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**ORIGINAL ARTICLE**

# Insulin-like growth factor-I regulates GPER expression and function in cancer cells

P De Marco[1], V Bartella[1], A Vivacqua[1], R Lappano[1], MF Santolla[1], A Morcavallo[2], V Pezzi[1], A Belfiore[2] and M Maggiolini[1]

Functional cross talk between insulin-like growth factor-I (IGF-I) system and estrogen signaling has been largely reported, although the underlying molecular mechanisms remain to be fully elucidated. As GPR30/GPER mediates rapid cell responses to estrogens, we evaluated the potential of IGF-I to regulate GPER expression and function in estrogen receptor (ER)α-positive breast (MCF-7) and endometrial (Ishikawa) cancer cells. We found that IGF-I transactivates the GPER promoter sequence and upregulates GPER mRNA and protein levels in both cells types. Similar data were found, at least in part, in carcinoma-associated fibroblasts. The upregulation of GPER expression by IGF-I involved the IGF-IR/PKCδ/ERK/c-fos/AP1 transduction pathway and required ERα, as ascertained by specific pharmacological inhibitors and gene-silencing. In both MCF-7 and Ishikawa cancer cells, the IGF-I-dependent cell migration required GPER and its main target gene *CTGF*, whereas the IGF-I-induced proliferation required both GPER and cyclin D1. Our data demonstrate that the IGF-I system regulates GPER expression and function, triggering the activation of a signaling network that leads to the migration and proliferation of cancer cells.

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## INTRODUCTION

Estrogens regulate the growth and differentiation of many normal and neoplastic tissues including breast, endometrial and ovarian tumors.[1,2] Although the biological responses to estrogens are mainly mediated by estrogen receptors (ER)α and ERβ,[3,4] the G protein-coupled receptor GPR30/GPER has been recently shown to also mediate estrogen signaling in a variety of normal and cancer cell types.[5–8] In particular, GPER has been involved in rapid events induced by estrogens, including the transactivation of epidermal growth factor receptor, the activation of the mitogen-activated protein kinase and phosphoinositide3-kinase (PI3K) transduction pathways, the stimulation of adenylylcyclase and the mobilization of intracellular calcium ($Ca^{2+}$).[7,9–11] Accumulating evidence has also indicated that GPER signaling triggers a typical gene expression profile, as evidenced by diverse reports including microarray analysis.[11–13] As far as the transcriptional regulation of GPER expression is concerned, our previous studies have demonstrated that EGFR ligands are able to transactivate the promoter of *GPER* and upregulate its mRNA and protein levels in cancer cells.[14,15] Of note, the upregulation of GPER was associated with higher risk of developing metastatic disease in patients with breast cancer[16] and lower survival rates in endometrial cancer patients.[17]

Insulin-like growth factor-I (IGF-I) belongs to a group of cellular and secreted factors (IGFs) that exert important roles in multiple biological systems.[18–21] The IGF-I action is mediated by IGF-IR, a transmembrane heterotetramer, whose cytoplasmic tyrosine kinase domain is linked to diverse transduction cascades.[22,23] Moreover, IGF-I is known to signal through AP-1 in breast cancer cells[24] and to stimulate the expression of genes involved in cell cycle progression, such as *cyclin D1*, by cross talking with estrogen-dependent pathways.[25]

The interaction between IGF-I and ERα in regulating breast development and carcinogenesis has been well established.[26,27] For instance, several studies have demonstrated that IGF-I induces the activation of ERα in a ligand-independent manner through the involvement of the mitogen-activated protein kinase signaling.[26,28] Recently, increasing evidences have also extended our knowledge regarding the functional cross talk between growth factors transduction pathways and GPCR-activated signaling.[29] In this regard, we have previously shown that the EGFR system is involved in the GPER expression and function.[14,15] In addition, a strict dependence of the IGF-I signaling on GPCRs was reported in many physiological functions[30] as well as in the development of diverse malignancies.[31]

In the present study, we evaluated the ability of IGF-I to regulate GPER expression and function in ER-positive breast (MCF-7) and endometrial (Ishikawa) cancer cells. Our results demonstrate that the upregulation of GPER by IGF-I is mediated by the IGF-IR/PKCδ/ERK/c-fos/AP1 transduction pathway and involves ERα. As GPER was required for the IGF-I-induced cell migration and proliferation, our findings contribute to better understand the functional cross talk between these two important factors toward innovative therapeutic intervention in estrogen-sensitive tumors.

## RESULTS

### IGF-I induces GPER expression

In order to provide novel insights into the cross talk between IGF-I system and estrogen signaling, we sought to evaluate the ability of IGF-I to regulate GPER expression in breast MCF-7 and endometrial Ishikawa cancer cells. Interestingly, we found that IGF-I upregulates the mRNA and protein levels of GPER in both cell types (Figures 1a–d). Next, we determined that the GPER protein induction is abrogated in the presence of the IGF-IR inhibitor AG1024 (AG), the MEK inhibitor PD98059 (PD), the PKC inhibitor GF109203X (GF) and the PKCδ inhibitor Rottlerin (Rot), but it still

[1]Department of Pharmaco-Biology, University of Calabria, Rende, Italy and [2]Endocrinology, Department of Health, University Magna Graecia of Catanzaro, Catanzaro, Italy. Correspondence: Professor M Maggiolini, Department Pharmaco-Biology, University of Calabria, Via P Bucci, Arcavacata di Rende, 87030 Rende (CS), Italy.
E-mail: marcellomaggiolini@yahoo.it
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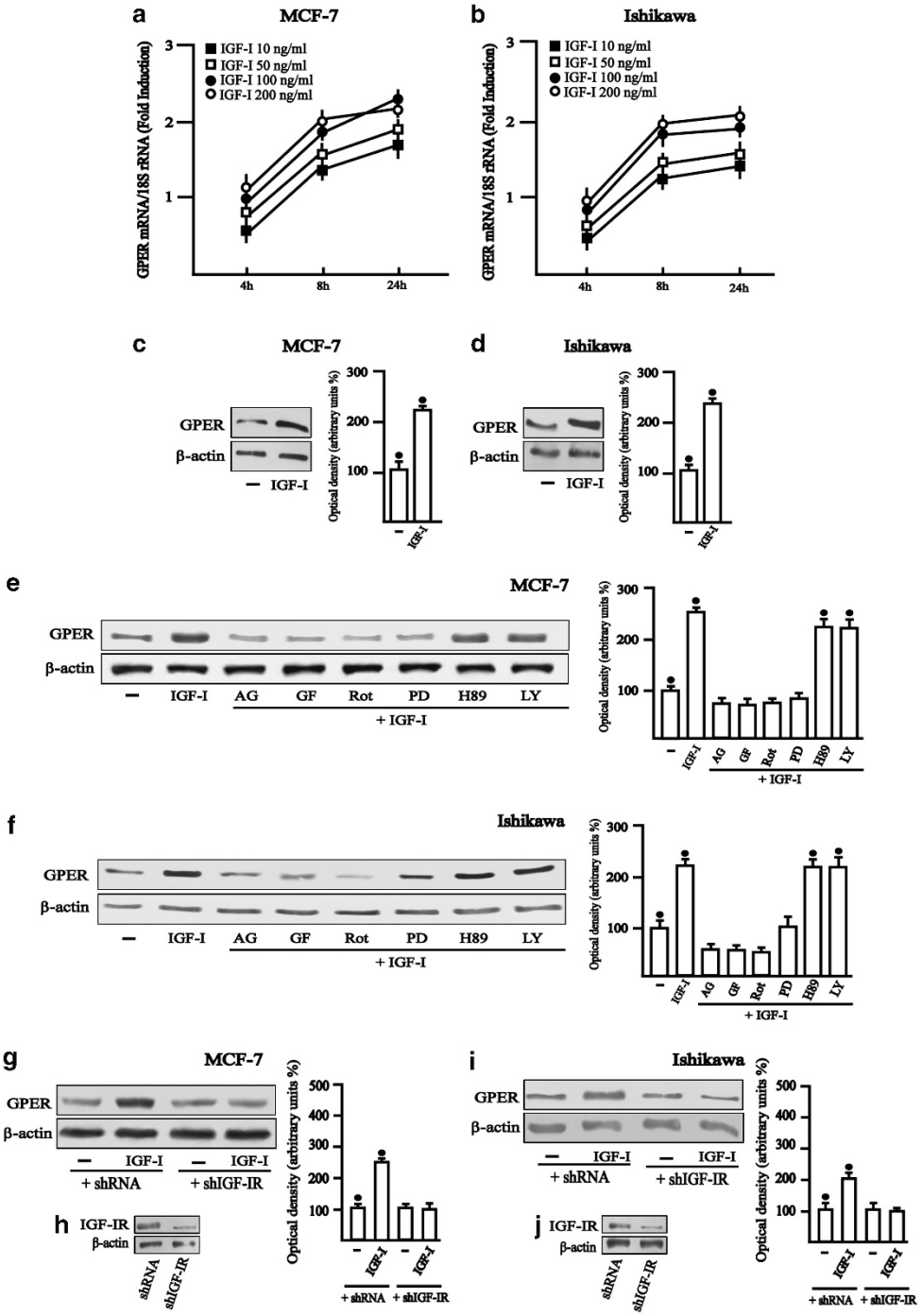


**Figure 1.** IGF-I upregulates GPER expression in MCF-7 and Ishikawa cells. (**a**, **b**) IGF-I induces GPER mRNA expression, as evaluated by real-time PCR. The mRNA expression of *GPER* was normalized to 18S expression. Results are shown as fold changes of mRNA expression upon treatment compared to cells treated with vehicle (−). (**c**, **d**) GPER protein levels were evaluated by immunoblotting in cells treated for 24 h with 100 ng/ml IGF-I. (**e**, **f**) GPER protein expression was evaluated by immunoblotting in cells treated for 24 h with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM IGF-IR inhibitor tyrphostin AG1024 (AG), 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD), 10 μM PKA inhibitor H89, 10 μM PI3K inhibitor LY294,002 (LY), as indicated. (**g**–**j**) The upregulation of GPER protein levels by 100 ng/ml IGF-I was abrogated in the presence of shIGF-IR. Side panels show densitometric analysis of the blots normalized to β-actin. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ○, ●, ■ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

persists using the PKA and phosphoinositide3-kinase inhibitors, H89 and LY294,002 (LY), respectively (Figures 1e–f). Corroborating the aforementioned findings, the upregulation of GPER was also prevented by silencing IGF-IR expression (Figures 1g–j). In immunofluorescence studies performed in MCF-7 cells, IGF-I further confirmed the ability to upregulate GPER expression (Figure 2), an effect that was no longer observed transfecting cells with shGPER (Figure 2). According to these observations, IGF-I

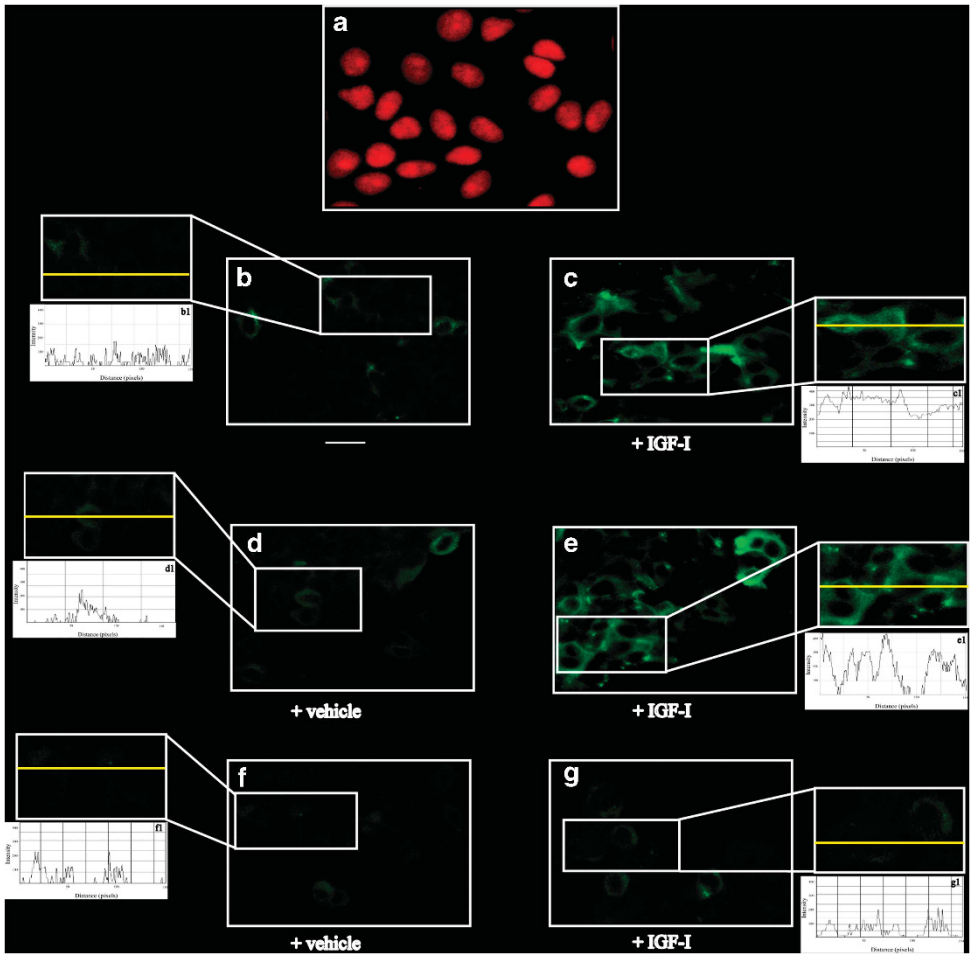


**Figure 2.** Representative fluorescence images of GPER immunolabelling. MCF-7 cells were fixed, permeabilized and stained with anti-GPER antibody. (**a**) Nuclei (red) were stained by propidium iodide. (**b**, **c**) Cells were treated for 24 h with vehicle (−) or 100 ng/ml IGF-I (as indicated), GPER accumulation was evidenced by the green signal. MCF-7 cells were transfected with a control shRNA (**d**, **e**) or with a shGPER (**f**, **g**) and treated as described above, then stained with GPER antibody. For descriptive purposes, Figures 1b–g, show the plot profiles obtained at the level of the yellow line of the corresponding inset using the program WCIF Image J for Windows. Note the higher values indicating zones of intense labeling. Each experiment shown is representative of 10 random fields. Data are representative of three independent experiments.

transactivated the *GPER* promoter construct transiently transfected in MCF-7 cells (Supplementary Figure 1).

### Molecular mechanisms involved in the upregulation of GPER by IGF-I

As the inhibitors of PKC and MEK transduction pathways prevented the upregulation of GPER induced by IGF-I, we then evaluated the activation of PKC and ERK in MCF-7 cells. IGF-I promoted rapid PKCδ phosphorylation, which was no longer evident in the presence of the PKC inhibitors GF and Rot, while it was still present using the MEK inhibitor PD (Figure 3a). Moreover, IGF-I also induced a rapid phosphorylation of ERK1/2, which was abolished by PD as well as by the PKC inhibitors GF and Rot (Figure 3b). Taken together, these data suggest that ERK1/2 activation is downstream PKCδ in MCF-7 cells treated with IGF-I.

It has been previously shown that the activation of the ERK transduction pathway leads to a rapid upregulation of c-fos,[11,13,32,33] which has a relevant role in the growth stimulation of normal and cancer cells mainly forming the AP1 transcription complex with jun family members.[34] Accordingly, the ERK activation upon exposure to IGF-I was paralleled by the induction of c-fos (Figure 3c). Of note, this response was abrogated using AG, GF, Rot and PD, suggesting that the IGF-IR/PKCδ/ERK signaling mediates the regulation of c-fos induced by IGF-I in MCF-7 cells (Figure 3c). Confirming the aforementioned data, IGF-I transactivated an AP1-luc-responsive collagenase promoter construct that was transiently transfected in MCF-7 cells, while the luciferase activity was abrogated in presence of AG, GF, Rot, PD (Figure 3d) or co-transfecting a dominant-negative form of c-fos (DN/c-fos) (Figure 3e). Of note, performing chromatin immunoprecipitation assay in MCF-7 cells, we ascertained that IGF-I induces the recruitment of c-fos to the AP1 site located within the promoter sequence of *GPER* (Figure 3f). In accordance with these results, the transactivation of the *GPER* promoter construct by IGF-I was abolished co-transfecting the DN/c-fos (Figure 3g) and the upregulation of the GPER protein levels was prevented in the presence of the DN/c-fos (Figure 3h). Taken together, these findings indicate that the IGF-IR/PKCδ/ERK/c-fos-AP1 transduction pathway mediates the transcription of GPER induced by IGF-I.

### GPER is involved in the migration and proliferation promoted by IGF-I

As *CTGF* is a major gene target of GPER activity,[12] we asked whether CTGF responds to IGF-I through GPER. In MCF-7 cells, IGF-I transactivated the *CTGF* promoter construct (Figure 4a) and this effect was prevented silencing *GPER* expression. Likewise, the

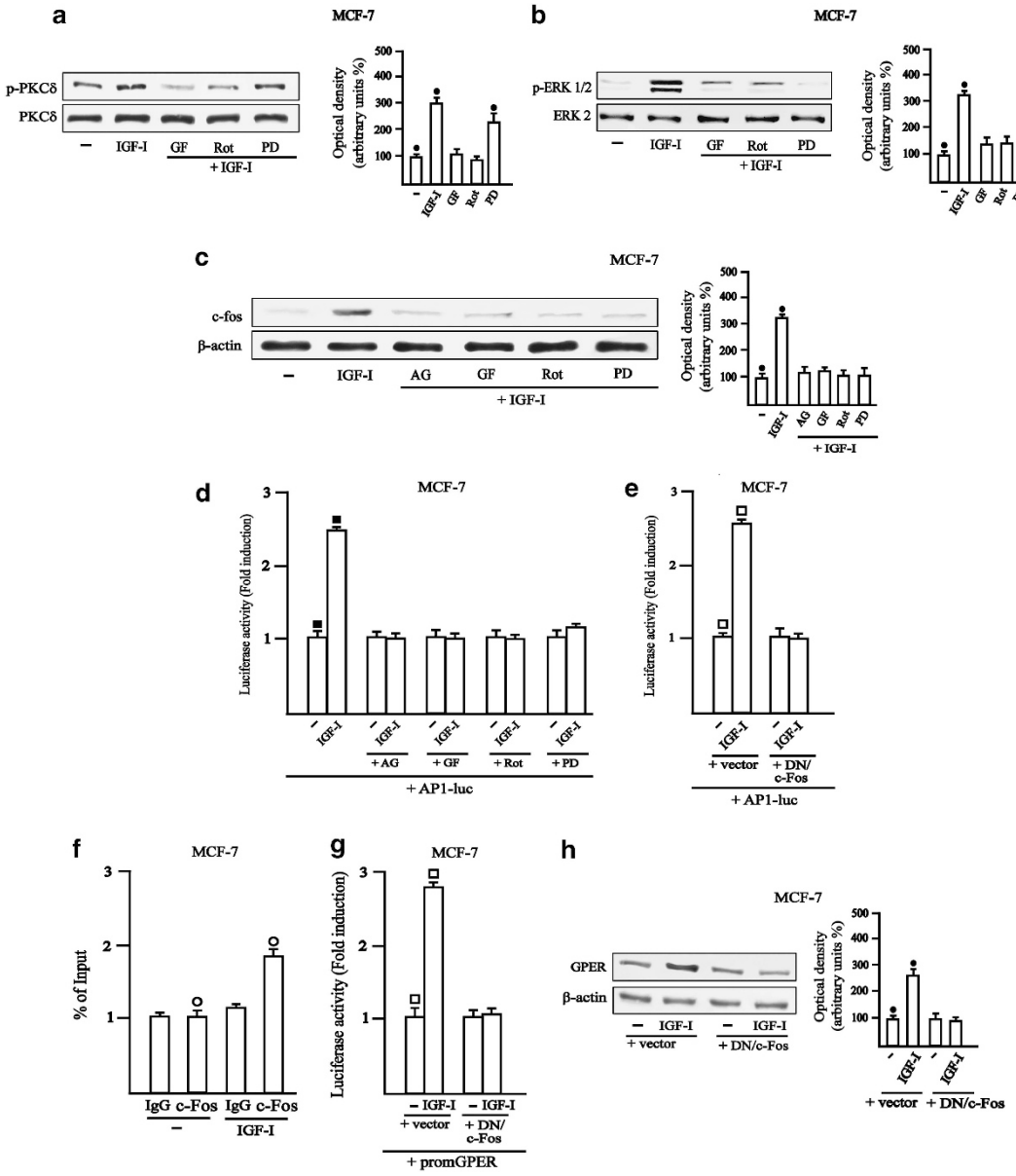


**Figure 3.** Transduction pathways mediating GPER upregulation by IGF-I in MCF-7 cells. Immunoblots of p-PKCδ (**a**) and p-ERK1/2 (**b**) from MCF-7 treated for 15 min with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD). Immunoblots shown are representative of experiments performed in triplicate. Side panels show densitometric analysis of the blots normalized to total ERK2 and PKCδ. (**c**) Immunoblotting of c-fos from MCF-7 cells treated for 3 h with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM IGF-IR inhibitor tyrphostin AG1024 (AG), 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD). (**d**) Cells were transfected with AP1-luc-responsive collagenase promoter and treated with 100 ng/ml IGF-I alone and in combination with AG, GF, Rot or PD, as indicated. (**e**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the transactivation of AP1-luc by 100 ng/ml IGF-I. (**f**) A 100-ng/ml IGF-I induces the recruitment of c-fos to the AP1 site located within the *GPER* promoter sequence. In control samples, non-specific IgG was used instead of the primary antibody. (**g**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the transactivation of the GPER promoter construct. (**h**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the upregulation of GPER protein levels by 100 ng/ml IGF-I. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activities induced by 100 ng/ml IGF-I were calculated. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ●, ■, □, ○ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments. Side panels in (**c**) and (**h**) show densitometric analysis of the blots normalized to β-actin.

induction of the CTGF protein levels by IGF-I in both MCF-7 and Ishikawa cells was no longer evident, abrogating the expression of *GPER* (Figures 4b and d). As a biological counterpart, the migration stimulated by IGF-I after both 6 h (data not shown) and 24 h of treatment (Figure 5) was abolished silencing either *GPER* or *CTGF* expression by transfecting MCF-7 and Ishikawa cells for 24 h with specific shGPER and shCTGF constructs (Supplementary Figure 2). Considering that both cell types used express ERα, we next determined that its expression is also required for the migration induced by IGF-I after both 6 h (data not shown) and 24 h of treatment (Figure 5 and Supplementary Figure 2) (see Discussion). On the basis of previous investigations showing that IGF-I

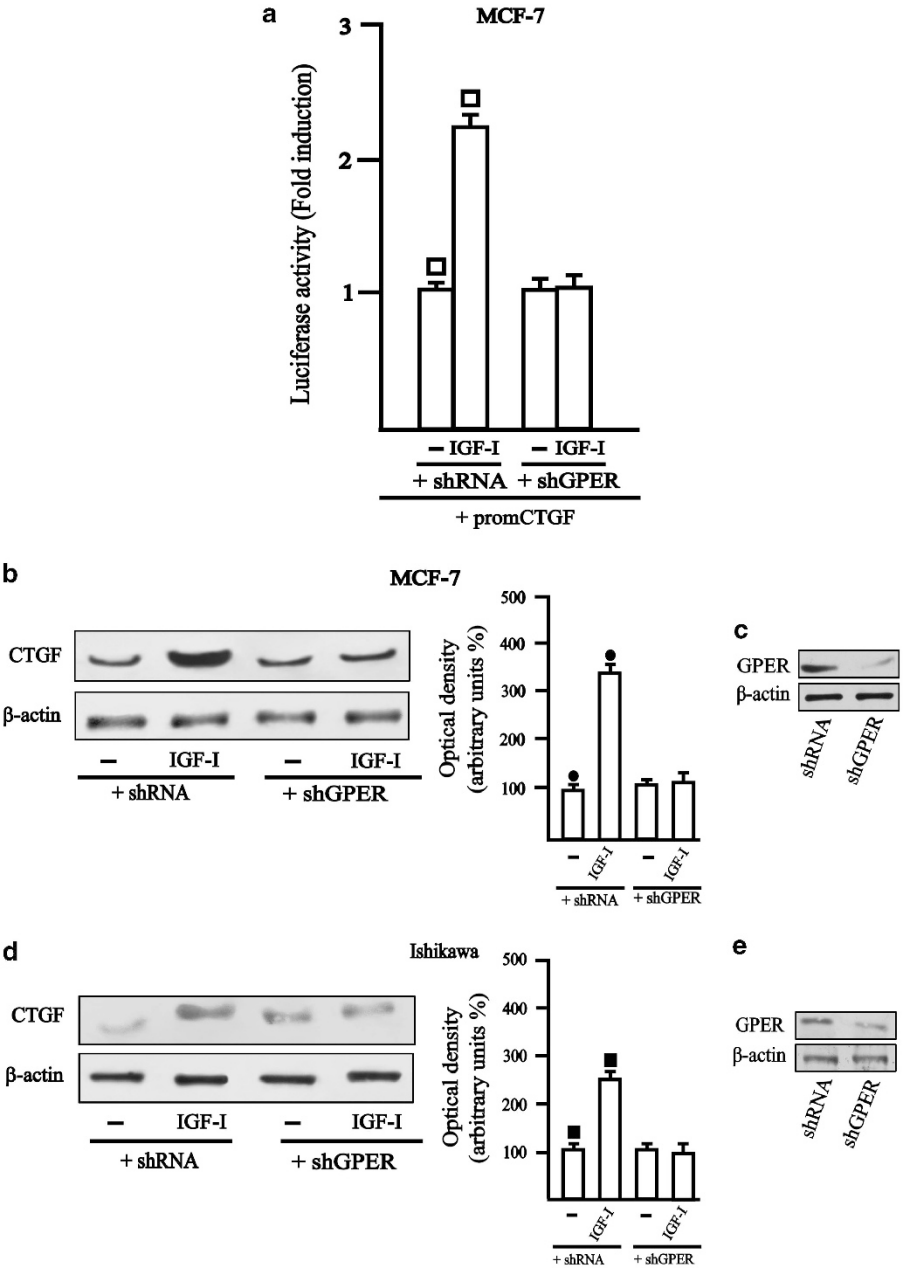


**Figure 4.** IGF-I transactivates the *CTGF* promoter construct and upregulates CTGF protein levels. (**a**) The IGF-I (100 ng/ml) induced transactivation of *CTGF* promoter construct is abrogated in presence of shGPER in MCF-7 cells. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activities induced by 100 ng/ml IGF-I were calculated. (**b**, **d**) In MCF-7 and Ishikawa cells treated for 3 h with 100 ng/ml IGF-I, the upregulation of CTGF protein levels was abrogated in presence of shGPER. Side panels show densitometric analysis of the immunoblots normalized to β-actin. (**c**, **e**) Efficacy of *GPER* silencing by shGPER. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. □, ●, ■ Indicate $P < 0.05$ for cells receiving vehicle (−) versus treatments.

promotes the proliferation of cancer cells,[25,26] we therefore asked whether a functional cross talk between IGF-I and GPER is involved in the growth responses to IGF-I. Notably, the proliferation induced by IGF-I in MCF-7 and Ishikawa cells was abolished by silencing *GPER* expression (Figures 6a and c). In accordance with these observations, the upregulation of cyclin D1 induced by IGF-I in MCF-7 cells was abrogated knocking down *GPER* expression (Figure 6e) or transfecting the DN/c-fos construct (Figure 6g). In addition, a direct interaction between GPER and cyclin D1 was found upon exposure to IGF-I in MCF-7 cells (Figures 6h–i). Collectively, these results suggest that GPER is involved in a signaling network that mediates the migration and proliferation induced by IGF-I in MCF-7 and Ishikawa cells.

### ERα is involved in the regulation of GPER by IGF-I

Considering the well known cross talk between IGF-I system and ERα in cancer cells,[27] we aimed at evaluating whether ERα is involved in the upregulation of GPER expression induced by IGF-I. Interestingly, the transactivation of the *GPER* promoter construct by IGF-I was prevented using the ERα inhibitor ICI 182,780 (ICI) (Figure 7a). Accordingly, the increase of GPER protein levels by

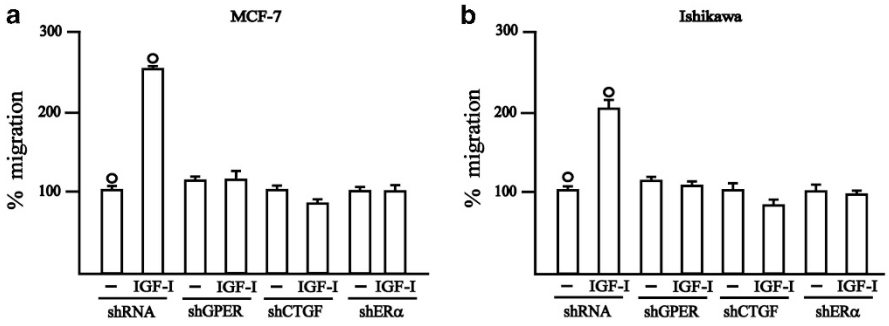


**Figure 5.** GPER, CTGF and ERα are involved in the migration of (**a**) MCF-7 and (**b**) Ishikawa cells induced by IGF-I. Cell migration promoted by IGF-I was abolished silencing *GPER*, *CTGF* or *ERα* expression. Bar graph shows a representative experiment with means of triplicate samples, standardized to the respective untreated controls set to100%. Error bars show standard deviations. ○ Indicates $P<0.05$ for cells receiving vehicle (−) versus treatments.

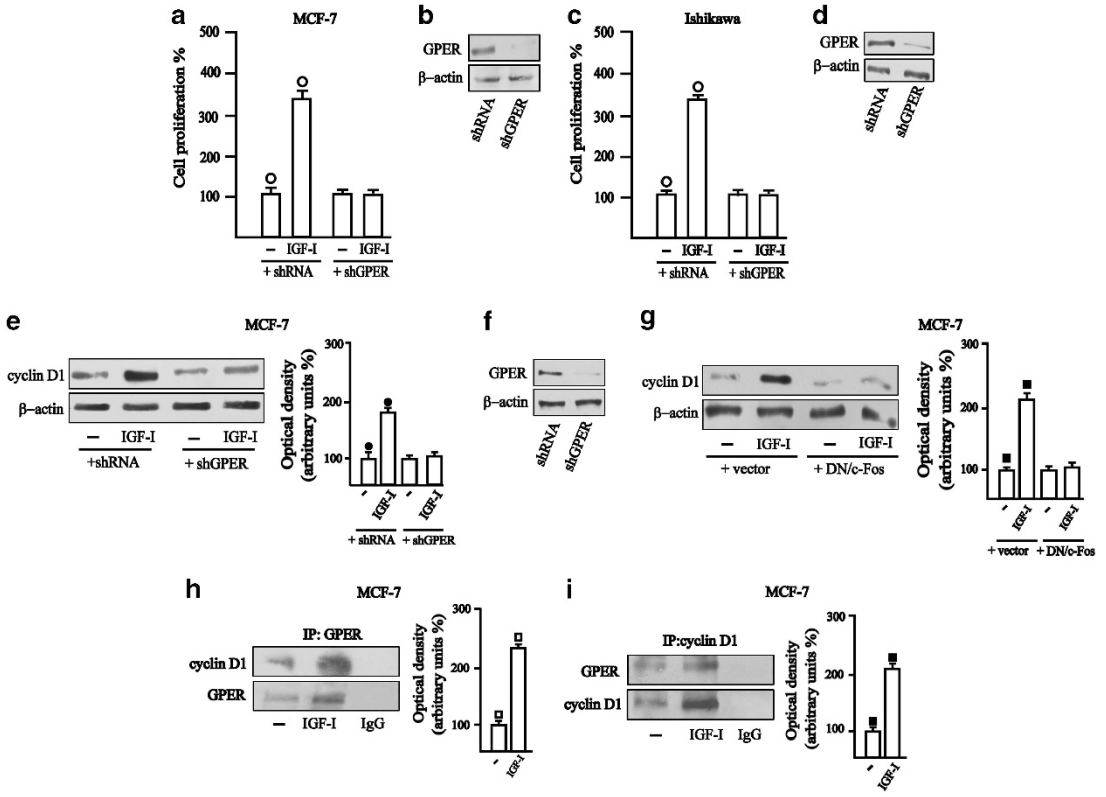


**Figure 6.** GPER is required for proliferation of MCF-7 and Ishikawa cells induced by IGF-I. (**a**, **c**) Cell proliferation induced by 100 ng/ml IGF-I was abrogated by silencing GPER expression. (**e**, **g**) The upregulation of cyclin D1 protein by 100 ng/ml IGF-I was abolished in the presence of shGPER and DN/c-fos. Side panels show densitometric analysis of the blots normalized to β-actin. (**b**, **d**, **f**) Efficacy of *GPER* silencing. (**h**, **i**) The treatment for 24 h with 100 ng/ml IGF-I strongly increases the coimmunoprecipitation of GPER with cyclin D1 in MCF-7 cells, as indicated. In control samples, non-specific IgG was used instead of the primary antibody. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ○, ●, ■, □ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

IGF-I was abolished in the presence of ICI or silencing *ERα* expression (Figures 7b–d). Further corroborating these results, in MCF-7 cells the recruitment of p-ER$\alpha^{Ser118}$ to an AP1 site located within the GPER promoter sequence induced by IGF-I (Figure 7e) was no longer evident transfecting cells with the DN/c-fos construct (Figure 7f).

Using carcinoma-associated fibroblasts obtained from breast tumors,[35] we further confirmed the ability of IGF-I to upregulate the expression of GPER and its target gene *CTGF*[12] (Supplementary Figure 3). Hence, the property of IGF-I to regulate GPER expression can be extended to these cancer surrounding cells, which mainly contribute to the malignant tumor features.[36]

## DISCUSSION

Cross talk between estrogen and IGF-I signaling has been involved in several biological functions in breast cancer cells, including proliferation, survival, transformation, migration, adhesion and invasion.[37,38] For instance, IGF-I transactivates ERα in a ligand-independent manner,[28] and activated IGF-IR interacts with ERα

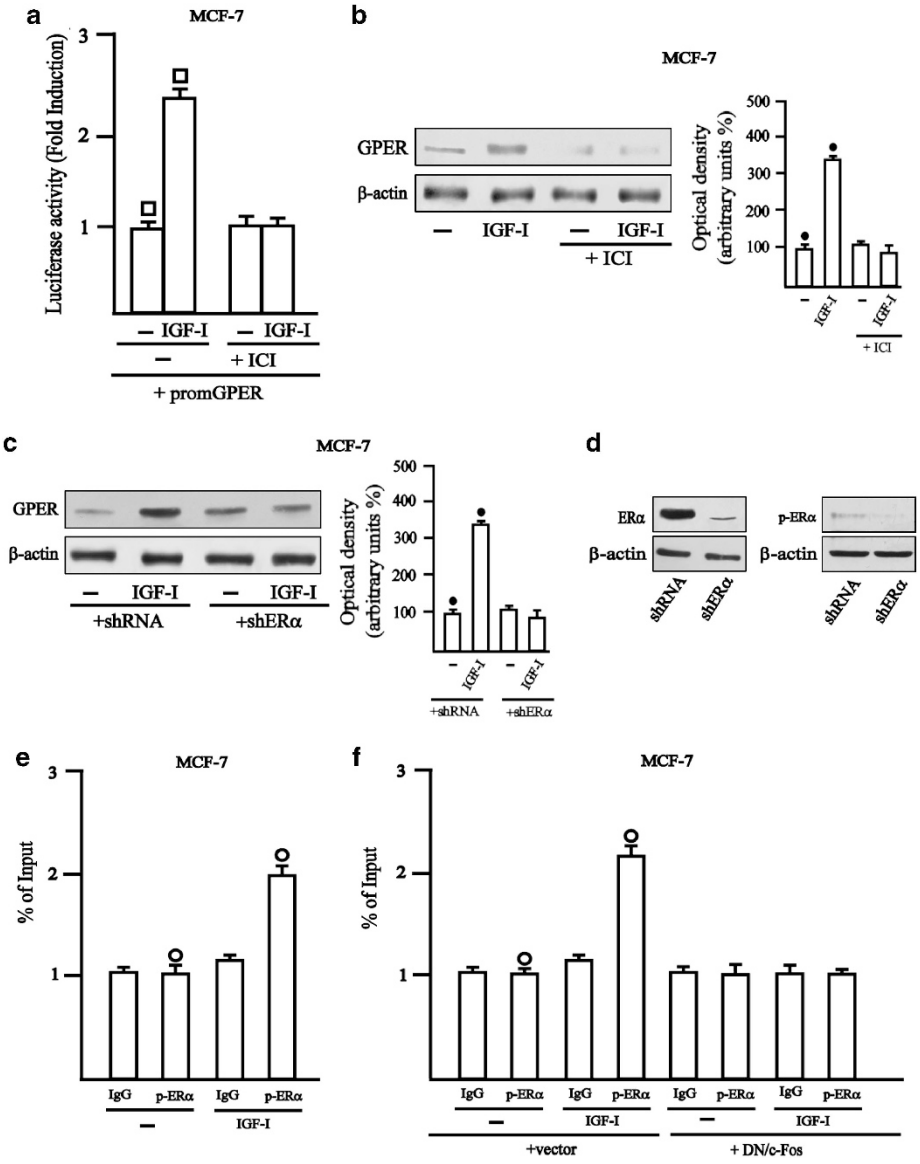


**Figure 7.** ERα is involved in the GPER transcription by IGF-I. (**a**) The transactivation of *GPER* promoter construct induced by 100 ng/ml of IGF-I is abrogated in presence of 10 μM ICI 182,780 (ICI). The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activity induced by treatments was calculated. (**b**, **c**) The IGF-I induced upregulation of GPER protein levels was abolished by 10 μM ICI and by silencing ERα expression. (**d**) Efficacy of *ERα* and p-ER$\alpha^{Ser118}$ silencing. (**e**, **f**) The recruitment of p-ER$\alpha^{Ser118}$ induced by 100 ng/ml IGF-I to the AP1 site located within the *GPER* promoter sequence is abolished in presence of an expression vector encoding a dominant negative form of c-fos (DN/c-fos). In control samples non-specific IgG was used instead of the primary antibody. Each column represents the mean ± s.d. of three independent experiments. □, ●, ○ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

contributing to breast cancer development and progression.[39,40] In addition, IGF-IR has been shown to be involved in non-genomic, membrane-associated ERα activity,[37] although many aspects remain to be further elucidated. In this regard, previous studies on the functional interaction between the IGF-I system and estrogens have focused only on estrogen signaling mediated by ERα. In recent years, the identification of GPER as a novel ER has opened a new scenario, which is still under investigation for the assessment of its full impact on the action of estrogens. Considering the functional cross talk between IGF-I and the estrogen-dependent pathways as well as the lack of data on the ability of IGF-I system to engage GPER in triggering biological responses, in the present study, we aimed to evaluate whether IGF-I could regulate GPER expression and function in MCF-7 and Ishikawa cancer cells. These cells express relatively low amounts of insulin receptors (IR) that may contribute to the formation of IR/IGF-IR hybrid receptors, which also bind to IGF-I with a slightly lower affinity than homotypic IGF-IR.[41–43] Therefore, both MCF-7 and Ishikawa cells have low IR/IGF-IR content;[42,43] hence, we believe that most of the IGF-I effects on GPER signaling are mediated by the homotypic IGF-IR.

Our results demonstrate that IGF-I transactivates the promoter of *GPER* and upregulates its expression at both mRNA and protein levels in MCF-7 and Ishikawa cancer cells. In particular, we show that the induction of GPER by IGF-I is mediated by sequential events such as the rapid activation of PKCδ and ERK1/2 and the stimulation of c-fos, which is then recruited to an AP1 site located within the *GPER* promoter sequence. The functional role elicited

by the AP1 complex is essential, as the transactivation of the *GPER* promoter sequence and the upregulation of GPER expression were abrogated by using a construct encoding for a dominant negative form of c-fos. Most importantly, GPER and its major target gene *CTGF* were required for cell migration induced by IGF-I. Given that CTGF primarily modulates and coordinates cell responses through the involvement of components of the extracellular matrix,[44,45] the GPER/CTGF signaling activated by IGF-I might contribute to cell motility and invasion during cancer progression. Likewise, we also show that cell proliferation in response to IGF-I, requires a functional cross talk between GPER and the main cell cycle regulator cyclin D1. On the basis of the aforementioned observations, GPER may contribute to the intricate transduction network engaged by IGF-I in triggering important biological effects in cancer cells.

It has been largely reported that ERα mediates biological responses leading to the progression of estrogen-sensitive tumors.[46] In the classical model of action, ERα bound to the estrogen-responsive elements located within the promoters of target genes recruits an array of co-factors involved in the regulation of the transcriptional machinery.[24,47] In addition, ERα modulates gene expression by a functional interaction with transcription factors, like AP1,[47] as well as with GPER as demonstrated in our previous studies.[11,17] In this regard, the present data reveal that ERα is also involved in the IGF-I-dependent regulation of GPER expression and therefore to GPER-mediated action. Hence, extending the current knowledge on the cross talk between ERα and GPER, our results indicate that these different ERs cooperate in mediating various extracellular stimuli leading to gene regulation and growth effects in cancer cells. Different forms of interaction between GPCRs and growth factor receptors have become increasingly evident in different cellular functions,[29] including cancer growth, angiogenesis and metastasis.[48] In particular, IGF-IR requires GPCRs in regulating many physiological functions.[49] Indeed, the cross talk between IGF-I and GPCR-dependent pathways occurs at multiple levels in a variety of malignancies.[50] For instance, a physical association between IGF-IR and the G protein subunits, Gα and Gβγ, has been involved in cell migration in breast cancer cells.[51] In addition, IGF-I cooperated with agonists of GPCRs in promoting cell responses such as DNA synthesis, proliferation and anchorage-independent growth in pancreatic cancer cells.[31] The identification of GPER has expanded our understanding on the regulation and action of GPCRs. In this vein, it is worth to note that EGFR ligands transactivated the promoter of *GPER* and upregulated its expression in cancer cells.[14,15] In addition, we have recently demonstrated that hypoxia, through the HIF-1α-mediated transduction pathway, induces GPER expression, hence suggesting a new mechanism by which estrogens may exert relevant biological effects under hypoxic conditions.[52] We have also found that GPER interacting with EGFR acts as a transcription factor in carcinoma-associated fibroblasts,[35] indicating the potential of GPER to affect cancer progression through the stimulation of cells surrounding tumors. Similarly, the present findings that GPER expression and function are regulated by IGF-I in cancer cells as well as in carcinoma-associated fibroblasts, shed new light on a novel mechanism by which the IGF-I system and estrogen signaling cooperate toward cancer development and progression. Of note, previous reports have shown that higher GPER levels were associated with worse clinical pathological features and lower survival rates in estrogen-sensitive tumors.[16,17] Therefore, the expression levels of GPER 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 adverse outcome and survival. Thus, a better understanding on the GPER regulation and function represents a new challenge for a more comprehensive therapeutic approach in estrogen-responsive malignancies including breast and endometrial tumors. In this regard, our results further indicate that GPER is involved in an intricate tumorigenic transduction network, which should be considered as a promising therapeutic target toward innovative intervention.

## MATERIALS AND METHODS

### Reagents

IGF-I, H89, LY294,002 (LY) and PD98059 (PD) were purchased from Sigma-Aldrich Corp. (Milan, Italy). Bisindolylmaleimide I (GF109203X) was bought from Santa Cruz Biotechnology (DBA, Milan, Italy), 3-bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024) and Rottlerin from Calbiochem (Milan, Italy). ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, UK). All compounds were solubilized in dimethylsulfoxide, except PD and IGF-I, which were dissolved in ethanol and in water, respectively.

### Cell culture

MCF-7 breast cancer cells were maintained in DMEM/F-12 (Invitrogen, Gibco, Milan, Italy) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma, Milan, Italy). Ishikawa endometrial cancer cells were maintained in DMEM (Invitrogen, Gibco) without phenol red supplemented with 10% fetal bovine serum 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma). Cells were switched to medium without serum the day before experiments; thereafter cells were treated as indicated. Primary fibroblast cells from breast cancer tissues were obtained as we have previously described[35] and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum, 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma).

### Plasmids

The GPER luciferase expression vector promGPER was previously described.[52] The CTGF luciferase reporter plasmid promCTGF (-1999/+36)-luc was a gift from Dr B Chaqour.[53] The luciferase reporter plasmid for AP-1-responsive collagen promoter was a kind gift from H van Dam (Department of Molecular Cell Biology, Leiden University, Leiden, Netherlands). As an internal transfection control, we cotransfected the plasmid pRL-TK (Promega, Milan, Italy) that expresses RenillaLuciferase. The plasmid DN/c-fos, which encodes a c-fos mutant that heterodimerizes with c-fos dimerization partners but does not allow DNA biding,[54] was a kind gift from Dr C Vinson (NIH, Bethesda, MD, USA). The Sure Silencing (sh) IGF-IR, (sh) ERα and the respective control plasmids (shRNA), generated in pGeneClip Puromycin Vector, were purchased from SA Bioscience Corp. (Frederick, MD, USA) and used according to the manufacturer's recommendations (more information is available at www.sabiosciences.com). Short hairpin constructs against human GPER (shGPER) and CTGF (shCTGF) were obtained and used as previously described.[12,14] In brief, they were generated in lentiviral expression vector pLKO.1 purchased by Euroclone, Milan, Italy. The targeting strand generated from the GPER shRNA construct is 5′-CGCTCCCTGCAAGCAGTCTTT-3′. The targeting strand generated from the CTGF shRNA construct is 5′-TAGTACAGCGATTCAAAGATG-3′.

### Transfections and luciferase assays

MCF-7 cells ($1 \times 10^5$) 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 FuGene6 reagent, as recommended by the manufacturer (Roche Diagnostics, Milan, Italy), with a mixture containing 0.5 μg 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 cells were incubated for an additional 24 h. Luciferase activity was then measured with the Dual Luciferase Kit (Promega Italia, Milan, Italy) 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 set

as onefold induction upon which the activity induced by treatments was calculated.

### Reverse transcription and real-time PCR

Total RNA was extracted using Trizol commercial kit (Invitrogen) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gel stained with ethidium bromide. Total cDNA was synthesized from RNA by reverse transcription 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 Step One (TM) sequence detection system (Applied Biosystems Inc, Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc.). Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. For GPER and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-ACACACC TGGGTGGACACAA-3′ (GPER forward); 5′-GGAGCCAGAAGCCACATCTG-3′ (GPER reverse); 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward); and 5′-GGG CATCACAGACCTGTTATT-3′ (18S reverse). Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression.

### Western blotting

Cells were grown in a 10-cm dishes and exposed to ligands before lysis in 500 μl of lysis buffer containing: 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% TritonX-100, 1% sodium dodecyl sulfate (SDS), 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 electrophoreted through a reducing SDS/10% (w/v) polyacrilamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy). Membranes were blocked and probed with primary antibodies against GPER (N-15), CTGF (L-20), c-Fos (H-125), phosphorylated ERK $\frac{1}{2}$ (E-4), and ERK2 (C-14), phosphorylated PKCδ (Thr 507), PKCδ (C-20), cyclin D1 (M-20), ERα (F-10), IGF-IR (7G11), and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA), and p-ERα$^{Ser118}$(16J4) purchased from Cell Signaling Technology. The levels of protein and phospho proteins were detected with appropriate secondary HRP-conjugated antibodies and the ECL (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). All experiments were performed in triplicate and blots shown are representative.

### Co-immunoprecipitation

For co-immunoprecipitation of GPER/cyclin D1 complex, cells were lysed using 500 μl of RIPA buffer (Sigma) with a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). A total of 125 μg proteins were incubated overnight with 2 μg of GPER (N-15) or cyclin D1 (M-20) antibody and 20 μl of protein A/G agarose immunopreciptation reagent (Santa Cruz Biotechnology, Inc.). After four washes in PBS, samples were resuspended in 20 μl of SDS-sample buffer (0.5 M Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 4% bromophenol blue). Western Blot analysis was performed as described above.

### Chromatin immunoprecipitation

Cells grown in 10 cm plates were shifted for 24 h to medium lacking serum and then treated with vehicle or 100 ng/ml IGF-I for 3 h. Chip assay was performed as we have previously described.[52] The immune cleared chromatin was immunoprecipitated with anti c-fos (H-125) and anti ERα (F-10) antibodies or non-specific IgG (Santa Cruz Biotechnology, DBA).

A 4-μl volume of each immunoprecipitated DNA sample was used as template to amplify, by RT–PCR, one fragment of 261 bp, containing an AP-1 site, located into the GPER promoter region. The primer pairs used to amplify this fragment were: 5′-CGTGCCCATACCTTCATTGCTTCC-3′ (forward) and 5′-CCTGGCCGGGTGTCTGTG-3′(reverse). Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 4 μl of the template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent differences with respect to the relative inputs.

### Immunostaining assay

Fifty percent confluent cultured MCF-7 cells grown on coverslips were serum deprived and transfected for 12 h with a control shRNA or a shRNA specific for GPER (shGPR30), using Fugene 6 reagent, as recommended by the manufacturer, and then treated for 24 h with vehicle or 100 ng/ml IGF-I. Thereafter cells were fixed in 4% paraformaldehyde; permeabilized with 0.2% TritonX-100, washed three times with PBS, blocked and incubated overnight with a rabbit primary antibody GPR30 (N-15) (1:500; purchased from Santa Cruz Biotechnology). 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). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS, GbH Mannheim, Germany) were used for experiments evaluation.

### Proliferation assay

For quantitative proliferation assay, cells ($1 \times 10^5$) were seeded in 12-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped fetal bovine serum; when used, 500 ng of the indicated shRNA were added to cells using Fugene 6 reagent as recommended by the manufacturer and then renewed every 2 days before counting; treatments were added every day. Evaluation of cell growth was performed after 4 days using automatic counter (Countess - Invitrogen). Data shown are representative of three independent experiments performed in triplicate.

### Migration assay

Migration assays were performed using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane). For knockdown experiments, cells were transfected with shRNA constructs directed against GPER or CTGF or ERα with an unrelated shRNA construct (3 μg DNA/well transfected with FuGene6 reagent in medium without serum). After 24 h, cells were seeded in the upper chambers. IGF-I was added to the medium without serum in the bottom wells. After 24 h, cells on the bottom side of the membrane were fixed and counted.

### 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.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)