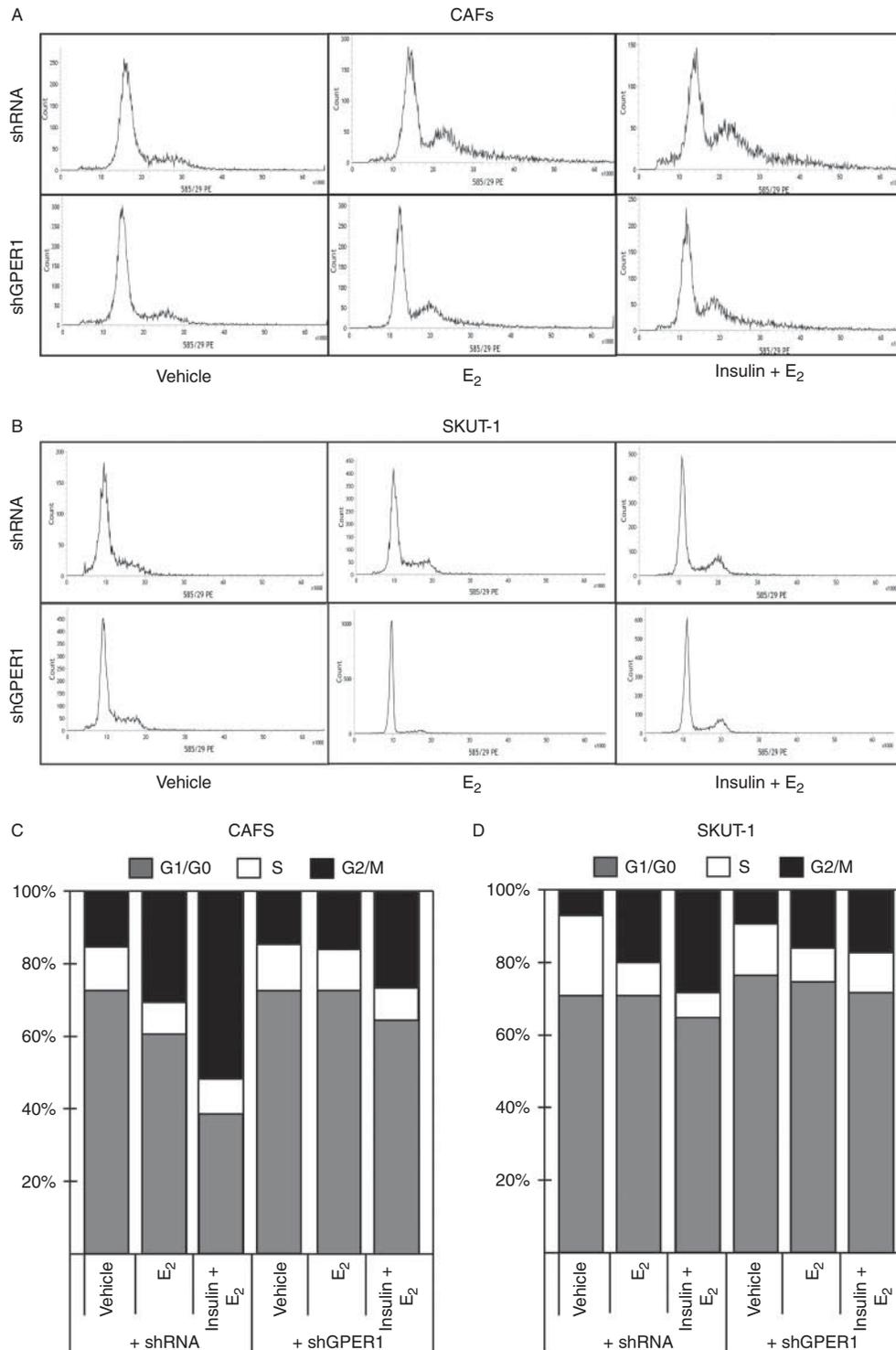
Previous studies have demonstrated a direct correlation between insulin levels and the occurrence of diverse

malignancies (Belfiore & Malaguarnera 2011). Also, GPER1 expression has been associated with negative biological features in patients with various types of tumors (Prossnitz & Barton 2011, Sjöström *et al.* 2014). Interestingly, we found a positive relationship between the insulin levels and the mRNA expression of GPER1 in CAFs obtained from breast cancer patients (Fig. 10), indicating that GPER1 may be regulated *in vivo* by insulin, at least in the main players of the breast tumor microenvironment, such as CAFs.

Discussion

Insulin is involved in the regulation of carbohydrate, lipid, and protein metabolism; however, it also exerts a stimulatory role in cancer progression, in particular upon pathophysiological conditions characterized by insulin resistance (Belfiore & Malaguarnera 2011). In accordance with this, previous experimental and epidemiological studies have demonstrated a positive correlation between insulin levels and cancer development (Belfiore & Malaguarnera 2011). As regards breast cancer, the potential of insulin to contribute to tumor progression has been highlighted in diverse investigations (Rose & Vona-Davis 2012, Sieri *et al.* 2012, Catsburg *et al.* 2014). Nicely supporting these data, in postmenopausal women within the framework of the Women's Health Initiative Observational Study (WHI-OS), the highest tertile of baseline insulin was associated with a twofold risk increase in breast cancer compared with the lowest tertile (Kabat *et al.* 2009). Estrogens perform a fundamental role in hormone-sensitive breast cancer, mainly activating the classical ERs (Hall *et al.* 2001). In addition, the stimulatory effects of estrogens may be mediated through GPER1, which activates a network of transduction pathways triggering diverse biological responses (Prossnitz & Maggolini 2009, Prossnitz & Barton 2014, Scaling *et al.* 2014).

Considering the well-known functional cross-talk between insulin and estrogen signaling, in the current study we evaluated whether insulin could regulate the expression and function of GPER1 in cancer progression. Using as model systems, the INSR-positive CAFs and leiomyosarcoma cells (SKUT-1), we have demonstrated that insulin transactivates the promoter of *GPER1* and upregulates its expression at both the mRNA and protein levels. In particular, we have shown that the induction of GPER1 by insulin is mediated by the rapid activation of PRKCD and MAPK1/2 signal transduction and the stimulation of *c-Fos*, which is recruited to the AP1 site located within the promoter sequence of GPER1.

**Figure 9**

(A and B) Cell-cycle analysis performed in CAFs and SKUT-1 cells transfected with shRNA or shGPER1 and then treated for 8 h with 10 nM E₂. The cells were also treated with 10 nM insulin for 8 h before the treatment for an additional 8 h with 10 nM E₂, as indicated. (C and D) The histograms show

the percentages of cells in G1/G0, S, and G2/M phases of the cell cycle, as determined by flow cytometry analysis (BD, FACS JAZZ, Milan, Italy). Values represent the mean \pm s.d. of three independent experiments.

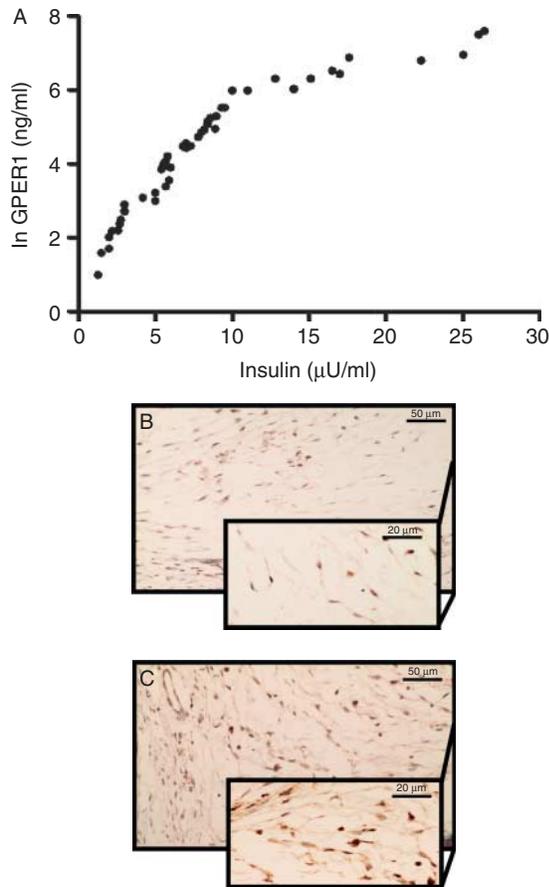


Figure 10

(A) Correlation between serum insulin levels ($\mu\text{U/ml}$) and log transformed *GPER1* mRNA expression (ng/ml) determined by real-time PCR in CAFs obtained from breast cancer patients ($n=47$; $r=0.83$, $P<0.001$). Representative images of *GPER1* expression in stromal fibroblasts of a breast cancer patient with low serum insulin levels (B) and a breast cancer patient with high serum insulin levels (C). Higher magnification images of specific areas are shown below the original images.

Moreover, we ascertained that the functional role performed by AP1 is essential, as the promoter transactivation as well as the expression of *GPER1* were abrogated using a construct encoding a dominant-negative form of c-Fos. Noteworthy, *GPER1* and one of its main target genes, *CTGF*, were required for cell migration induced by insulin. As *CTGF* has been mainly involved in cell motility (Chu *et al.* 2008, Pandey *et al.* 2009), the *GPER1/CTGF* signaling activated by insulin might contribute to the invasion abilities of cancer cells during cancer development and metastasis.

Results from previous studies have indicated that estrogens increase insulin sensitivity and stimulate glucose uptake upon acute and chronic exposure to these steroids in target tissues as well as in ESR1-positive breast

cancer cells (Alonso *et al.* 2006, Moreno *et al.* 2010, Garrido *et al.* 2013). On the basis of these findings and considering that *GPER1* has been shown to be involved in insulin-regulated metabolic functions in both mice and humans (Mårtensson *et al.* 2009, Kumar *et al.* 2011, Sharma & Prossnitz 2013), we have also ascertained that *GPER1* mediates the glucose uptake induced by estrogens in CAFs and SKUT-1 cells. When these cells were treated with insulin before E_2 , the glucose uptake was further boosted consequently to the upregulation of *GPER1* triggered by insulin, given that this response was abrogated by silencing *GPER1* expression. Paralleling the aforementioned results, treatment with insulin before E_2 increased the percentage of cells in the G2/M phase, whereas after knocking-down the expression of *GPER1* the effects of E_2 were no longer evident. We found a positive relationship between the levels of insulin and the expression of *GPER1* in CAFs obtained from breast cancer patients, indicating that insulin may regulate *GPER1* *in vivo*. It would be worthwhile to assess in future *in vivo* studies if insulin may act through regulating *GPER1* under different pathophysiological conditions.

The interaction between various G-protein-coupled receptors (GPCRs) and growth factor receptors has become increasingly evident in cancer growth, angiogenesis, and metastasis (Lappano & Maggiolini 2012). Moreover, experimental, epidemiological, and clinical data indicate that a cross-talk between different GPCRs and insulin signaling plays a critical role in the regulation of normal physiological functions as well as in the pathogenesis of a variety of abnormal processes, including cancer (Kisfalvi *et al.* 2007, Young & Rozengurt 2010). In this regard, the current study further highlights the stimulatory role of insulin in tumor progression elicited through the upregulation of *GPER1*, which consequently strengthens the action of estrogens. Noteworthy, the functional interaction between *GPER1* and insulin signaling has been recently extended to the potential of *GPER1* to facilitate the metabolic functions of insulin (Prossnitz & Barton 2014). In accordance with these findings, *GPER1*-deficient mice displayed a reduced estrogen-stimulated release of insulin *in vitro* as well as a decreased response to glucose challenges *in vivo* (Martenson *et al.* 2009, Kumar *et al.* 2011). In addition, ligand-activated *GPER1* protected β -cells from apoptosis in mouse and human islets, thus *GPER1* may play a functional role in insulin secretion as well as in β -cell survival (Liu *et al.* 2009, Prossnitz & Barton 2014).

In our previous studies, we have highlighted the upregulation of *GPER1* expression induced by growth factors such as EGF and IGF1, as well as by one main factor

involved in tumor aggressiveness such as hypoxia, in the proliferation and migration of breast cancer cells and CAFs (Albanito *et al.* 2008, Vivacqua *et al.* 2009, Recchia *et al.* 2011, De Francesco *et al.* 2013, 2014, De Marco *et al.* 2013). Likewise, estrogens have been shown to stimulate growth effects in tamoxifen-resistant breast cancer cells through both an increased expression of GPER1 and the GPER1-mediated transactivation of EGFR (Ignatov *et al.* 2011). Notably, high levels of expression of GPER1 in breast, endometrial, and ovarian tumors have been associated with a higher risk of developing metastatic disease and poor survival rates (Prossnitz & Barton 2011). High levels of GPER1 were also identified in IBC, an aggressive and commonly hormone-independent form of breast cancer (Arias-Pulido *et al.* 2010). Recently, the overexpression of GPER1 and its plasma membrane localization have been suggested to be critical events in breast cancer progression, whereas the lack of GPER1 in the plasma membrane was associated with excellent long-term prognosis in ER-positive tamoxifen-treated breast tumors (Sjöström *et al.* 2014). Therefore, the expression of GPER1 may characterize not only the estrogen sensitivity and the response to endocrine pharmacological intervention in these tumors, but could also be predictive of biologically aggressive phenotypes consistent with an adverse outcome and low survival. Further supporting the involvement of GPER1 in breast cancer progression, its activation led to certain deformations of breast glandular structure, which characterize the malignant transformation of the breast tissue (Marchese & Silva 2012). GPER1-dependent proliferation of nontumorigenic breast epithelial cells was also recently assessed, indicating a role for GPER1 in breast physiology and pathology (Scaling *et al.* 2014).

The present study extends our knowledge regarding the functional interaction between insulin and GPER1 transduction pathways. In this regard, it should be pointed out that in cancer patients affected by insulin resistance, increased insulin levels combine with frequent INSR overexpression leading to abnormal stimulation of non-metabolic effects mediated by INSR, such as cell survival, proliferation, and migration (Belfiore & Malaguarnera 2011). For instance, high insulin levels have been associated with an increased risk of breast cancer and breast cancer relapses in diabetic and nondiabetic women (Duggan *et al.* 2011, Cohen & Le Roith 2012, Sieri *et al.* 2012). Taking into account these data and the results of the present study, it would be interesting to evaluate in future studies the actual role performed by estrogenic GPER1 signaling in different pathophysiological conditions characterized by insulin resistance. Altogether,

our findings provide novel insights into the potential of GPER1 to contribute to the intricate tumorigenic transduction network triggered by insulin not only in cancer cells but also through the major cells in the tumor microenvironment such as CAFs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0245>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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