Università della Calabria

Dipartimento di Farmacia e Scienze della Nutrizione e della Salute

DOTTORATO DI RICERCA IN BIOCHIMICA CELLULARE E ATTIVITA' DEI FARMACI IN ONCOLOGIA

(XXVIII CICLO)

Settore Scientifico Disciplinare MED/04

Novel molecular mechanisms involved in the stimulatory action of zinc in breast cancer

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A.A. 2015/2016

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Abstract

Lo Zinco (Zn), minerale essenziale che regola diverse funzioni biologiche, è coinvolto nella progressione del tumore mammario. In particolare, è stato dimostrato che in seguito all'attivazione indotta dallo Zn di recettori ad attività tirosin-chinasica, come IGF-IR, EGFR e IR, vengono innescate vie di trasduzione del segnale che sono alla base della progressione tumorale, quali la via delle MAP-chinasi (MAPK) e la via del fosfatidilinositolo3-chinasi (PI3-K)/AKT. Numerosi studi hanno inoltre dimostrato il coinvolgimento di recettori accoppiati a proteina G (GPCRs) nello sviluppo dei tumori e la loro azione sinergica con vari recettori di membrana . Alla luce di tali osservazioni, una migliore comprensione dei processi attraverso cui i recettori per i fattori di crescita cooperano con segnali mediati da GPCRs potrebbe contribuire allo sviluppo di nuove strategie terapeutiche volte a prevenire e/o ritardare la crescita tumorale. Nel presente studio, è stato dimostrato che lo Zn è coinvolto nel cross-talk funzionale tra IGF-IR, EGFR e GPER in cellule di tumore mammario e fibroblasti tumore-associati (CAFs). In particolare, è stato dimostrato che GPER, IGF-IR e EGFR contribuiscono agli effetti stimolatori indotti da ZnCl2 nella progressione del ciclo cellulare, nella proliferazione e nella migrazione di cellule di carcinoma mammario e dei CAFs. I nostri risultatievidenziano nuovi meccanismi molecolari attraverso i quali lo Zn può indurre effetti stimolatori in cellule di tumore mammario e nei CAF, suggerendo pertanto nuovi potenziali approcci farmacologici nel trattamento del carcinoma mammario.

Abstract

Zinc (Zn) contributes to the regulation of several cellular functions, however it may be also implicated in the progression of breast cancer through different mechanisms. For instance, Zn may activate tyrosine kinase receptors, as insulin-like growth factor receptor I (IGF-IR), epidermal growth factor receptor (EGFR) and the insulin receptor (IR), which then trigger the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/AKT transduction signaling. These pathways have been largely implicated in cancer growth and invasion together with other important signal molecules like the G-protein coupled receptors (GPCRs). In this regard, the cross-talk between GPCRs and growth factor receptors has been shown to contribute to cancer growth, angiogenesis and metastasis. Recently, both EGF and IGF-I mediated pathways were demonstrated to interact with the G protein estrogen receptor (GPER, also known as GPR30), which has been involved in the proliferation and migration of several types of cancer and stromal cells. Overall, these findings indicate that a better understanding of the molecular mechanisms by which growth factor receptors cooperate with GPCR signals would be highly relevant toward new therapeutic strategies aimed to prevent or delay the progression of several tumors. In the present study, we ascertained that zinc chloride ($ZnCl_2$) triggers a functional crosstalk of GPER with IGF-IR and EGFR in breast cancer cells and in main components of the tumor microenvironment like cancer-associated fibroblasts (CAFs) derived from breast cancer patients. Further corroborating these data, we assessed that GPER along with IGF-IR and EGFR contribute to the stimulatory effects induced by $ZnCl_2$ on cell-cycle progression, proliferation, and migration of breast cancer cells and CAFs. Our results provide novel mechanistic insights through which Zn may induce stimulatory effects in breast tumor cells and CAFs, suggesting further molecular targets in the treatment of breast malignancy.

Chapter 1

Introduction

1.1 Introduction

Zinc (Zn) is the second most abundant heavy metal in human tissues and contributes to the regulation of crucial cellular functions [1]. As an essential mineral, Zn is required for protein, nucleic acid, carbohydrate and lipid metabolism and is involved in gene transcription, growth, development and differentiation [1]. Zn is normally found in air, water and soil, however, Zn concentrations may be boosted by several industrial activities including mining, coal and waste combustion and steel processing [2]. For instance, soils located in areas where Zn is mined, refined or used as fertilizer, are heavily contaminated with the metal [2]. The Recommended Daily Allowance of Zn in adults is 8–11mg/day, with a tolerable upper intake level of 40mg/day [3-5]. The adverse effects associated with a high Zn intake include acute gastrointestinal effects and headache, impaired immune function, changes in lipoprotein and cholesterol levels, reduced copper levels and zinc-iron interactions as well as various other disorders [6-8]. In addition, Zn has been involved in the development of several types of tumors including breast cancer [9-10]. In this regard, previous studies have reported an association between dysregulated Zn homeostasis and breast cancer progression together with higher Zn levels in breast tumor specimens as compared to normal mammary tissues [11-12]. Compelling evidence has also linked an aberrant expression of Zn transporter proteins with the proliferation and migration of breast cancer cells [13-15]. A recent study has also suggested that specific dysregulations of Zn transporters may characterize grade, invasiveness, metastatic potential and response to therapy in breast cancer [16]. Of note, zinc

regulated transporters (ZIP) that control Zn influx into the cytosol, were found to be up-regulated by estrogens [17], and increased ZIP levels in breast tumors resulted to be associated with a poor prognosis [15]. Noteworthy, Zn may activate tyrosine kinase receptors as EGFR, IGF-IR and the insulin receptor, which then trigger the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/AKT signalling [18-20]. These transduction pathways have been largely implicated in cancer growth and invasion together with other important signal molecules like the G protein-coupled receptors (GPCRs) [21]. Notably, both EGF and IGF-I mediated signalling were shown to functionally interact with the G protein estrogen receptor (GPER, previously known as GPR30) transduction pathway in breast cancer cells [22-23]. In this regard, it has been reported that GPER activation induces important responses like proliferation and migration in several types of cancer cells and stromal cells that contribute to the malignant progression like cancer-associated fibroblasts (CAFs) [24].

1.2 Breast cancer

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women worldwide. Whereas localized disease is largely curable, metastatic or recurrent disease carries an unfavorable prognosis [25].As a greater percentage of breast cancers are being diagnosed at an earlier stage, the medical community has been challenged to develop diagnostic and treatment modalities that maximize benefit reducing the morbidity associated with therapy [26]. The management of breast cancer has changed considerably in the last two decades with improvements in systemic therapy and advances in surgical techniques [27].

There are two main types of breast cancer:

- Ductal carcinoma, it starts in the ducts that move milk from the breast to the nipple. Most breast cancers are of this type.

- Lobular carcinoma, it starts in the parts of the breast, called lobules that produce milk. In rare cases, breast cancer can start in other areas of the breast. Breast cancer may be invasive or non-invasive. Non-invasive breast cancer is also called "in situ."
- Ductal carcinoma in situ (DCIS) or intraductal carcinoma, is breast cancer in the lining of the milk ducts that has not yet invaded nearby tissues. It may progress to invasive cancer if untreated.
- Lobular carcinoma in situ (LCIS) is a marker for an increased risk of invasive cancer in the same or both breasts (Fig. 1.1).

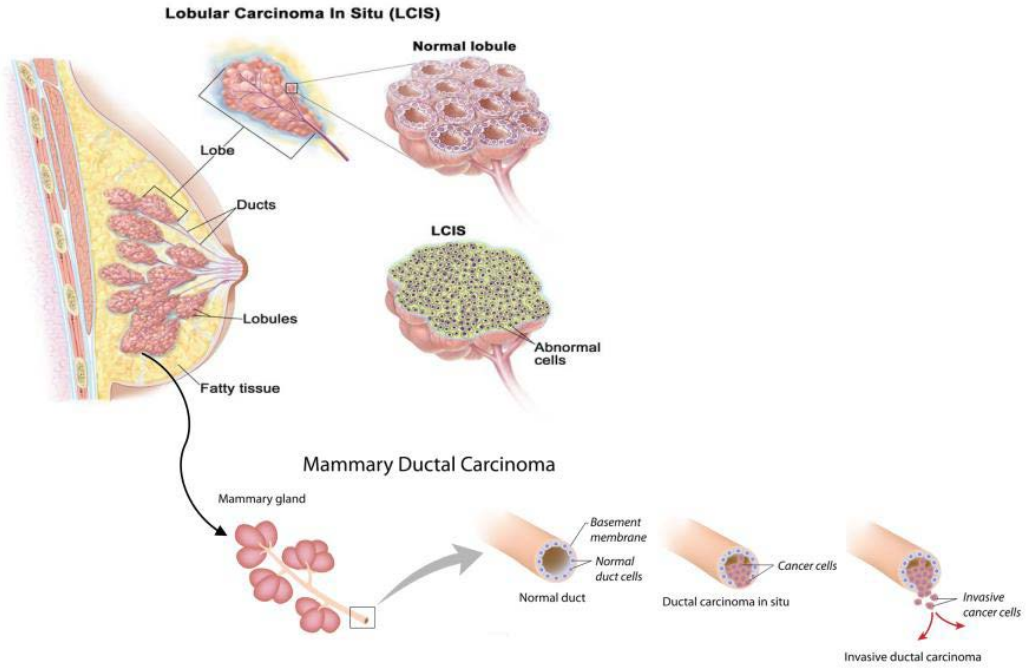

Figure 1.1 | Representation of the anatomy of the Lobular Carcinoma and Mammary Ductal Carcinoma.

There are many risk factors involved in the development of breast tumor:

- *Age and gender*. The risk of developing breast cancer increases with age. Most advanced breast cancer cases are found in women over age 50 [28]. Women are 100 times more likely to get breast cancer than men.

- *Family history of breast cancer*. You may also have a higher risk for breast cancer if you have a close relative who has had breast, uterine, ovarian, or colon cancer. About 20-30% of women with breast cancer have a family history of the disease.
- *Genes*. The most common gene defects are found in the BRCA1 and BRCA2 genes. These genes normally produce proteins that protect you from cancer. If a parent passes you a defective gene, you have an increased risk for breast cancer. Women with one of these defects have up to an 80% chance of getting breast cancer sometime during their life [29].
- *Menstrual cycle*. Women who got their periods early (before age 12) or went through menopause late (after age 55) have an increased risk for breast cancer [30].

Other risk factors include:

- *Alcohol use.* Drinking more than 1-2 glasses of alcohol a day may increase your risk for breast cancer [31].
- *Childbirth*. Women who have never had children or who had them only after age 30 have an increased risk for breast cancer. Being pregnant more than once or becoming pregnant at an early age reduces your risk of breast cancer [32].
- *Hormone replacement therapy (HRT)*. You have a higher risk for breast cancer if you have received hormone replacement therapy with estrogen for several years or more [33].
- *Obesity* has been linked to breast cancer, although this link is controversial. The theory is that obese women produce more estrogen, which can fuel the development of breast cancer [34].
- *Radiation.* The radiation therapy to treat cancer of the chest area, increase higher risk to develop breast cancer [34].Treatment is based on many factors, including: type and stage of the cancer, whether them cancer is sensitive to certain hormones, whether the cancer over-expresses a gene called HER2/neu.

In general, cancer treatments may include: surgery to remove cancerous tissue, lumpectomy removes the breast lump; mastectomy removes all or part of the breast; chemotherapy medicines to kill cancer cells, radiation therapy to destroy cancerous tissue, hormonal therapy. Most women receive a combination of treatments. For women with stage I, II, or III breast cancer, the main aim is to treat the cancer and prevent it from returning. For women with stage IV cancer, the objective is to improve symptoms and help them live longer. In most cases, stage IV breast cancer cannot be cured.

- Stage 0 and DCIS Lumpectomy plus radiation or mastectomy is the standard treatment. There is some controversy on how best to treat DCIS.
- Stage I and II Lumpectomy plus radiation or mastectomy with some sort of lymph node removal is the standard treatment. Hormone therapy, chemotherapy, and biologic therapy may also be recommended following surgery.
- Stage III Treatment involves surgery, possibly followed by chemotherapy, hormone therapy, and biologic therapy.
- Stage IV Treatment may involve surgery, radiation, chemotherapy, hormonal therapy or a combination of these treatments. After treatment, some women will continue to take medications such as tamoxifen for a period of time. All women will continue to have blood tests, mammograms, and other tests after treatment. Women who have had a mastectomy may have reconstructive breast surgery, either at the same time as the mastectomy or later [35].

1.2.1 Tumor Microenvironment

The breast cancer microenvironment is a complex combination of several different cell types and molecules and it is a key contributor to malignant progression [36]. The role of tumor microenvironment is becoming more and more important in breast cancer. Several stromal cell

types are implicated in promoting 'hallmarks' of cancer cells [37]. The microenvironment includes fibroblasts, macrophages, immune cells, adipocytes, endothelial cells, and antigenic vascular cells. Stromal cells surround and interact with tumor cells. Over the last years, a robust body of evidence has highlighted the importance of the crosstalk between tumor and stoma. Tumor microenvironment has been shown to play a crucial role in tumorigenesis, from initiation to progression. Stromal cells promote cancer growth and invasion through the chemokine–chemokine receptor axis [38, 39]. Infiltrating immune cells energize the immune effectors and vascular cells permit nutrients and oxygen uptake by tumors. In a normal mammary duct, there are luminal epithelial cells internally and myoepithelial cells externally delimited by a basement membrane, which maintains the luminal cell polarity [40]. The extracellular matrix (ECM) allows communication with the surrounding stroma. Genetic and epigenetic alterations lead to luminal cell proliferation, loss of epithelial polarity and decrease of myoepithelial cells, and changes in the ECM/basal membrane, finally resulting in mammary tumor development [41]. As opposed to normal fibroblasts, cancer-associated fibroblasts (CAFs) [42]. improve tumor growth and metastasis by producing growth factors and ECM proteins, as well as by modulating immune polarization [43]. Also, the number of CAFs is increased during tumor progression [44]. Accordingly, growth factors, cytokines, chemokines, and matrix metalloproteinases secreted by stromal cells lead to the recruitment of macrophages, endothelial precursor cells, and regulatory lymphocytes, which sustain tumor progression [45]. It is worth noting that stroma has been correlated with clinical outcomes and response to therapy in breast cancer [46]. The expression of ECM genes, uniformly expressed in both neoplastic and adjacent stromal cells, may divide breast cancers into different subgroups with different clinical outcomes [47, 48]. A study performing hierarchical clustering of the gene-expression profile of ECM-related genes classified breast cancer samples into four groups associated with different clinical outcomes [49]. Stromal signatures are highly informative for patients with breast cancer. A

serum activated gene-expression signature from activated fibroblasts was identified as a negative prognostic factor in patients with breast cancer [50]. Also, a 26-gene signature called the stroma-derived prognostic predictor was generated by tumor-associated stroma and matched normal stroma from breast cancer samples [51]. This signature was found to be an independent prognostic factor [50]. So tumor microenvironment influences patient outcomes and stromal gene expression signatures represent a strong prognostic value recapitulating the immune, angiogenic, and hypoxic responses [50]. The stromal cells can be divided into three general classes (Fig. 1.2):

- Infiltrating immune cells
- Angiogenic vascular cells
- Cancer-associated fibroblastic cells.

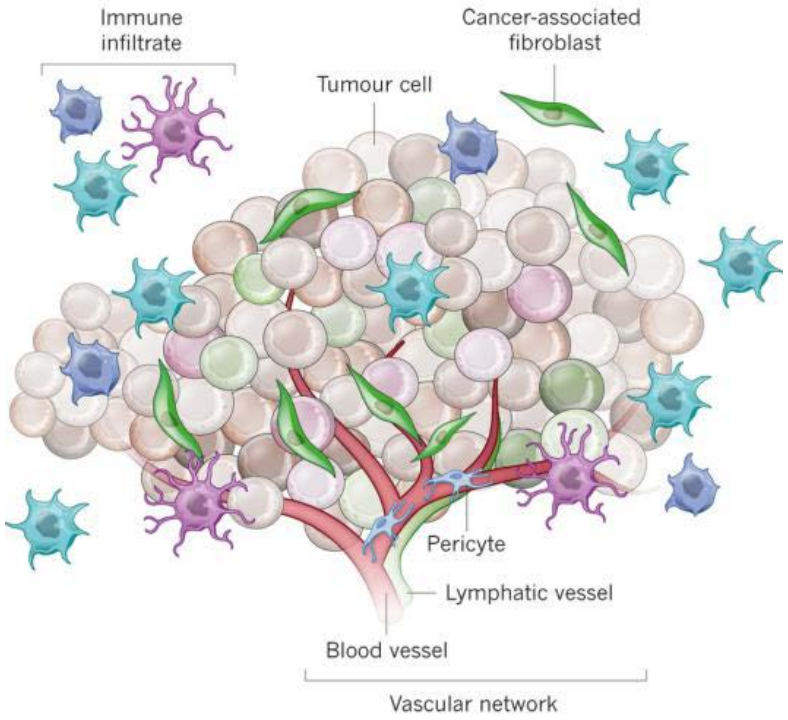

Figure 1.2 | Tumor formation involves the co-evolution of neoplastic cells together with extracellular matrix and vascular endothelial, stromal and immune cells. The tumor niche is a dynamic physical topography in which structural support, access to growth factors, vascular supply and immune cell interactions can vary drastically even within the same lesion. The immune infiltrate can include multiple cell types, these cell populations can have both pro- and anti-tumor functions and can vary in their activation status and their localization within the tumor. The vascular network can differ in regard to the vessel's tissue of origin, maturity (extent of pericyte coverage), interstitial pressure and functionality. Cancer-associated fibroblasts can have significant plasticity and diverge with regard to activation status, localization within the tissue, stress response and origin.

1.2.2 Cancer-associated fibroblasts (CAFs)

During tumorigenesis, the normal microenvironment 'niche' changes to an altered (ie, reactive or desmoplastic) stroma which is composed of non-malignant supporting cells (ie, blood vessels, infiltrating inflammatory cells and blast-like cells) [51, 52]. This altered microenvironment functions by influencing homeostasis of cancer cells via paracrine regulators (eg, growth factors, cytokines and chemokines) and exosomes containing nucleic acids [51, 53-54]. Cancer associated fibroblasts (CAFs), prominent stromal elements in most types of human carcinomas, are α-smooth muscle actin positive, spindle-shaped, blast-like cells. Differentiation of CAFs from other cell types, such as local fibroblasts, hepatic stellate cells, mesenchymal stem cells, endothelial and epithelial cells, is mainly mediated by transforming growth factor-β1 (TGF-β1), but other factors, such as growth hormones (ie, epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), chemokines, epigenetic regulators and oxidative stress also may play a role in CAF differentiation. [54, 56, 57] (Fig. 1.3). CAFs, phenotypically, closely resemble normal myofibroblasts, but they express specific markers (ie, fibroblast activation protein (FAP), fibroblast-specific protein 1, neuronglial antigen-2, vimentin, Thy-1, tenascin (TN)-C, periostin (POSTN), palladin or podoplanin (PDPN) and display an increased proliferation and migratory behaviour in vitro [58,59]. CAFs produce and secrete various extracellular matrix (ECM) proteins (ie, collagens I, III, IV), proteoglycans (ie, fibronectin, laminin, TN), chemokines (eg, CXCL and CCL), cytokines (eg, interleukin (IL)-6 and IL-8) and other tumor-promoting factors which affect vascularization (ie, PDGF, vascular endothelial growth factor (VEGF), stromal-derived factor-1 (SDF-1), matrix metalloproteinase (MMPs)), proliferation capacity, tumor cell invasiveness and survival (ie, TGF-β, EGF, hepatocyte growth factor (HGF) or FGF) [51, 60-63]. Regarding anticancer therapy, the frequency of genetic mutations in CAFs is one of the most important issues.

Cells with genetic stability may be less prone to escape or resistance to chemotherapy than those with genomic instability [64]. Several studies demonstrated that high percentage of CAFs undergo genetic alterations, such as loss of heterozygosis or mutation of tumor suppressor genes (ie, phosphatase and tensin homolog and P53) [65-68]. The theory of genetic coevolution of CAF and the neighbouring cells (ie, random mutation of CAF generated independently from neoplastic epithelial cells that may support tumor progression) is under debate, due to the potential artefacts caused by the analytical methods used for the identification of these genetic alterations [68]. Interestingly, CAF derived proteins which [51] may have an important role in the development of environment-mediated drug resistance, [52] may act as powerful prognostic markers and [53] may be promising targets of anticancer therapy [68].

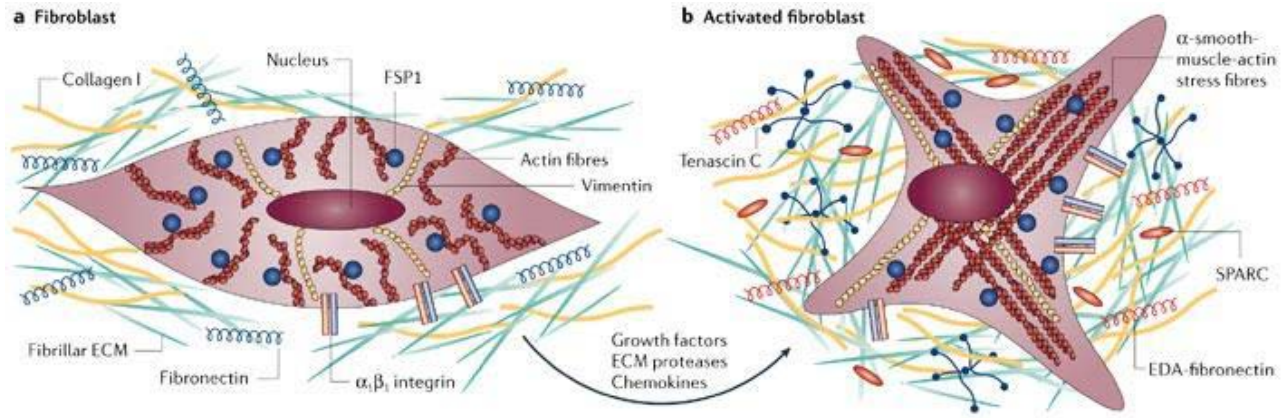

Figure 1.3 | A) Normal fibroblasts are embedded within the fibrillar extracellular matrix (ECM) of connective tissue, which consists largely of type I collagen and fibronectin. Fibroblasts interact with their surrounding microenvironment through integrins such as the α1 and β1 integrin. Typically, fibroblasts appear as fusiform cells with a prominent actin cytoskeleton and vimentin intermediate filaments. B) fibroblasts can acquire an activated phenotype, which is associated with an increased proliferative activity and enhanced secretion of ECM proteins such as type I collagen and tenascin C, and also fibronectin that contains the extra domain a (EDA-fibronectin) and SPARC (secreted protein acidic and rich in cysteine). Phenotypically, activated fibroblasts are often characterized as expressing α-smooth-muscle actin. Numerous growth factors such as TGFβ, chemokines such as MCP1, and ECM-degrading proteases have been shown to mediate the activation of fibroblasts

1.3 The G protein-coupled estrogen receptor (GPR30/GPER)

Among the GPCR family members, GPR30/GPER, was recently shown to mediate the multifaceted actions of estrogens in different tissues including cancer cells [69]. GPER was first identified as an orphan member of the 7-transmembrane receptor family by multiple groups in the late 1990s [70-72]. It belongs to the rhodopsin-like receptor superfamily and its gene is mapped to chromosome 7p22.3 [70]. There are four alternate transcriptional splicing variants encoding the same protein which is comprised of 375 amino acids and contains seven transmembrane spanning segments [72]. Although GPER is a seven-transmembrane receptor, its subcellular localization remains to be fully elucidated. Indeed, several studies have reported the presence of GPER at the plasma membrane, in the endoplasmic reticulum, in the Golgi apparatum as well as in the nucleus of CAFs extracted from mammary biopsies [73-75]. Several studies demonstrated that the ligand-dependent activation of GPER trigger the activation of the heterotrimeric G proteins and subsequently Src and adenylyl cyclase (AC), resulting in intracellular cAMP production. Src is involved in matrix metalloproteinases (MMP) activation, which cleave pro-heparan-bound epidermal growth factor (pro-HB-EGF) releasing free HB-EGF. The latter activates EGF receptor (EGFR), leading to multiple downstream events, for example the activation of phospholipase C (PLC), PI3K and MAPK [70]. Activated PLC produces inositol triphosphate (IP3), which further binds to IP3 receptor and leads to intracellular calcium mobilization [76]. The downstream signal of PI3K is AKT pathway, closely related to cancer cell growth as involved in cell survival and proliferation [77]. The activation of MAPK and PI3K results in the activation of numerous cytosolic pathways and nuclear proteins, which in turn regulate transcription factors such as SRF, CREB and members of the E26 transformation specific (ETS) family by direct phosphorylation [78]. These promotes the expression of a second wave of transcription factors such as FOS, JUN, EGR1, ATF3, C/EBPδ, and NR4A2. Cells are literally reprogrammed under the effect of this

network of transcription factors and a series of GPER target genes such as CTGF are up-regulated [79] (Fig. 1.4).

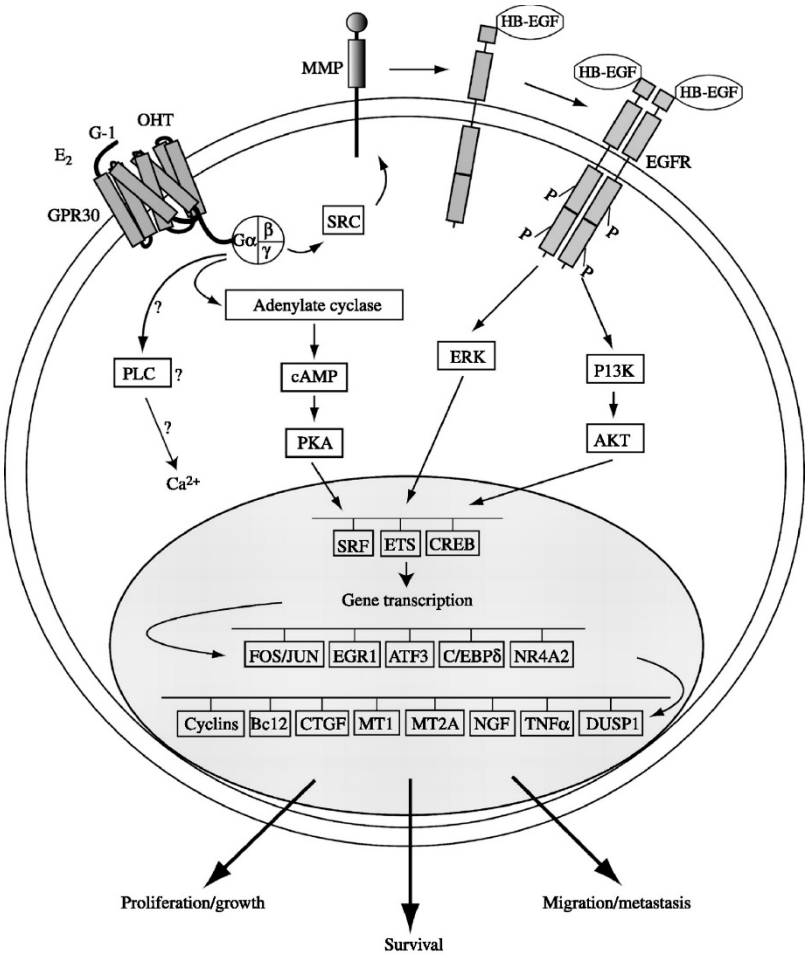

Figure 1.4 | Schematic representation of the GPER signaling network

In addition, there may be a variety of signaling crosstalk pathways and both negative and positive feedback loops. For example, it has been demonstrated that EGF up-regulates GPER expression through the EGFR/MAPK pathway in ER-negative breast cancer cells, most likely by promoting the recruitment of the c-FOS-containing transcription factor AP-1 to the GPER promoter [80]. Considering that GPER signaling uses the EGFR/MAPK pathway, a positive feedback loop is conceivable. This mechanism is also operational for EGF and the related growth factor TGFα in ERα-positive breast cancer cells [81]. GPER gene expression has been detected in at least four

kinds of human tumor specimens or cell lines, including breast cancer [71, 81-83], endometrial cancer [82-84], ovarian cancer [85-87], thyroid cancer [88], and a rat pheochromocytoma cell line PC-12 [89]. In addition, there is a growing body of evidence supporting that GPER is strongly associated with cancer proliferation [90], migration [91], invasion [92], metastasis [93,94], differentiation [95], and drug resistance [96, 97]. Indeed, as estrogens stimulate the progression of breast cancer in approximately two-thirds of patients who express ER [98, 99], some selective estrogen receptor modulators (SERMs), such as tamoxifen, have been clinically used to antagonize the binding of estrogen to its classic ERs, which is an effective therapeutic strategy in attenuating the growth of ER-positive breast cancers. However, there are around 25% of ER-positive breast cancer patients who do not respond to anti-estrogen therapy (Early Breast Cancer Trialists' Collaborative Group 2005). It implies that the blockade of classic ERs alone may be not enough to completely abolish estrogen-induced breast cancer cell growth, since estrogen may promote it through other receptors besides classic ERs. Such hypothesis is further supported by the discovery of GPER as the third specific ER with different structure and function respect to ERα and ERβ. GPER has a high binding affinity to not only for estrogen, but also for some ER antagonists, such as tamoxifen and ICI 182,780. Notably, estrogen and the aforementioned anti-estrogens stimulate GPER signaling [100]. These important findings provide a further possible mechanism for the progression of estrogen-related cancers, and raise a novel potential target for anti-estrogen therapy. As it concerns clinical findings, GPER overexpression, was associated with lower survival rates in endometrial and ovarian cancer patients [101, 102] as well as with a higher risk of developing metastatic disease in breast cancer patients [103]. Moreover, in a previous extensive survey, GPER was found to be highly expressed and significantly associated with tumor size (>2 cm), with the presence of distant metastases and increased human EGFR-2 (HER-2)/neu expression [104]. Likewise, a recent study demonstrated that the majority of the aggressive inflammatory breast

tumors examined resulted GPER positive [105], suggesting that the expression of the receptor may be considered a predictor of an aggressive disease. In addition to the aforementioned studies on the potential functions of GPER in cancer and possibly other pathological conditions, this receptors was implicated in a broad range of physiological functions regarding reproduction, metabolism, bone, cardiovascular, nervous and immune systems [102]. Estrogen binds to GPER with a high affinity of a reported Kd 2.7 nM (84) or 6 nM [103]. Moreover, in the last few years a great attention was focused on the identification of synthetic ligands of GPER acting as agonists or antagonists. In particular different compounds named G-1 [104] G-15 [105], GPER-L1 and GPER-L2 [106] and MIBE [110], were identified using virtual and bio-molecular screening and are used to evaluate the GPER-mediated signaling and functions. In addition, different studies shows that ICI 182,780 [109] and 4-hydroxytamoxifen (OHT) [109-110] are also able to bind GPER and mimic estrogen effects. It has been reported that a variety of xenoestrogens, including bisphenol A, can bind and activate GPER leading to important biological responses [111].

1.4 EGFR/IGF-IR cross-talk

RTKs are transmembrane proteins involved in the control of multiple physiological processes [112]. These proteins consist of an extracellular ligand binding domain, a single transmembrane domain and a highly conserved cytosolic tyrosine kinase domain [112]. The RTK family includes the majority of receptors for growth factors, like the EGFR, commonly known as ErbBs/HER and the IGF-IR, which belongs to the IGF system. In particular, ErbB family comprises four members: EGFR (ErbB1, also known as HER1), ErbB2 (also known as Neu or HER2), ErbB3 (HER3), and ErbB4 (HER4). ErbBs exist as monomers and upon ligand activation or when overexpressed, form homo- and heterodimers [113,114]. The four ErbB receptors are specifically activated by soluble small peptides: EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth

factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), epigen (EPG) and the neuregulins (NRGs 1–6) often referred as heregulins [115]. The ligand binding and the subsequent formation of an ErbB dimer promote the cross-phosphorylation of the dimer partner, generating a network of intracellular signals that control numerous biological processes [116, 117]. In particular, the ErbB family dimers activate various transduction pathways including the MAPK cascade, which leads to gene transcription, cell proliferation, migration, differentiation, angiogenesis [118, 119], and the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling, which mainly promotes cell survival [120]. Likewise, the IGF system plays a key role in cell growth, differentiation, survival as well as in cell transformation and metastasis [121, 122]. It comprises ligands (IGF-I, IGF-II and insulin), cell surface receptors as the IGF-I receptor (IGF-IR), the IGF-II receptor (IGF-IIR), the insulin receptor (IR) and six soluble IGF-binding proteins (IGF-BPs) [122, 123]. Because of the high sequence similarity between the IR and the IGF-IR, IGFs and insulin are able to cross-bind to each other's receptor, even though with much weaker binding affinity [124]. The ligand binding induces the receptor phosphorylation and the recruitment of numerous docking proteins, including IRS family members (IRS-1, IRS-2) and adaptors molecules as Shc and Grb2 [125]. These substrates are then involved in the activation of transduction pathways like MAPK, PI3-K and the Janus kinase/signal transduce rand activator of transcription pathway (JAK/STAT), which mediate important biological responses as glucose metabolism, cell growth, survival and inhibition of apoptosis [126] It is generally accepted that the growth-promoting activities of IGF-I, IGF-II and insulin are mainly mediated by IGF-IR, whereas IGF-IIR serves as a clearance receptor as it removes IGF-II from the cell surface [127]. Accordingly, elevated IGF-IR expression has been associated with multiple biological features of cancer progression including the development of metastatic processes and the resistance to chemotherapeutics [128]. The IGF system also acts influencing the signaling and the biological responses mediated by other cell membrane proteins

such as EGFR [129]. For instance, in vitro and in vivo studies have indicated that the cross-talk between IGF-IR and EGFR can lead to the resistance against EGFR-targeted drugs with relevant implications for cancer therapy [130, 131]. Moreover, EGFR and IGF-IR induce the proliferation of cancer cells through different mechanisms which include the interaction with GPCRs as well as with downstream transduction molecules [129]. Several studies have suggested that the transduction signaling generated by EGFR and the IGF system interacts with different mediators of the estrogen action toward the resistance to endocrine therapy and cancer progression [132]. For instance, the overexpression of EGFR and IGF-IR, the lack of hormonal targeted cancer therapy, the low survival rate and the poor patient prognosis are hallmark features of the estrogen receptor alpha-negative (ERα-) breast cancer subtype [133-137]. Recent studies have shown that ligand-activated EGFR and IGF-IR mediate the regulation and function of GPER in cancer cells, adding a novel mechanism by which these growth factor receptors may contribute to the estrogen stimulation of malignant cells. The aforementioned findings have also extended our knowledge on the functional cross-talk between EGFR and IGF-IR with a member of the GPCR family, supporting the hypothesis that interfering with such interactions may be an effective pharmacological approach in cancer patients. These data suggest the significance of IGF-IR/EGFR activation via the cross-talk with estrogen receptor to regulate cancer cell phenotype and properties (Fig. 1.5). A better understanding of the molecular mechanisms involved in the cross-talk among these receptors may provide new therapeutical approaches that could guarantee major benefits in patients with hormone-sensitive tumors [138].

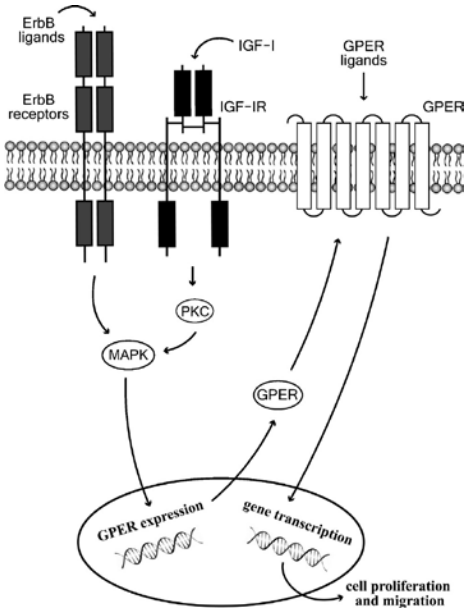

Figure 1.5 | Schematic representation of the cross-talk between GPER and both ErbBreceptors and IGF-IR. Ligand-induced activation of growth factor receptors mediatesthe up-regulation of GPER through diverse kinase transduction pathways. Agonistactivated GPER triggers stimulatory effects like gene expression changes, cell prolif-eration and migration. Note that GPER is placed somewhat arbitrarily at the plasmamembrane, but signaling would presumably work the same way with GPER localizedwithin cells.

1.5 Action of Zinc in breast cancer

Zinc (Zn) essential for many cellular processes, has also been reported to play a potential role as second messenger in signal pathway linked with various physiological actions [139, 140]. The catalytic activity of hundreds of enzymes of every type is dependent on the participation of Zn [1]. Recently, a regulatory role of Zn in cell signaling has also been recognized [19]. Hershfinkel and colleagues [141] have reported the existence of a Zn-sensing receptor that triggers calcium dependent signaling. Evidence that Zn acts extracellularly to induce this effect, combined with the ability of Zn to organize protein domains in a multidentate manner [142], raises mechanistic questions concerning the mode of interaction of the Zn ligand with its putative receptor. The adverse effects associated with a high Zn intake include acute gastrointestinal effects and headache, impaired immune function, changes in lipoprotein and cholesterol levels, reduced copper levels and zinc-iron interactions as well as various other disorders [143, 144]. Furthermore, the imbalances in

Zn homeostasis cause disease states including Alzheimer's disease [145], diabetes [146, 147, 148], cancer [149] and others [150]. In breast cancer patients, the level of Zn has been found to be lower in serum than healthy subjects and elevated in malignant tissues [151-154], whereas in liver, prostate and gallbladder cancers, the level of Zn in the malignant tissues has been found to be decreased [151, 152, 155-157]. These recent reports suggest that Zn is implicated in breast cancer development. The intracellular Zn level is tightly controlled by several protein molecules called zinc-binding protein and zinc transporters [158]. These behaviors of Zn in and out of cell across membranes are maintained through two families including the ZRT IRT-like protein (ZIP) family that facilitates Zn influx into the cytosol, and zinc transporter (ZnT) family that facilitates Zn efflux from the cytosol. Some ZIP family members are reported to involve in aggressive cancer progression [146, 151, 159-166]. ZIP6 is associated with histological grade of estrogen-receptor-positive breast cancer, and is positively related with the lymph node metastasis [151, 167-169]. ZIP6 also has been reported to regulate epithelial-mesenchymal transition (EMT) [151, 156, 159]. Moreover, ZIP10 has been reported to mediate the migration and invasive behaviors of breast cancer cells [170]. Moreover, the degree of Zn accumulation is associated with cancer progression [139] and malignancy [140], but the mechanisms responsible for Zn accumulation, and the relationship between Zn accumulation and breast cancer subtype are not well understood. Of note, Zn regulated transporters (ZIP) that control its influx into the cytosol, were found to be up-regulated by estrogens [152], and increased ZIP levels in breast tumors resulted to be associated with a poor prognosis [150].

1.6 Aim of the study

In the present study, we have evaluated whether Zn might trigger the transduction signaling mediated by GPER through a crosstalk with IGF-IR and EGFR in breast cancer cells and in main

components of the tumor microenvironment like CAFs. In particular, we have determined the molecular mechanisms through which zinc chloride ($ZnCl_2$) involves GPER in the activation of IGF-IR/EGFR-mediated signalling, which in turn triggers downstream pathways like ERK and AKT in breast cancer cells and in CAFs. Further corroborating these findings, we have evidenced that $ZnCl_2$ stimulates a functional crosstalk of GPER with IGF-IR and EGFR toward the transcription of diverse GPER target genes. In addition, we have shown that GPER contributes to the stimulatory effects induced by $ZnCl_2$ on cell-cycle progression, proliferation and migration of breast cancer cells and migration of CAFs.

Chapter 2

Matherials and Methods

2.1 Reagents

We purchased zinc chloride ($ZnCl_2$), zinc sulfate ($ZnSO_4$), wortmannin (WM), N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN), N-acetyl-L-cysteine (NAC) and 2′,7′-dichlorofluorescin diacetate (DCFDA) from Sigma-Aldrich (Milan, Italy); tyrphostin AG1478 from Biomol Research Laboratories (Milan, Italy); PD98059 (PD), and 3-bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024) from Calbiochem (Milan, Italy); (3aS,4R,9bR)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G15) from Tocris Bioscience (Bristol, UK); human Connective Tissue Growth Factor (CTGF) Recombinant Protein from MBL International (Eppendorf, Milan, Italy). All compounds were solubilized in DMSO except $ZnCl_2$, $ZnSO_4$, NAC and human CTGF recombinant protein, which were dissolved in water. Treatments with the inhibitors AG1478, AG1024, G15, NAC, PD, TPEN and WM were performed concomitantly with $ZnCl_2$ exposure, as indicated.

2.2 Cell Cultures

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% FBS and 100 mg/mL penicillin/streptomycin (Life Technologies, Milan, Italy). CAFs were extracted from breast cancer primary tumors of patients who had undergone surgery. The specimens were collected and signed informed consent was obtained from all the patients and from the institutional review board(s) of

the Regional Hospital of Cosenza. Tissues from tumors were placed in digestion solution (400 IU collagenase, 100 IU hyaluronidase and 10% serum, containing antibiotics and antimycotic) and incubated overnight at 37°C. Cells were separated by differential centrifugation at 90×g for 2min. Supernatant containing fibroblasts was centrifuged at 485×g for 8min, pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1, supplemented with 10% FBS and antibiotics) and cultured at 37°C in 5% CO_2. All cell lines were switched to medium without serum the day before immunoblots and reverse transcription-PCR experiments.

2.3 Isolation, cultivation, and characterization of CAFs

CAFs were obtained from surgical specimens of breast cancer tissues of 47 patients who underwent mastectomy at the Regional Hospital in Cosenza (Italy). Samples were immediately incised in 5 ml of medium and incubated over-night in digestion solution (400 IU collagenase, 100 IU hyaluronidase and 10% FBS, containing antibiotic and antimycotic solutions). Cells were then separated by differential centrifugation at 90×g for 2 min. The supernatant containing fibroblasts were centrifuged at 485×g for 8 min, the pellet obtained was 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, 5% CO2. At 80% of confluence 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, Milan, Italy). In order to assess fibroblasts activation, anti-fibroblast activated protein α (FAPα) antibody (H-56, Santa Cruz Biotechnology, DBA, Milan, Italy) was used (Figure 2.1). All experiments were performed in a mixed population of CAFs obtained from 5 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 approval by the Institutional Ethical Committee Board (Regional Hospital of Cosenza, Italy).

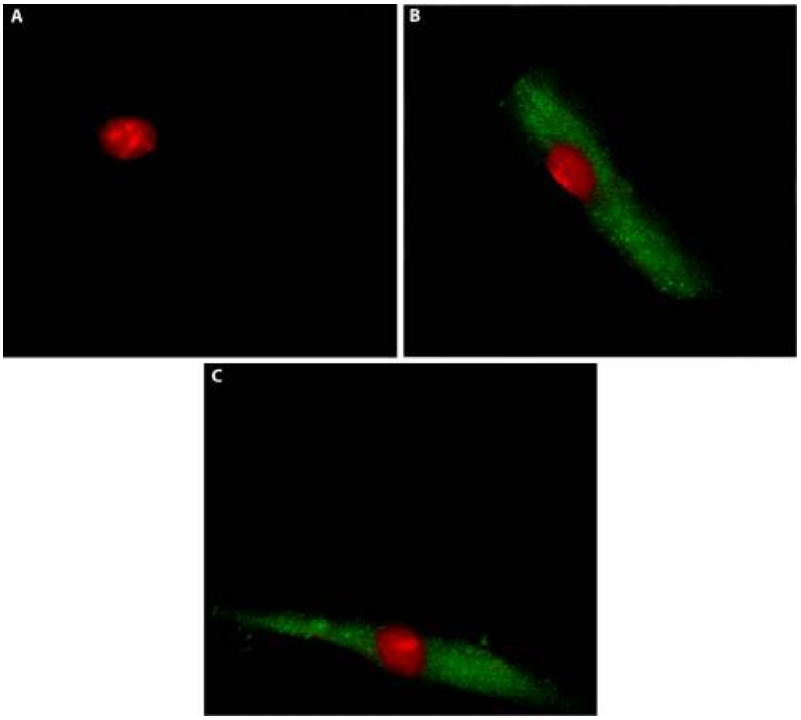

Figure 2.1 | CAFs were immunostained by anti-cytokeratin 14 (A), anti-vimentin (B) and anti FAPα (C)antibody.

2.4 Plasmids and luciferase assays

The luciferase reporter plasmid for c-fos encoding a 2.2-kb 5´ upstream fragment of human c-fos was a gift from Dr. K. Nose (Hatanodai, Shinagawa-ku, Tokyo). EGR1-luc plasmid, containing the -600 to +12 5'-flanking sequence from the human EGR1 gene, was kindly provided by Dr. Safe (Texas A&M University). The Renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as internal transfection control. Cells ($1x10^5$) were plated into 24-well dishes with 500 µl/well culture medium containing 10% FBS. Transfection were performed using X-treme GENE 9 DNA transfection reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy), with a mixture containing 0.5 µg of reporter plasmid and 10 ng of pRL-TK. After 24 h, treatments were added and cells were incubated for 18 h. Luciferase activity was measured using the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla

luciferase activity. 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.

2.5 Gene silencing experiments

SkBr3 cells and CAFs were plated in 10-cm dishes and transiently transfected by X-treme GENE 9 DNA Transfection Reagent for 24 h before treatments with a control vector, a specific shRNA sequence for each target gene. The short hairpin (sh)RNA constructs to knock down the expression of GPER and CTGF and the unrelated shRNA control constructs have been described previously [27/122]. Short hairpin (sh)RNA constructs against human GPER were bought from Open Biosystems (www.Biocat.de) with catalog no. RHS4533-M001505. The targeting strands generated from the shRNA vectors sh1, sh2, sh3, sh4, and unrelated control are complementary to the following sequences, respectively: CGAGTTAAAGAGGAGAAGGAA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCAAGCAGTCTTT, GCAGTACGTGATCGGCCTGTT, and CGACATGAAACCGTCCATGTT. Considering that sh3 showed the highest efficacy, after the first use it has been referred to as shGPER. The shRNA construct for CTGF was obtained from the same supplier (Open Biosystems; www.Biocat.de). It has clone ID TRCN0000061950 and is based on the same lentiviral expression vector pLKO.1 as the other shRNA constructs. The targeting strand generated from the CTGF shRNA construct is TAGTACAGCGATTCAAAGATG.

2.6 Gene expression studies

Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described [28]. The expression of selected genes was quantified by real-time PCR using Step One sequence detection system (Applied Biosystems, Milan, Italy). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc. Milan, Italy) and are

as follows: GPER Fwd 5′- ACACACCTGGGTGGACACAA-3′ and Rev 5′-GGAGCCAGAAGCCACATCTG-3’; HES-1 Fwd 5′-TCAACACGACACCGGATAAA-3′ and Rev 5′-CCGCGAGCTATCTTTCTTCA-3′; NOTCH 1 Fwd 5′-AATGGCGGGAAGTGTGAAGC-3′ and Rev 5′-GCATAGTCTGCCACGCCTCT-3′; MTN-2 Fwd 5′-CTCCGAGTGGGCCAGTAAAG-3′ and Rev 5′- CTGGCTCAGATTCTGTTGGCT-3′; FBN-1 Fwd 5′-GCCGCATATCTCCTGACCTC-3′ and Rev 5′-GTCGATACACGCGGAGATGT-3′; 18S Fwd 5′- GGCGTCCCCCAACTTCTTA-3′ and Rev 5′-GGGCATCACAGACCTGTTATT-3′. Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression. For c-fos, CTGF, Cyr61, EGR1, MT1X, MT2A, cyclin D1, cyclin A, GPER and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5'-CGAGCCCTTTGATGACTTCCT-3' (c-fos forward), 5'-GGAGCGGGCTGTCTCAGA-3' (c-fos reverse); 5'-ACCTGTGGGATGGGCATCT-3' (CTGF forward), 5'-CAGGCGGCTCTGCTTCTCTA-3' (CTGF reverse); 5′-GAGTGGGTCTGTGACGAGGAT-3′ (Cyr61 forward) and 5′-GGTTGTATAGGATGCGAGGCT-3′ (Cyr61 reverse); 5'-GCCTGCGACATCTGTGGAA-3’ (EGR1 forward), 5'-CGCAAGTGGATCTTGGTATGC-3’ (EGR1 reverse); 5'-TGTCCCGCTGCGTGTTT-3' (MT1X forward) and 5'-TTCGAGGCAAGGAGAAGCA-3' (MT1X reverse); 5'-CCCGCTCCCAGATGTAAAGA-3' (MT2A forward) and 5'-GGTCACGGTCAGGGTTGTACATA-3' (MT2A reverse); 5'-GTCTGTGCATTTCTGGTTGCA-3' (cyclin D1 forward) and 5'-GCTGGAAACATGCCGGTTA-3' (cyclin D1 reverse); 5'-GCATGTCACCGTTCCTCCTTG -3' (cyclin A forward) and 5'-GGGCATCTTCACGCTCTATTTT -3' (cyclin A reverse); 5'-CCTGGACGAGCAGTATTACGATATC-3' (GPER forward) and 5'-TGCTGTACATGTTGATCTG-3' (GPER reverse) and 5’- GGCGTCCCCCAACTTCTTA -3’ (18S forward) and 5’- GGGCATCACAGACCTGTTATT -3’ (18S reverse), respectively. Assays were

performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression.

2.7 Western blot analysis

Cells were processed according to a previously described protocol [29] to obtain protein lysate that was electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel, electroblotted onto a nitrocellulose membrane and probed with primary antibodies against antiphosphotyrosine antibody (4G10) (Merck Millipore, Milan, Italy), pEGFR Tyr 1173 (sc-12351), EGFR (1005), phosphorylated ERK1/2 (E-4), ERK2 (C-14), phosphorylated p-AKT1/2/3 (Ser 473)-R, AKT/1/2/3 (H-136), IGF-IR (7G11), GPER (N-15), c-fos (H-125), EGR1 (C-19), CTGF (L-20), cyclin D1 (M-20), cyclin A (H-432) and β-actin (C2) (Santa Cruz Biotechnology, DBA, Milan, Italy). Proteins were detected by horseradish peroxidase-linked secondary antibodies (DBA, Milan, Italy) and revealed using the ECL System (GE Healthcare, Milan, Italy).

2.8 Immunoprecipitation Assay

Cells were lysed using 200 µl RIPA buffer with a mixture of protease inhibitors containing 1.7mg/ml aprotinin, 1mg/ml leupeptin, 200mmol/L phenylmethylsulfonyl fluoride, 200mmol/L sodium orthovanadate, and 100mmol/L sodium fluoride. A total of 100 µg proteins were incubated for 2 h with 2 µg of the appropriate antibody (GPER, N-15; IGF-1R, 7G11) and 20 µl of protein A/G agarose immunopreciptation reagent (Santa Cruz Biotechnology). Samples were centrifuged at 13,000 rpm for 5 min at 4°C to pellet beads. After four washes in PBS, samples were resuspended in RIPA buffer with protease inhibitors and SDS sample buffer. Western Blot analysis was performed as described above.

2.9 ROS production

The non-fluorescent 2′,7′-dichlorofluorescin diacetate (DCFDA) probe, which becomes highly fluorescent on reaction with ROS, was used to evaluate intracellular ROS production. Briefly, cells (2×10^5) were incubated with 10 μM DCFDA (Sigma Aldrich, Milan, Italy) at 37 °C for 30 min, washed with PBS and then exposed to treatments, as indicated. Cells were washed with PBS and the fluorescent intensity of DCF was measured (excitation at 485 nm and emission at 530 nm).

2.10 Cell cycle analysis

Cells synchronized for 24 h in serum-free medium were transfected, treated and subjected to fluorescence-activated cell sorting (FACS) analysis. Adherent and floating cells were centrifuged and resuspended in PBS containing 20 μg/mL propidium iodide plus 40 μg/mL ribonuclease (Sigma-Aldrich) for 1 h. Cells were then subjected to FACS analysis (FACS Jazz, BD, Milan, Italy) and results were expressed in terms of percentage.

2.11 Proliferation assay

Cells were seeded in 24-well plates in regular growth medium. After cells attached, they were incubated in medium containing 2.5% charcoal-stripped FBS, transfected for 24 h, and treated as indicated, with transfection and treatments renewed every 2 days. Cells were counted using an automated cell counter (Life Technologies) following the manufacturer's recommendations.

2.12 Migration assays

Migration assays were performed using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane, Sigma Aldrich, Milan, Italy). Cells were transfected in in regular growth medium. After 24 h, cells were trypsinized and seeded in the upper chambers. Treatments were added to the medium without serum in the bottom wells where applicable, cells on the bottom side of the membrane were fixed and counted 6 hours after seeding. Wound-healing assays were also

performed in order to further assess cell migration. Cells were seeded into 12-well plates in regular growth medium. When at 70% to 80% confluence, cells were transfected in medium without serum. After 24 h, medium was replaced with 2.5% charcoal-stripped FBS and cells were treated. We then used a p200 pipette tip to scratch the cell monolayer. Cells were allowed to migrate for 24 h, the gap area was then photographed and migration distances were measured.

2.13 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.

Chapter 3

Results

3.1 GPER is involved in the activation of EGFR and IGF-IR by Zn in breast cancer cells

As a dysregulated Zn homeostasis may contribute to breast carcinogenesis through different mechanisms [148], including the activation of growth factors transduction pathways [154-156], we began our study by ascertaining that Zn chloride ($ZnCl_2$) triggers the rapid phosphorylation of EGFR and IGF-IR (Fig. 3.1A) as well as the activation of downstream kinases such as ERK and AKT (Fig. 3.1B) in a dose-dependent manner. Similar results were obtained using Zn sulfate ($ZnSO_4$) (data not shown). On the basis of these findings and considering that Zn serum concentration is approximately 15 μM [170], in subsequent assays 10 μM $ZnCl_2$ were used. As our previous studies have shown that, in cancer cells, both EGFR and IGF-IR transduction signalling are involved in GPER regulation [110, 81-82], we evaluated whether the activation of EGFR and IGF-IR by $ZnCl_2$ may involve GPER. By co-immunoprecipitation studies performed in SkBr3 cells, we ascertained that $ZnCl_2$ increases a direct interaction of GPER with EGFR and IGF-IR, while the Zn chelator TPEN prevented this response (Fig. 3.1C).

Next, we determined that the $ZnCl_2$-dependent activation of EGFR and IGF-IR as well as ERK and AKT requires GPER, as observed silencing its expression (Fig. 3.2A-D). Using the EGFR inhibitor AG1478, the IGF-IR inhibitor AG1024 and the GPER antagonist G15, we also established that EGFR, IGF-IR and GPER are involved in ERK and AKT activation by $ZnCl_2$ (Fig. 3.2E). Likewise, in the presence of the Zn chelator TPEN and the scavenger of reactive oxygen species (ROS) NAC, the phosphorylation of ERK and AKT by $ZnCl_2$ was no longer evident (Fig. 2E), suggesting that the production of ROS may be involved in these effects [155-156]. Therefore, we assessed that the

generation of ROS triggered by $ZnCl_2$ in SkBr3 cells is no longer evident in the presence of TPEN or NAC (Fig. 3.2F), nicely confirming the aforementioned findings. Collectively, these observations indicate that $ZnCl_2$ activates a complex transduction signalling that may involve GPER together with EGFR and IGF-IR and downstream effectors like ERK and AKT, hence leading to important biological outcomes.

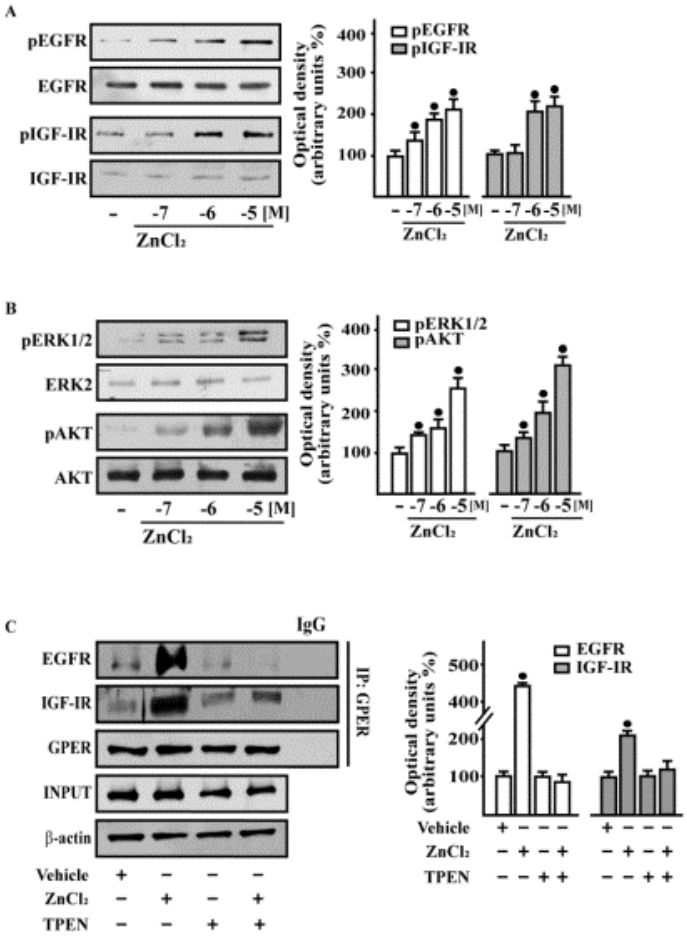

Figure 3.1 | $ZnCl_2$ triggers rapid responses and stimulates the co-immunoprecipitation of EGFR and IGF-IR with GPER in breast cancer cells. (B) in SkBr3 cells treated for 15 min with vehicle (-) and increasing concentrations of ZnCl2, as indicated. Side panels show densitometric analysis of the blots normalized to EGFR, IGFIR, ERK2, and AKT that served as loading controls, respectively for pEGFR, pIGF-IR, pERK1/2, and pAKT. (C) Co-immunoprecipitation assays performed in SkBr3 cells treated with 10mM ZnCl2 for 15 min using the antibody against GPER followed by immunoblotting for EGFR or IGF-IR, as indicated. In control samples, nonspecific IgG was used instead of the primary antibody. IP, Immunoprecipitation. Input represents the blots probed with the antibody against GPER. Side panels show densitometric analysis of the blots normalized to b-actin. Data shown are the mean ±SD of three independent experiments. (*) indicates $P<0.05$ for cells treated with vehicle (-) versus treatments

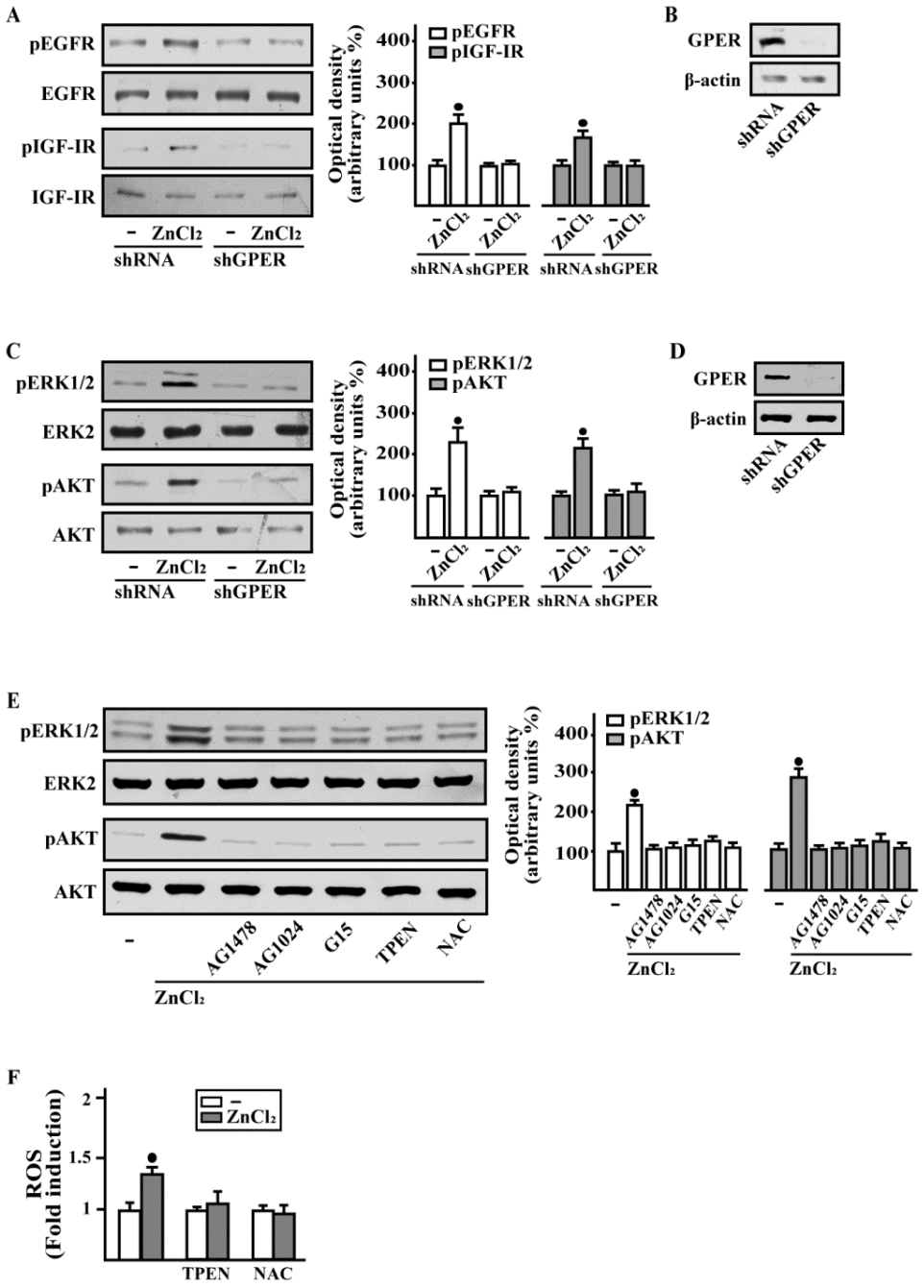

Figure 3.2 | GPER is involved in the rapid action of $ZnCl_2$ in breast cancer cells. (A–D) Phosphorylation of EGFR (A), IGF-IR (A), ERK1/2 (C) and AKT (C) in SkBr3 cells after silencing GPER expression. Cells were transfected with control shRNA or shGPER, and treated for 15 min with vehicle (-) and 10mM ZnCl2. (B and D) Efficacy of GPER silencing. (E) ERK1/2 and AKT activation in SkBr3 cells treated for 15 min with vehicle (-) or 10mMZnCl2 alone or in combination with 10mMEGFR inhibitor AG1478, 10 mM IGF-IR inhibitor tyrphostin AG1024, 100nM GPER antagonist G15, 20mM zinc chelator TPEN, and 300mM free radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to EGFR, IGFIR, ERK2, and AKT that served as loading controls, respectively for pEGFR, pIGF-IR, pERK1/2, and pAKT. (F) ROS production determined as DCF fluorescence in SkBr3 cells treated for 1 h with vehicle (-) or 10mM ZnCl2 alone and in combination with 20 mM zinc chelator TPEN, and 300 mM free radical scavenger NAC. DCF fluorescence obtained in cells treated with vehicle (-) was set as onefold induction upon which ROS levels induced by treatments was calculated. Data shown are the mean ±SD of three independent experiments. (*) indicates $P<0.05$ for cells treated with vehicle (-) versus treatments.

3.2 GPER contributes to gene expression changes and growth responses induced by Zn in breast cancer cells

Considering that GPER triggers a specific gene signature [171], we then assessed that in SkBr3 cells $ZnCl_2$ regulates the mRNA expression of certain GPER target genes like c-fos, CTGF, Cyr61, EGR1, MT1X and MT2A, as evaluated by real-time PCR (Fig. 3.3A). Next, we ascertained that the treatment with $ZnCl_2$ does not alter the mRNA expression of GPER (data not shown), however its silencing prevented the mRNA induction of c-fos and EGR1 (Fig.3.3B-C), two main GPER target genes [171]. In accordance with these findings, the transactivation of c-fos and EGR1 promoter constructs upon $ZnCl_2$ exposure was no longer evident knocking down GPER expression (Fig. 3.3D-E). Moreover, the EGFR inhibitor AG1478, the IGF-IR inhibitor AG1024, the GPER antagonist

G15, the MEK inhibitor PD, the PI3K inhibitor WM, the zinc chelator TPEN and the ROS scavenger NAC abolished the luciferase activity of c-fos and EGR1 reporter plasmids induced by $ZnCl_2$ (Fig. 3.3F-G).

In accordance with these findings, c-fos and EGR-1 protein expression triggered by $ZnCl_2$ was prevented by GPER silencing (Fig. 3.4A-B) and in the presence of each of the aforementioned inhibitors (Fig. 3.4C).

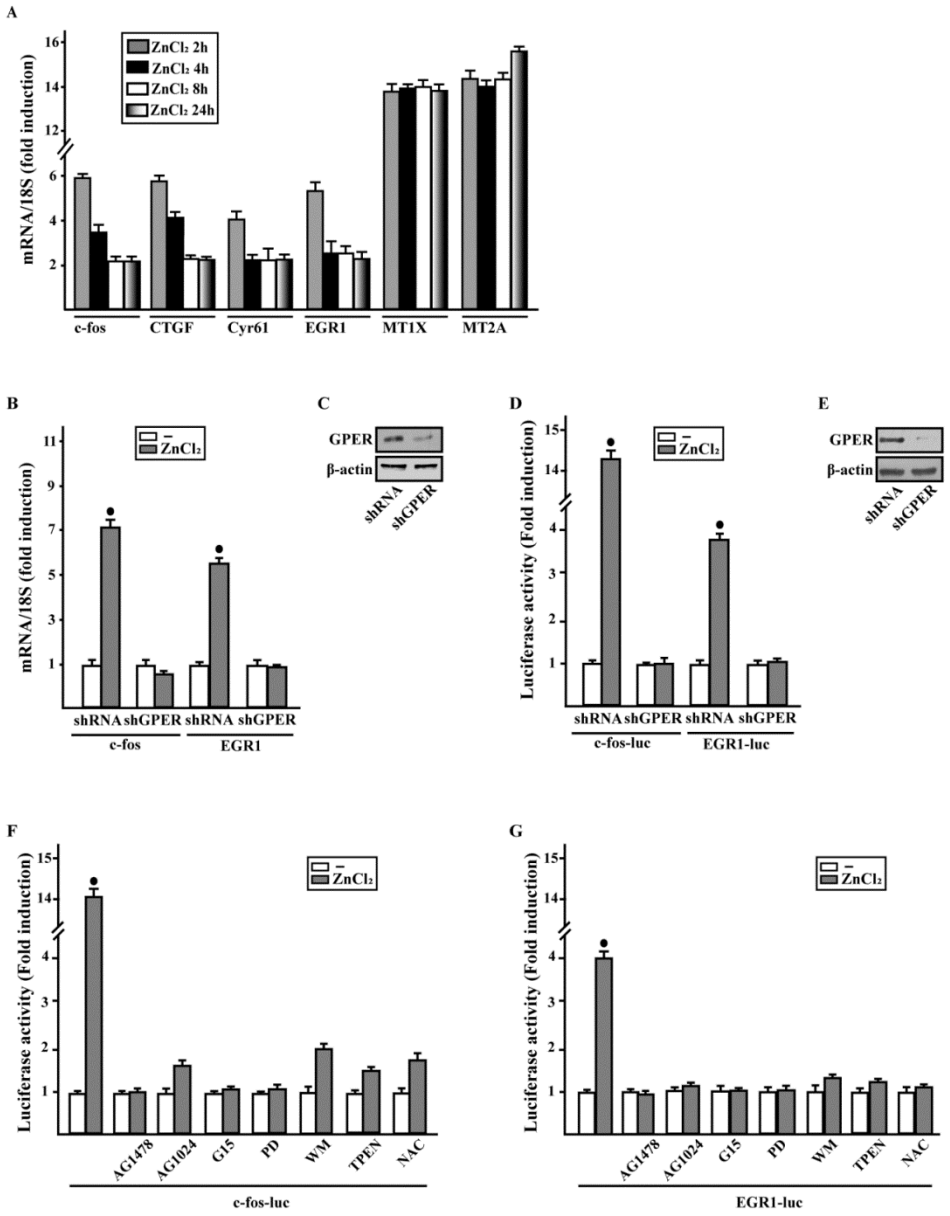

Figure 3.3 | $ZnCl_2$ regulates the expression of GPER target genes in breast cancer cells. ZnCl2 regulates the expression of GPER target genes in breast cancer cells. (A) The mRNA expression of c-fos, CTGF, Cyr61, EGR1, MT1X, MT2A and GPER was evaluated by real-time PCR in SkBr3 cells treated with vehicle (-) and 10mM ZnCl2, as indicated. (B) Evaluation of c-fos andEGR1mRNAexpression in SkBr3 cells transfected with shRNA or shGPER, and treated for 2 h with vehicle (-) and 10mM ZnCl2. (C) Efficacy of GPER silencing. Results obtained from experiments performed in triplicate were normalized for 18 S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (D) Evaluation of c-fos and EGR1 luciferase reporter genes in SkBr3 cells transfected with shRNA or shGPER, and treated for 18 hwith vehicle (-) and 10mM ZnCl2. (E) Efficacy of GPER silencing. (F and G) Evaluation of c-fos and EGR1 luciferase reporter genes in SkBr3cells treated for 18 h with vehicle (-) or 10mM ZnCl2 alone or in combination with 10mM EGFR inhibitor AG1478, 10mM IGF-IR inhibitor tyrphostin AG1024, 100nM GPER antagonist G15, 10mM MEK inhibitor PD98089 (PD), 1mM PI3K inhibitor wortmannin (WM), 20mM Zn chelator TPEN, and 300mM free radical scavenger NAC. Luciferase activity was normalized to the internal transfection control; values are presented as fold change (mean ±SD) of vehicle control and represent three independent experiments, each performed in triplicate. (*) indicates $P<0.05$ for cells receiving vehicle (-) versus treatments.

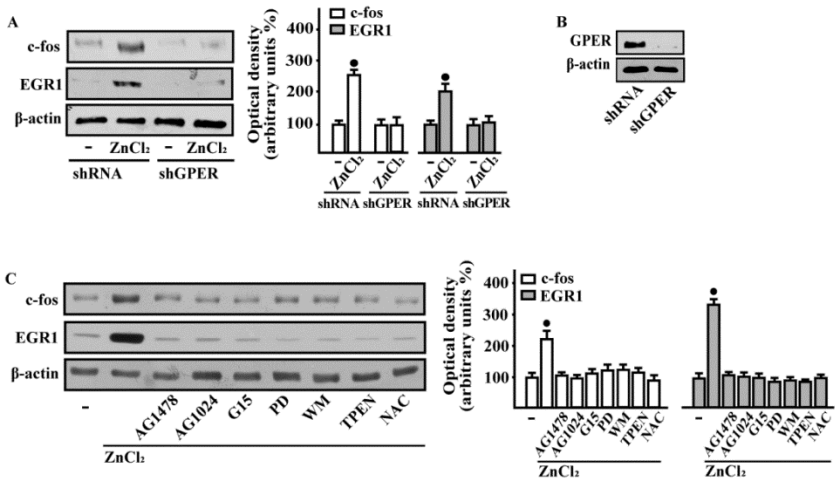

Figure3.4 | GPER is involved in c-fos and EGR1 protein increase induced by $ZnCl_2$ in breast cancer cells. in breast cancer cells. (A and B) Protein levels of c-fos and EGR1 in SkBr3 cells transfected with shRNA or shGPER, and treated with vehicle (-) or 10mM ZnCl2 for 4 h. (B) Efficacy of GPER silencing. (C) Immunoblots showing c-fos and EGR1 protein expression in SkBr3 cells treated for 4 h with vehicle (-), and 10mM ZnCl2 alone or in combination with 10mM EGFR inhibitor AG1478, 10mM IGF-IR inhibitor tyrphostin AG1024, 100nM GPER antagonist G15, 10mM MEK inhibitor PD98089 (PD), 1 mM PI3K inhibitor wortmannin (WM), 20 mMZn chelator TPEN, and 300mMfree radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to b-actin. Values represent the mean ±SD of three independent experiments. (*) indicates $P<0.05$ for cells treated with vehicle (-) versus treatments.

As cyclin D1 and cyclin A have been implicated in the development of several tumors including breast cancer [172], we also evaluated the potential of $ZnCl_2$ to induce these cell cycle regulators. We found that $ZnCl_2$ stimulates the expression of both cyclins (Fig. 3.5A-B), however this response was abrogated silencing GPER (Fig. 3.5B-C) as well as in the presence of AG1478, AG1024, G15, PD, WM, TPEN (Fig. 3.5D). As it concerns NAC, its inhibitory action was mainly exerted on cyclin D1 protein increase by $ZnCl_2$ whereas the up-regulation of cyclin A upon NAC treatment was blunted but still evident (Fig. 3.5D). Indeed, although the chelator TPEN does not act in a selective manner, its ability to prevent the aforementioned responses to Zn may further confirm our findings on the biological properties of this metal. On the basis of the results obtained, it could be therefore argued that GPER is involved in Zn-dependent gene expression that occurs through the EGFR and IGF-IR transduction pathways.

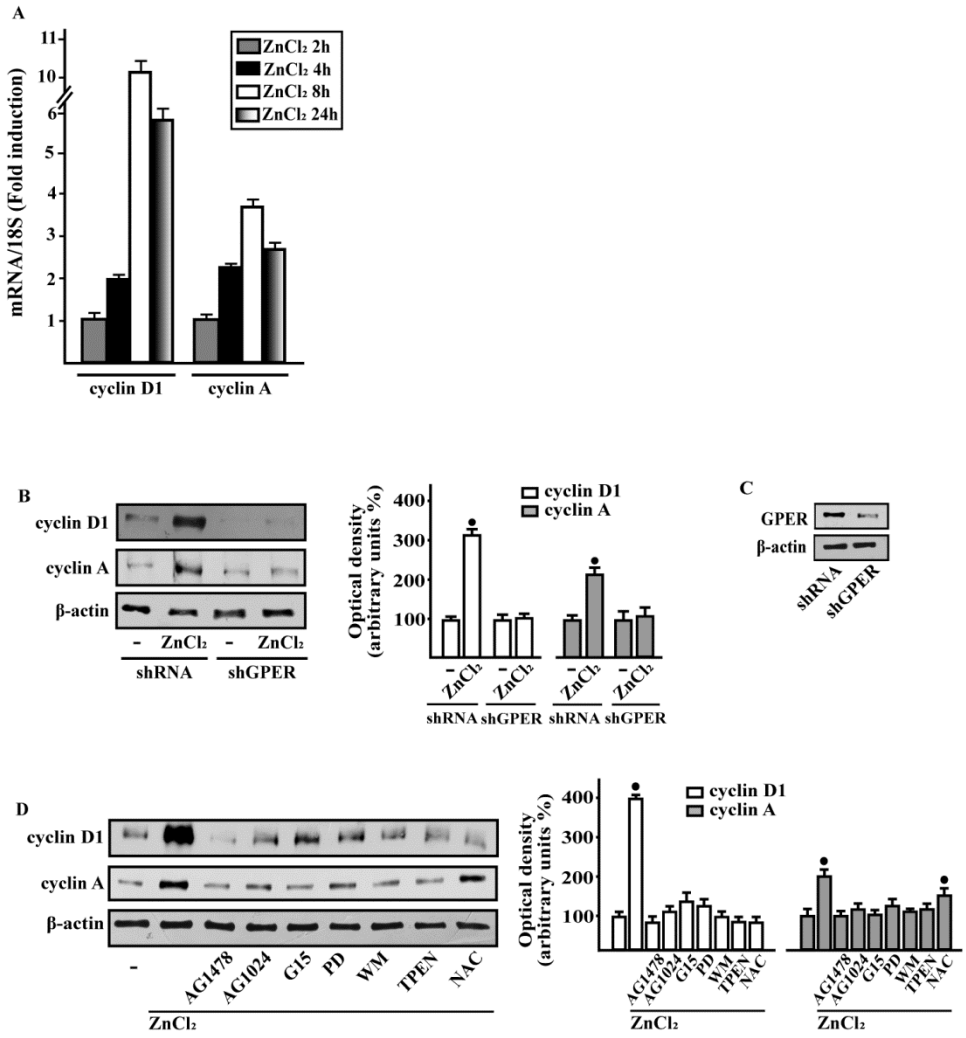

Figure 3.5 | GPER is involved in the up-regulation of cyclins by ZnCl2 in breast cancer cells. (A) The mRNA expression of cyclin D1 and cyclin A was evaluated by real-time PCR in SkBr3 cells treated with vehicle (-) or 10 mM ZnCl2, as indicated. Results obtained from experiments performed in triplicate were normalized for 18 S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (B) Cyclin D1 and cyclin A protein levels in SkBr3 cells transfected with shRNA or shGPER. and treated with vehicle (-) and 10 mM ZnCl2 for 12 h. (C) Efficacy of GPER silencing. (D) Cyclin D1 and cyclin A immunoblots in SkBr3 cells treated for 12 h with vehicle (-), and 10 mM ZnCl2 alone or in combination with 10mM EGFR inhibitor AG1478, 10mM IGF-IR inhibitor tyrphostin AG1024, 100nM GPER antagonist G15, 10mM MEK inhibitor PD98089 (PD), 1 mM PI3K inhibitor wortmannin (WM), 20mM zinc chelator TPEN, and 300 mM free radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to b-actin. Values represent the mean ±SD of three independent experiments. (*) indicates $P<0.05$ for cells treated with vehicle (-) versus treatments.

We also found that $ZnCl_2$ triggers SkBr3 cell cycle progression and proliferation through the involvement of GPER (Fig. 3.6A-E), thus paralleling the activation of both cyclin D1 and cyclin A. Likewise, we determined that SkBr3 cell migration induced by $ZnCl_2$ is no longer evident silencing GPER as determined by Boyden chamber assay (Fig. 3.6F-G). Taken together, these data extend the current knowledge on the molecular mechanisms through which Zn may induce stimulatory effects in breast cancer cells.

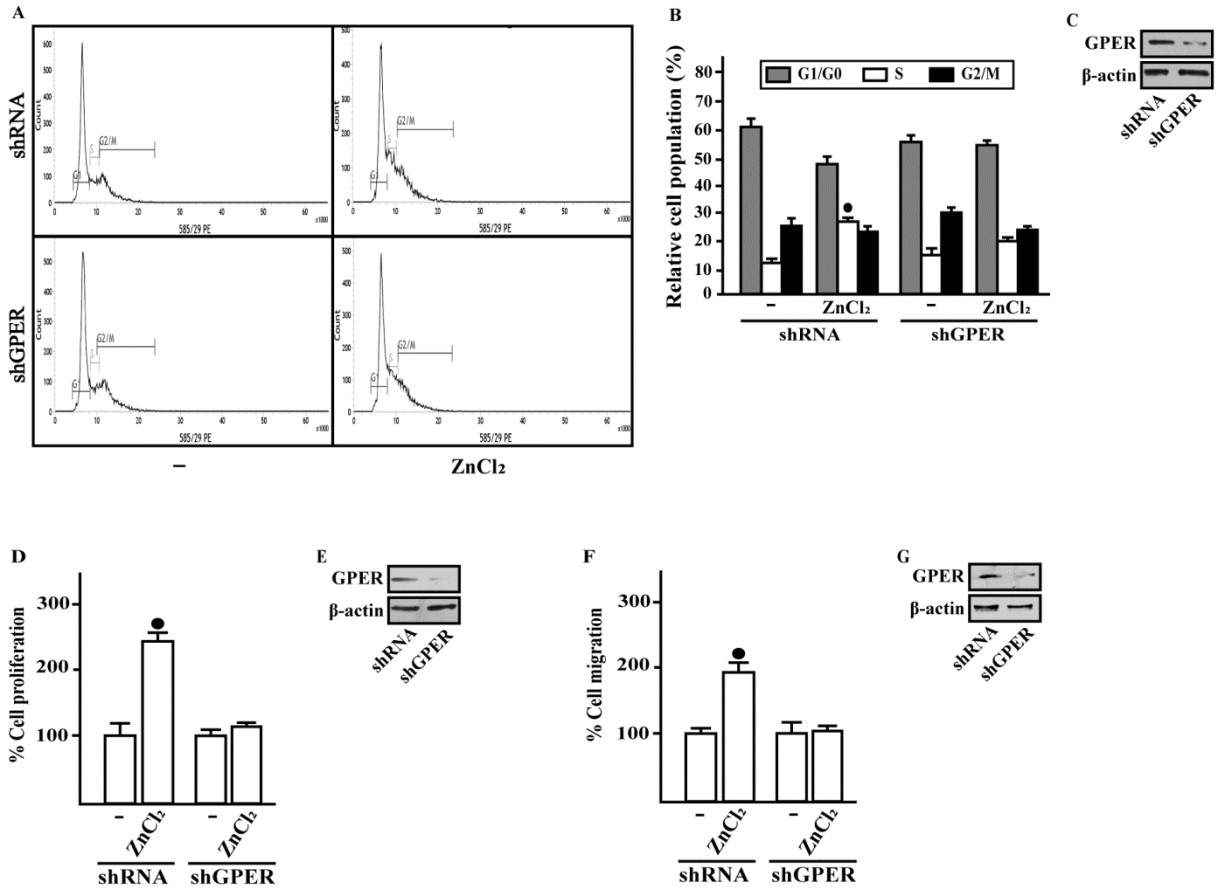

Figure 3.6 | GPER contributes to ZnCl2 induced cell-cycle progression and proliferation of breast cancer cells. (A) Cell-cycle analysis performed in SkBr3 cells transfected with shRNA or shGPER, and treated with vehicle (-) and 10mM ZnCl2 for 18 h. (B) The histograms show the percentages of cells in G1/G0, S, and G2/M phases of the cell cycle, as determined by flow cytometry analysis. (D) The proliferation of SkBr3 cells upon treatment with 10 mM ZnCl2 is prevented knocking down GPER expression. Cells were transfected with shRNA or shGPER and treated every 2 d with vehicle (-) or ZnCl2 as indicated, and then counted on day 6. Proliferation of cells treated with vehicle was set as 100% upon which cell growth induced by treatments was calculated. (F) The migration of SkBr3 cells upon 6 h treatment with 10 mM ZnCl2 is abrogated knocking down GPER expression, as evaluated by Boyden Chamber assay. (C, E, and G) Efficacy of GPER silencing. Each data point is the mean ±SD of three independent experiments performed in triplicate. (*) indicates $P<0.05$ for cells treated with vehicle (-) versus treatments.

3.3 GPER contributes to Zn action in CAFs.

In order to further ascertain whether GPER may contribute to the action of Zn, we used CAFs that play an active role toward the growth, expansion and dissemination of breast cancer cells [173-174]. Remarkably, $ZnCl_2$ increased the mRNA levels of diverse GPER target genes like c-fos, CTGF, Cyr61, EGR1, MT1X and MT2A in CAFs obtained from breast cancer specimens (Fig.7A). Gene expression profile displayed responses to $ZnCl_2$ similar to those observed in SkBr3 cells (Fig. 3A), as the induction of c-fos, CTGF, Cyr61 and EGR1 was rapid (2-4 h) but declined thereafter,

whereas the expression of MT1X and MT2A was still evident up to 24 h. Then, we observed that the up-regulation of CTGF protein levels upon $ZnCl_2$ treatment is prevented knocking down GPER expression in CAFs (Fig. 3.7B-C).

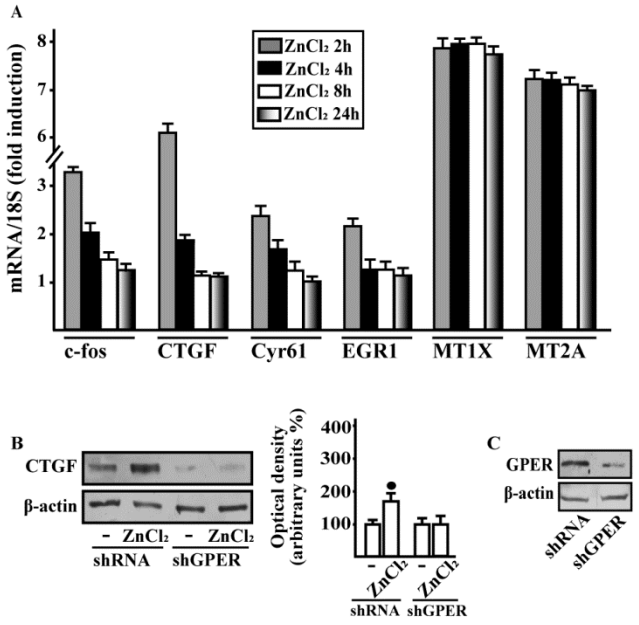

Figure 3.7 | **GPER is involved in gene expression changes induced by ZnCl2 in CAFs**. (A) The mRNA expression of c-fos, CTGF, Cyr61, EGR1, MT1X, and MT2A was evaluated by real-time PCR in CAFs treated with vehicle (-) and 10 mM ZnCl2, as indicated. Results obtained from experiments performed in triplicate were normalized for 18 S expression and shown as fold change of RNAexpression compared to cells treated with vehicle. (B) Immunoblots showing CTGF protein expression in CAFs transfected with shRNA or shGPER, and treated for 4 h with vehicle (-) and 10 mM ZnCl2. Side panel shows densitometric analysis of the blot normalized to b-actin. (C) Efficacy of GPER silencing. Values represent the mean ±SD of three independent experiments. (*) indicates P <0.05 for cells treated with vehicle (-) versus treatments.

As CTGF exerts an acknowledged role in migratory properties of different cell types [171, 175], we evaluated whether GPER signalling through CTGF may trigger the migration of CAFs. Scratch experiments and Boyden chamber assays revealed that $ZnCl_2$-stimulated migration of CAFs is abolished silencing GPER or CTGF expression, whereas adding CTGF the migratory response was rescued (Fig. 3.8). Collectively, the aforementioned results indicate that Zn-activated GPER signaling mediates a similar gene expression profile as well as important biological responses in both breast cancer cells and CAFs. On the basis of these findings, it could be argued that Zn may trigger through GPER a functional interplay between cancer cells and CAFs toward breast tumor progression.

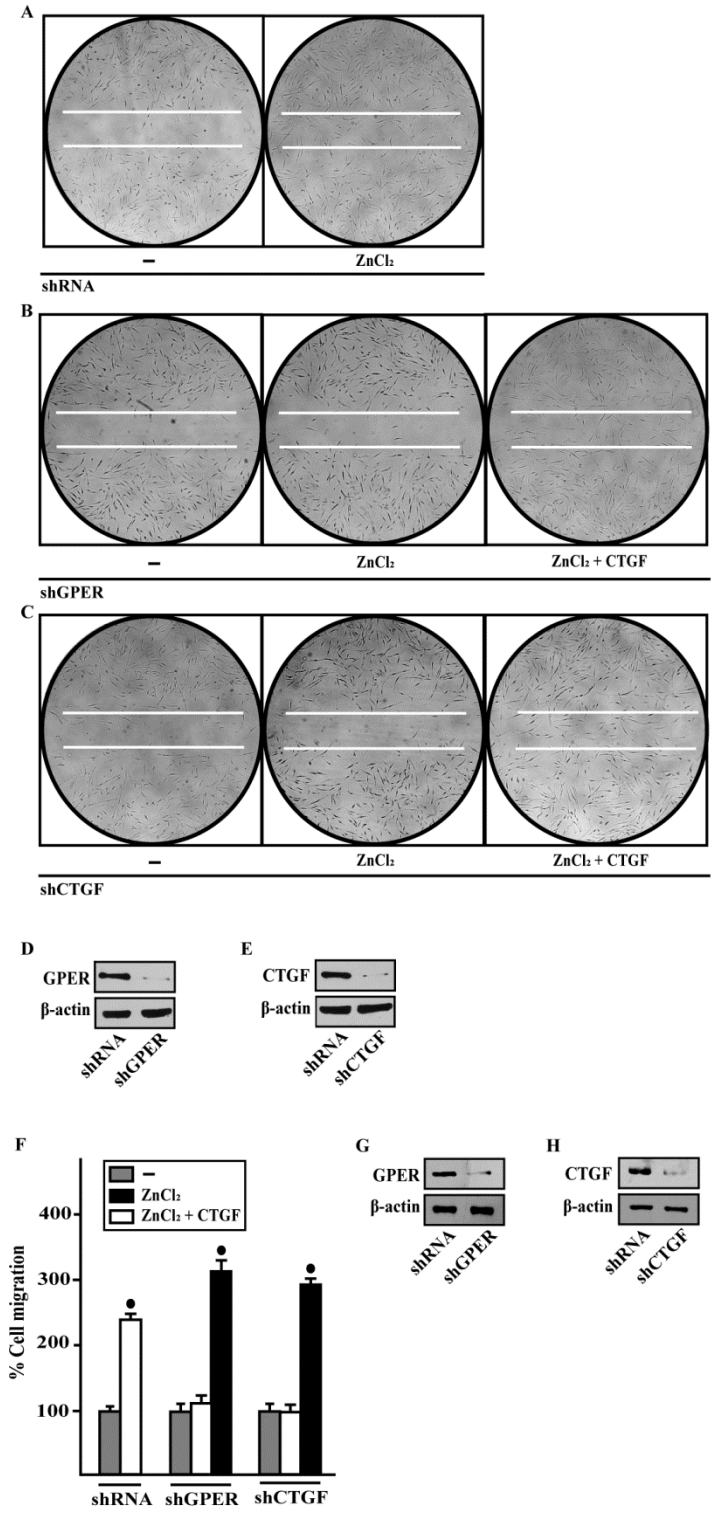

Figure 3.8 | GPER and its target gene CTGF contribute to the migration of CAFs induced by ZnCl2. (A–C) The migration of CAFs upon treatment with 10 mM ZnCl2 for 24 h is prevented knocking down GPER and CTGF expression, as assessed by wound-healing assay. Cell migration is rescued in CAFs transfected with shGPER (B) or shCTGF (C) exposed to 10 mM ZnCl2 for 24 h and treated with 100 ng/ml CTGF. Images shown are representative of three independent experiments. (F) The migration of CAFs induced by a 6 h treatment with 10 mM ZnCl2 is prevented knocking down GPER and CTGF expression, as evaluated by Boyden Chamber assay. Cell migration is rescued in CAFs transfected with shGPER and shCTGF, exposed to 10 mM ZnCl2 for 6 h and treated with 100 ng/ml CTGF. Efficacy of GPER (D and G) and CTGF (E and H) silencing. Values represent the mean ±SD of three independent experiments. (*) indicates $P<0.05$ for cells treated with vehicle(-) versus treatments.

Chapter 4

Discussion

Several human activities as well as natural events can lead to heavy metals pollution and, therefore, increased incidence of various tumors [164-166]. In the present study, we have demonstrated that one important pollutant like Zn may trigger a functional interplay of GPER with EGFR and IGF-IR, which leads to the activation of main transduction pathways, gene expression changes, and important biological responses like proliferation and migration in breast cancer cells and CAFs (Figure 3.9).

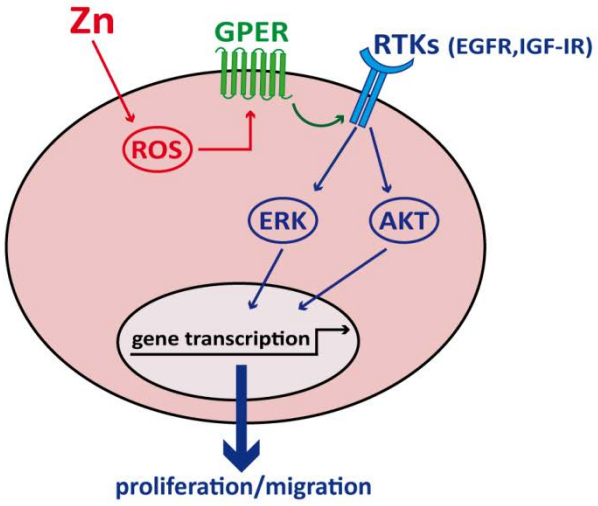

Figure 3.9 | Schematic representation of the functional cooperation of GPER with IGF-IR and EGFR upon zinc exposure**.**

Breast cancers have been reported to show an increased Zn uptake and tissue concentration as compared to the normal breast tissue [10, 167], while patients with advanced breast tumors show decreased serum Zn levels. Thus, the determination of serum Zn levels has been proposed as a prognostic marker in breast cancer patients [145, 168-169]. Of note, tamoxifen-resistant breast

cancer cells show increased levels of Zn and its transporter ZIP7 that are involved in the activation of EGFR and IGF-IR transduction signaling toward cell proliferation and invasion [15]. In accordance with these findings, growth factors-mediated effects of Zn promoted the activation of kinases, gene expression changes and growth responses [19-20].

Numerous studies have shown that GPER contributes to the progression of certain tumors including breast cancer [69, 177-180]. In addition, clinical studies have indicated that GPER may be a predictor of aggressive cancer behavior as its expression has been associated with negative clinical outcomes in several cancer histotypes [91, 95, 104, 107]. The activation of GPER has been shown to trigger EGFR transactivation, subsequent transduction events such as the activation of MAPK and PI3K cascades, gene expression changes, and relevant biological responses such as proliferation, migration and angiogenesis in diverse cancer cell types and CAFs [181-182]. In this context, it should be mentioned that the metal cadmium may induce cAMP increase, ERK1/2 activation and proliferation of breast cancer cells in a GPER-dependent manner [183]. Recently, we also demonstrated that copper activates the HIF-1α/GPER/VEGF signalling in cancer cells leading to angiogenesis and tumor progression [182]. Further extending these findings, in the present study we have demonstrated that in breast cancer cells exposed to Zn the activation of GPER leads to rapid signalling events such as the phosphorylation of EGFR and IGF-IR and their downstream effectors ERK and AKT, the up-regulation of c-fos and EGR1, two main GPER target genes largely involved in growth responses. It is worth noting that Zn induced also GPER targets namely metallothioneins MT1X and MT2A, whose overexpression correlates with chemoresistance and poor prognosis in breast tumors [184-185]. Moreover, in line with the known capability of GPER to trigger the transcription of genes associated with cell growth [171], we assessed the potential of Zn to regulate the expression of two members of the cyclin family as cyclin D1 and A. According to their regulatory role of cell-cycle progression, proliferation and notably migration [186], we assessed also that Zn through GPER significantly increases the percentage of SkBr3 cells in the S phase of the cell cycle as well as stimulates cell proliferation and migration.

Several studies have suggested the active role exerted by the cancer microenvironment on the growth and spread of neoplastic cells [187]. For instance, CAFs contribute to breast cancer aggressiveness through the production of secreted factors that promote migration, invasion and angiogenesis [187]. Further extending these findings, we have ascertained that Zn promotes the migration of CAFs through GPER and the induction of its target gene CTGF, which has been widely involved in cancer cells dissemination and metastasis [171, 175]. Moreover, we have assessed that Zn may influence analogous transcriptional and functional responses in both breast cancer cells and main components of the reactive stroma like CAFs toward more aggressive tumor features.

Altogether, the present data provide novel insights into the molecular mechanisms through which Zn may elicit stimulatory effects in breast cancer cells and tumor microenvironment components like CAFs. In particular, our findings indicate that GPER may be included together with EGFR and IGF-IR among the transduction mediators of relevant biological responses to Zn in breast cancer cells and the surrounding stroma.

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GPER, IGF-IR, and EGFR Transduction Signaling Are Involved in Stimulatory Effects of Zinc in Breast Cancer Cells and Cancer-Associated Fibroblasts

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Zinc (Zn) is an essential trace mineral that contributes to the regulation of several cellular functions; however, it may be also implicated in the progression of breast cancer through different mechanisms. It has been largely reported that the classical estrogen receptor (ER), as well as the G protein estrogen receptor (GPER, previously known as GPR30) can exert a main role in the development of breast tumors. In the present study, we demonstrate that zinc chloride ($ZnCl_2$) involves GPER in the activation of insulin-like growth factor receptor I (IGF-IR)/epidermal growth factor receptor (EGFR)-mediated signaling, which in turn triggers downstream pathways like ERK and AKT in breast cancer cells, and main components of the tumor microenvironment namely cancer-associated fibroblasts (CAFs). Further corroborating these findings, $ZnCl_2$ stimulates a functional crosstalk of GPER with IGF-IR and EGFR toward the transcription of diverse GPER target genes. Then, we show that GPER contributes to the stimulatory effects induced by $ZnCl_2$ on cell-cycle progression, proliferation, and migration of breast cancer cells as well as migration of CAFs. Together, our data provide novel insights into the molecular mechanisms through which zinc may exert stimulatory effects in breast cancer cells and CAFs toward tumor progression.

Key words: zinc; breast cancer cells; cancer-associated fibroblasts; EGFR; GPER; IGF-IR

INTRODUCTION

Zinc (Zn) is the second most abundant heavy metal in human tissues and contributes to the regulation of crucial cellular functions [1]. As an essential mineral, Zn is required for protein, nucleic acid, carbohydrate, and lipid metabolism and is involved in gene transcription, growth, development, and differentiation [1]. Zn is normally found in air, water, and soil; however, Zn concentrations may be boosted by several industrial activities including mining, coal, and waste combustion and steel processing [2]. For instance, soils located in areas where Zn is mined, refined, or used as fertilizer, are heavily contaminated with the metal [2]. The Recommended Daily Allowance of Zn in adults is 8–11 mg/day, with a tolerable upper intake level of 40 mg/day [3–5]. The adverse effects associated with a high Zn intake include acute gastrointestinal effects and headache, impaired immune function, changes in lipoprotein and cholesterol levels, reduced copper levels, and zinc–iron interactions as well as various other disorders [6–8]. In addition, Zn has been involved in the development of several types of tumors including breast cancer [9,10]. In this regard, previous studies have reported an association between dysregulated Zn homeostasis and breast cancer progression together with higher Zn levels in breast tumor specimens as compared to normal mammary tissues [11,12]. Compelling evidence has also linked an aberrant expression of Zn transporter proteins with the proliferation and migration of

Abbreviations: CAFs, cancer associated fibroblasts; CTGF, connective tissue growth factor; EGFR, epidermal growth factor receptor; EGR-1, early related gene; ERK, extracellular signal-regulated kinase; GPER, G protein estrogen receptor; IGF-IR, insulin-like growth factor I; PI3 K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; $ZnCl_2$, zinc chloride.

Assunta Pisano and Maria Francesca Santolla contributed equally to the work.

Grant sponsor: Associazione Italiana per la Ricerca sul Cancro; Grant number: 16719/2015 14066/13; Grant sponsor: Programma Operativo Nazionale "Ricerca e Competitività 2007–2013"; Grant number: PON 01_01078; Grant sponsor: Ministero della Salute; Grant number: 67/GR-2010-2319511; Grant sponsor: International Cancer Research Fellowship iCARE; Grant sponsor: European Union (Marie Curie Actions)

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Received 29 January 2016; Revised 8 June 2016; Accepted 22 June 2016

DOI 10.1002/mc.22518

Published online in Wiley Online Library (wileyonlinelibrary.com).

breast cancer cells [13–15]. A recent study has also suggested that specific dysregulations of Zn transporters may characterize grade, invasiveness, metastatic potential, and response to therapy in breast cancer [16]. Of note, zinc regulated transporters (ZIP) that control Zn influx into the cytosol, were found to be up-regulated by estrogens [17], and increased ZIP levels in breast tumors resulted to be associated with a poor prognosis [15]. Noteworthy, Zn may activate tyrosine kinase receptors as EGFR, IGF-IR, and the insulin receptor, which then trigger the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/AKT signaling [18–20]. These transduction pathways have been largely implicated in cancer growth and invasion together with other important signal molecules like the G protein-coupled receptors (GPCRs) [21]. Notably, both EGF and IGF-I mediated signaling were shown to functionally interact with the G protein estrogen receptor (GPER, previously known as GPR30) transduction pathway in breast cancer cells [22,23]. In this regard, it has been reported that GPER activation induces important responses like proliferation and migration in several types of cancer cells, and stromal cells that contribute to the malignant progression like cancer-associated fibroblasts (CAFs) [24].

In the present study, we therefore, aimed to evaluate whether Zn might trigger the transduction signaling mediated by GPER through a crosstalk with IGF-IR, and EGFR in breast cancer cells and CAFs. Our results provide novel mechanistic insights regarding a multifaceted network through which Zn may lead to stimulatory effects in breast tumor cells and CAFs derived from breast cancer patients.

METHODS

Reagents

We purchased zinc chloride ($ZnCl_2$), zinc sulfate ($ZnSO_4$), wortmannin (WM), N,N,N′,N′-tetrakis (2-pyridylmethyl)ethane-1,2-diamine (TPEN), N-acetyl-L-cysteine (NAC), and 2′,7′-dichlorofluorescin diacetate (DCFDA) from Sigma–Aldrich (Milan, Italy); tyrphostin AG1478 from Biomol Research Laboratories (Milan, Italy); PD98059 (PD), and 3-bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024) from Calbiochem (Milan, Italy); (3aS,4R,9bR)-4-(6-Bromo-1, 3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G15) from Tocris Bioscience (Bristol, UK); human Connective Tissue Growth Factor (CTGF) Recombinant Protein from MBL International (Eppendorf, Milan, Italy). All compounds were solubilized in DMSO except $ZnCl_2$, $ZnSO_4$, NAC, and human CTGF recombinant protein, which were dissolved in water. Treatments with the inhibitors AG1478, AG1024, G15, NAC, PD, TPEN, and WM were performed concomitantly with $ZnCl_2$ exposure, as indicated.

Cell Cultures

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% FBS, and 100 mg/ml penicillin/streptomycin (Life Technologies, Milan, Italy). CAFs were extracted as previously described in Ref. [25]. Briefly, breast cancer specimens were collected from primary tumors of patients who had undergone surgery. Signed informed consent was obtained from all the patients and from the institutional review board(s) of the Regional Hospital of Cosenza. Tissues from tumors were placed in digestion solution (400 IU collagenase, 100 IU hyaluronidase, and 10% serum, containing antibiotics and antimycotic) and incubated overnight at 37°C. Cells were separated by differential centrifugation at 90*g* for 2 min. Supernatant containing fibroblasts was centrifuged at 485*g* for 8 min, pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1, supplemented with 10% FBS, and antibiotics), and cultured at 37°C in 5% CO_2. The characterization of primary cells cultures of breast fibroblasts was assessed as described previously in Ref. [26]. Cells were switched to medium without serum the day before immunoblots and reverse transcription-PCR experiments.

Plasmids and Luciferase Assays

The luciferase reporter plasmid for c-fos encoding a 2.2-kb 5′ upstream fragment of human c-fos was a gift from Dr. K. Nose (Hatanodai, Shinagawa-ku, Tokyo). EGR1-luc plasmid, containing the −600 to +12 5′-flanking sequence from the human EGR1 gene, was kindly provided by Dr. Safe (Texas A&M University). The *Renilla* luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as internal transfection control. Cells (1×10^5) were plated into 24-well plates with 500 μl of regular growth medium/well the day before transfection. Cell medium was replaced on the day of transfection with serum-free medium and transfection was performed using X-tremeGENE 9 DNA Transfection Reagent (Sigma–Aldrich), and a mixture containing 0.5 μg of each reporter plasmid and 5 ng of pRL-TK. After 6 h, treatments were added and cells were incubated for 18 h. Luciferase activity was measured using the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase activity. 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.

Gene Silencing Experiments

SkBr3 cells and CAFs were plated in 10-cm dishes and transiently transfected by X-treme GENE 9 DNA

Transfection Reagent for 24 h before treatments with a control vector, a specific shRNA sequence for each target gene. The short hairpin (sh)RNA constructs to knock down the expression of GPER and CTGF, and the unrelated shRNA control constructs have been described previously in Ref. [27]. Short hairpin (sh) RNA constructs against human GPER were bought from Open Biosystems (www.Biocat.de) with catalog no. RHS4533-M001505. The targeting strands generated from the shRNA vectors sh1, 2, 3, 4, and unrelated control are complementary to the following sequences, respectively: CGAGTTAAAGAGGAGAAGGAA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCAAGCAGTCTTT, GCAGTACGTGATCGGCCTGTT, and CGACATGAAACCGTCCATGTT. Considering that sh3 showed the highest efficacy, after the first use it has been referred to as shGPER. The shRNA construct for CTGF was obtained from the same supplier (Open Biosystems; www.Biocat.de). It has clone ID TRCN0000061950 and is based on the same lentiviral expression vector pLKO.1 as the other shRNA constructs. The targeting strand generated from the CTGF shRNA construct is TAGTACAGCGATTCAAAGATG.

Gene Expression Studies

Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described in Ref. [28]. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc, Milan, Italy). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc). For c-fos, CTGF, Cyr61, EGR1, MT1X, MT2A, cyclin D1, cyclin A, GPER, and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-CGAGCCCTTTGATGACTTCCT-3′ (c-fos forward), 5′-GGAGCGGGCTGTCTCAGA-3′ (c-fos reverse); 5′-ACCTGTGGGATGGGCATCT-3′ (CTGF forward), 5′-CAGGCGGCTCTGCTTCTCTA-3′ (CTGF reverse); 5′-GAGTGGGTCTGTGACGAGGAT-3′ (Cyr61 forward) and 5′-GGTTGTATAGGATGCGAGGCT-3′ (Cyr61 reverse); 5′-GCCTGCGACATCTGTGGAA-3′ (EGR1 forward), 5′-CGCAAGTGGATCTTGGTATGC-3′ (EGR1 reverse); 5′-TGTCCCGCTGCGTGTTT-3′ (MT1X forward) and 5′-TTCGAGGCAAGGAGAAGCA-3′ (MT1X reverse); 5′-CCCGCTCCCAGATGTAAAGA-3′ (MT2A forward) and 5′-GGTCACGGTCAGGGTTGTACATA-3′ (MT2A reverse); 5′-GTCTGTGCATTTCTGGTTGCA-3′ (cyclin D1 forward) and 5′-GCTGGAAACATGCCGGTTA-3′ (cyclin D1 reverse); 5′-GCATGTCACCGTTCCTCCTTG -3′ (cyclin A forward) and 5′-GGGCATCTTCACGCTCTATTTT -3′ (cyclin A reverse); 5′-CCTGGACGAGCAGTATTACGATATC-3′ (GPER forward) and 5′-TGCTGTACATGTTGATCTG-3′ (GPER reverse) and 5′- GGCGTCCCCCAACTTCTTA -3′ (18S forward) and 5′- GGGCATCACAGACCTGTTATT -3′ (18S reverse), respectively. Assays were performed in triplicate and the results were normalized for 18S expression, and then calculated as fold induction of RNA expression.

Western Blot Analysis

Cells were grown in 10 cm dishes, exposed to ligands, and then lysed as previously described in Ref. [29]. Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, GE Healthcare, Milan, Italy), which were probed with primary antibodies against antiphosphotyrosine antibody (4G10) (Merck Millipore, Milan, Italy), pEGFR Tyr 1173 (sc-12351), EGFR (1005), phosphorylated ERK1/2 (E-4), ERK2 (C-14), p-AKT1/2/3 (Ser 473)-R, AKT/1/2/3 (H-136), IGF-1R (7G11), GPER (N-15), c-fos (H-125), EGR1 (C-19), CTGF (L-20), cyclin D1 (M-20), cyclin A (H-432), and β-actin (C2) (Santa Cruz Biotechnology, DBA, Milan, Italy) and then revealed using the ECL system from GE Healthcare (Milan, Italy).

Immunoprecipitation Assays

Cells were lysed using 200 μl RIPA buffer with a mixture of protease inhibitors containing 1.7 mg/ml aprotinin, 1 mg/ml leupeptin, 200 mmol/L phenylmethylsulfonyl fluoride, 200 mmol/L sodium orthovanadate, and 100 mmol/L sodium fluoride. A total of 100 μg proteins were incubated for 2 h with 2 μg of the appropriate antibody (GPER, N-15; IGF-1R, 7G11) and 20 μl of protein A/G agarose immunopreciptation reagent (Santa Cruz Biotechnology). Samples were centrifuged at 13 000 rpm for 5 min at 4°C to pellet beads. After four washes in PBS, samples were resuspended in RIPA buffer with protease inhibitors and SDS sample buffer. Western Blot analysis was performed as described above.

ROS Production

The non-fluorescent 2′,7′-dichlorofluorescin diacetate (DCFDA) probe, which becomes highly fluorescent on reaction with ROS, was used to evaluate intracellular ROS production. Briefly, cells (2×10^5) were incubated with 10 μM DCFDA (Sigma–Aldrich) at 37°C for 30 min, washed with PBS, and then exposed to treatments, as indicated. Cells were washed with PBS and the fluorescent intensity of DCF was measured (excitation at 485 nm and emission at 530 nm).

Cell Cycle Analysis

Cells synchronized for 24 h in serum-free medium were transfected, treated, and subjected to fluorescence-activated cell sorting (FACS) analysis. Adherent and floating cells were centrifuged, and resuspended in PBS containing 20 μg/ml propidium iodide plus 40 μg/ml ribonuclease (Sigma–Aldrich) for 1 h. Cells were then subjected to FACS analysis (FACS Jazz, BD, Milan, Italy) and results were expressed in terms of percentage.

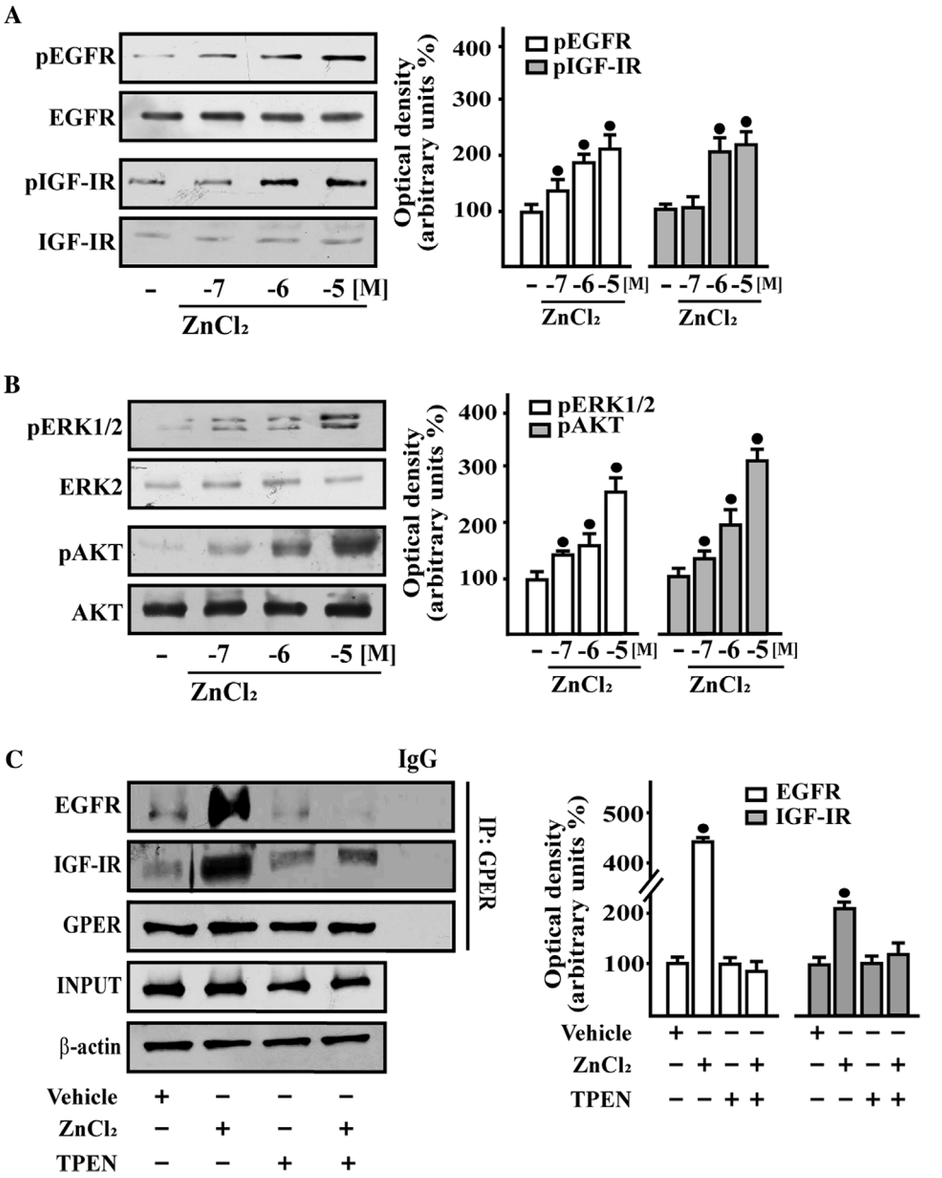

Figure 1. $ZnCl_2$ triggers rapid responses and stimulates the co-immunoprecipitation of EGFR and IGF-IR with GPER in breast cancer cells. (A and B) Phosphorylation of EGFR (A), IGF-IR (A), ERK1/2 (B) and AKT (B) in SkBr3 cells treated for 15 min with vehicle (–) and increasing concentrations of $ZnCl_2$, as indicated. Side panels show densitometric analysis of the blots normalized to EGFR, IGFIR, ERK2, and AKT that served as loading controls, respectively for pEGFR, pIGF-IR, pERK1/2, and pAKT. (C) Co-immunoprecipitation assays performed in SkBr3 cells treated with 10 μM $ZnCl_2$ for 15 min using the antibody against GPER followed by immunoblotting for EGFR or IGF-IR, as indicated. In control samples, nonspecific IgG was used instead of the primary antibody. IP, Immunoprecipitation. Input represents the blots probed with the antibody against GPER. Side panels show densitometric analysis of the blots normalized to β-actin. Data shown are the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

Proliferation Assay

Cells were seeded in 24-well plates in regular growth medium. After cells attached, they were incubated in medium containing 2.5% charcoal-stripped FBS, transfected for 24 h, and treated as indicated, with transfection and treatments renewed every 2 d. Cells were counted using an automated cell counter (Life Technologies) following the manufacturer's recommendations.

Migration Assays

Migration assays were performed using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane, Sigma–Aldrich). Cells were transfected in in regular growth medium. After 24 h, cells were trypsinized and seeded in the upper chambers. Treatments were added to the medium without serum in the bottom wells where applicable, cells on the bottom side

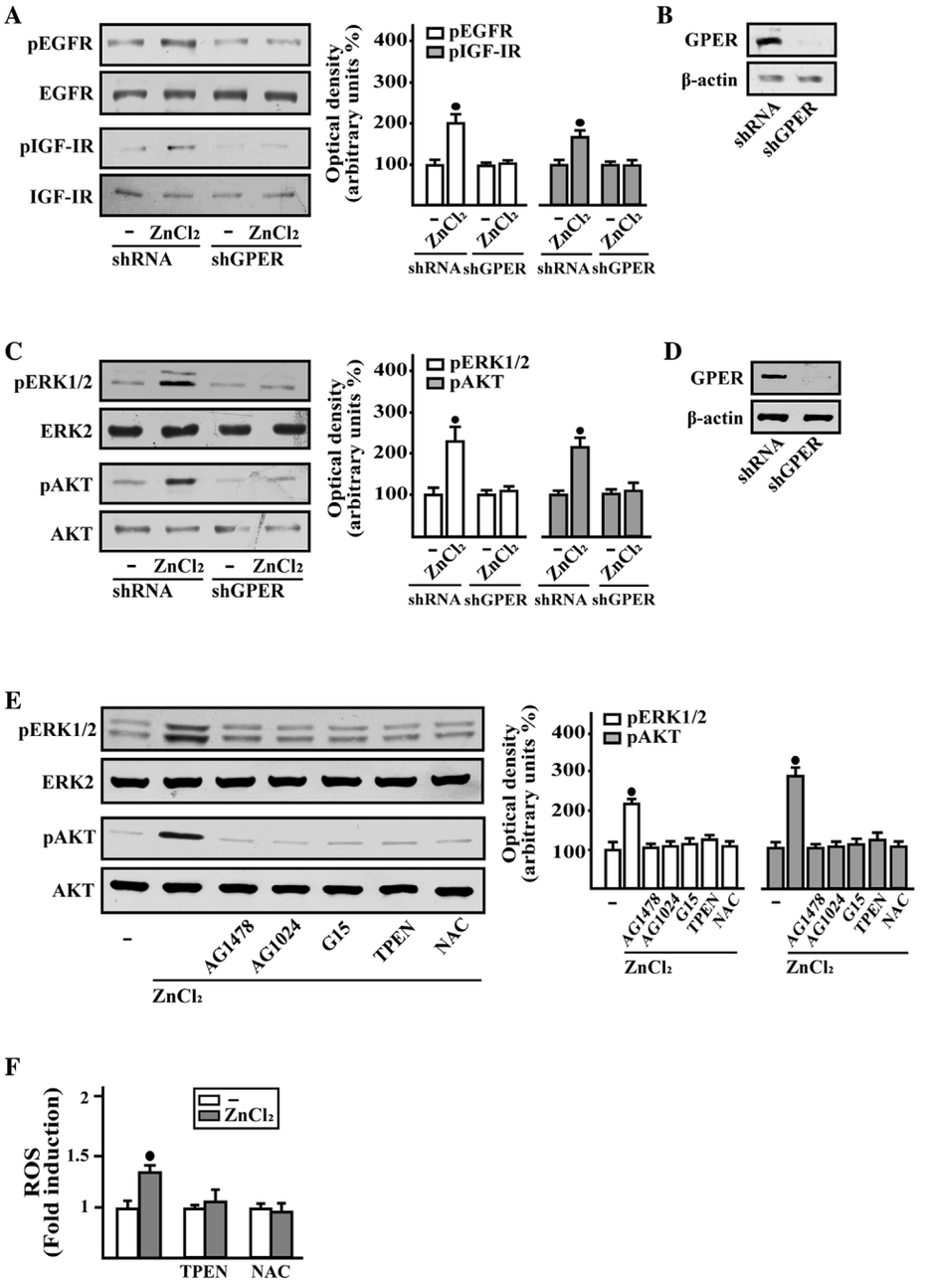

Figure 2. GPER is involved in the rapid action of $ZnCl_2$ in breast cancer cells. (A–D) Phosphorylation of EGFR (A), IGF-IR (A), ERK1/2 (C) and AKT (C) in SkBr3 cells after silencing GPER expression. Cells were transfected with control shRNA or shGPER, and treated for 15 min with vehicle (–) and 10 μM $ZnCl_2$. (B and D) Efficacy of GPER silencing. (E) ERK1/2 and AKT activation in SkBr3 cells treated for 15 min with vehicle (–) or 10 μM $ZnCl_2$ alone or in combination with 10 μM EGFR inhibitor AG1478, 10 μM IGF-IR inhibitor tyrphostin AG1024, 100 nM GPER antagonist G15, 20 μM zinc chelator TPEN, and 300 μM free radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to EGFR, IGFIR, ERK2, and AKT that served as loading controls, respectively for pEGFR, pIGF-IR, pERK1/2, and pAKT. (F) ROS production determined as DCF fluorescence in SkBr3 cells treated for 1 h with vehicle (–) or 10 μM $ZnCl_2$ alone and in combination with 20 μM zinc chelator TPEN, and 300 μM free radical scavenger NAC. DCF fluorescence obtained in cells treated with vehicle (–) was set as onefold induction upon which ROS levels induced by treatments was calculated. Data shown are the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

of the membrane were fixed and counted 6 h after seeding. Wound-healing assays were also performed in order to further assess cell migration. Cells were seeded into 12-well plates in regular growth medium. When at 70–80% confluence, cells were transfected in medium without serum. After 24 h, medium was replaced with 2.5% charcoal-stripped FBS and cells were treated. We then used a p200 pipette tip to scratch the cell

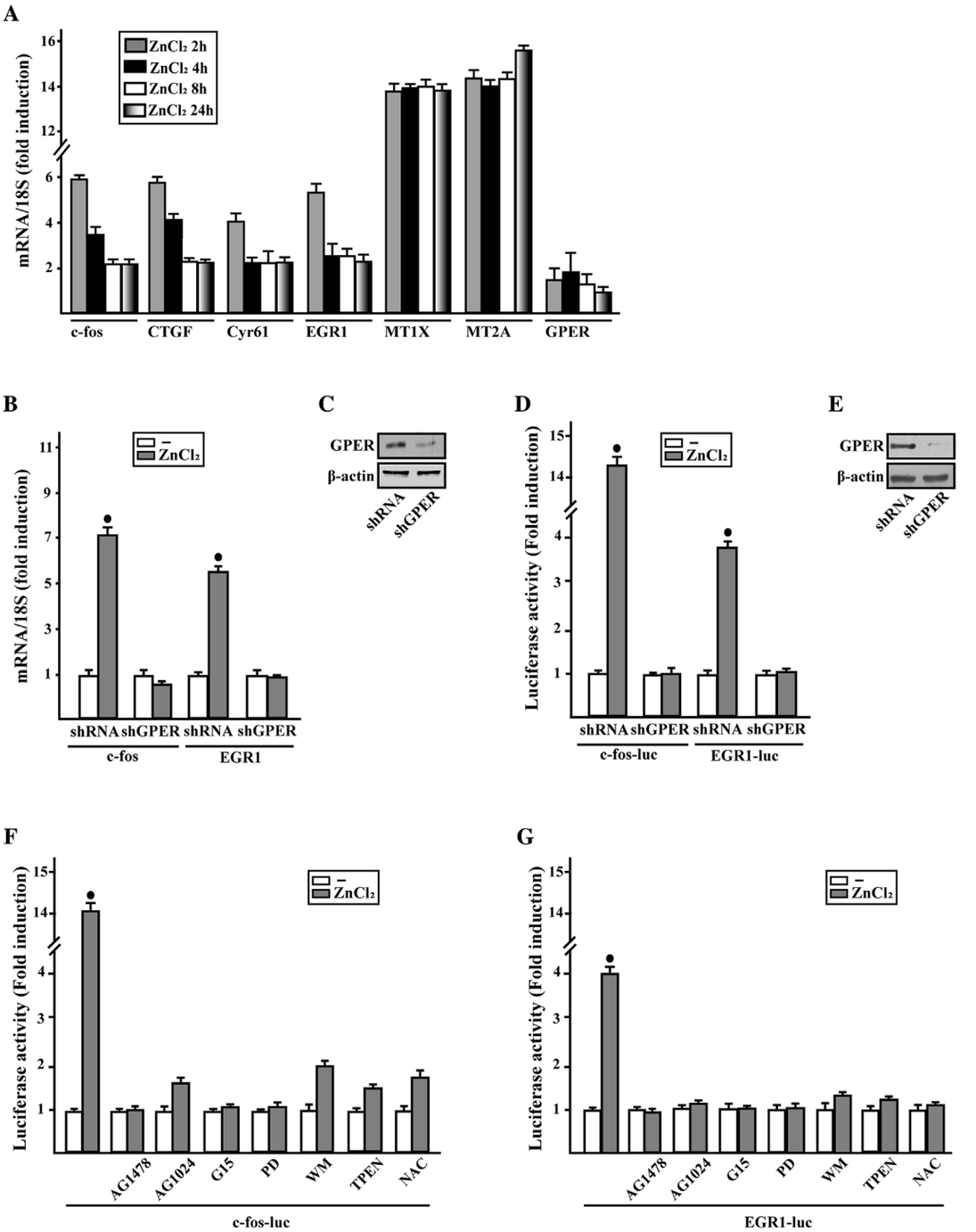

Figure 3. $ZnCl_2$ regulates the expression of GPER target genes in breast cancer cells. (A) The mRNA expression of c-fos, CTGF, Cyr61, EGR1, MT1X, MT2A and GPER was evaluated by real-time PCR in SkBr3 cells treated with vehicle (–) and 10 μM $ZnCl_2$, as indicated. (B) Evaluation of c-fos and EGR1 mRNA expression in SkBr3 cells transfected with shRNA or shGPER, and treated for 2 h with vehicle (–) and 10 μM $ZnCl_2$. (C) Efficacy of GPER silencing. Results obtained from experiments performed in triplicate were normalized for 18 S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (D) Evaluation of c-fos and EGR1 luciferase reporter genes in SkBr3 cells transfected with shRNA or shGPER, and treated for 18 h with vehicle (–) and 10 μM $ZnCl_2$. (E) Efficacy of GPER silencing. (F and G) Evaluation of c-fos and EGR1 luciferase reporter genes in SkBr3 cells treated for 18 h with vehicle (–) or 10 μM $ZnCl_2$ alone or in combination with 10 μM EGFR inhibitor AG1478, 10 μM IGF-IR inhibitor tyrphostin AG1024, 100 nM GPER antagonist G15, 10 μM MEK inhibitor PD98089 (PD), 1 μM PI3 K inhibitor wortmannin (WM), 20 μM Zn chelator TPEN, and 300 μM free radical scavenger NAC. Luciferase activity was normalized to the internal transfection control; values are presented as fold change (mean ± SD) of vehicle control and represent three independent experiments, each performed in triplicate. (●) indicates $P < 0.05$ for cells receiving vehicle (–) versus treatments.

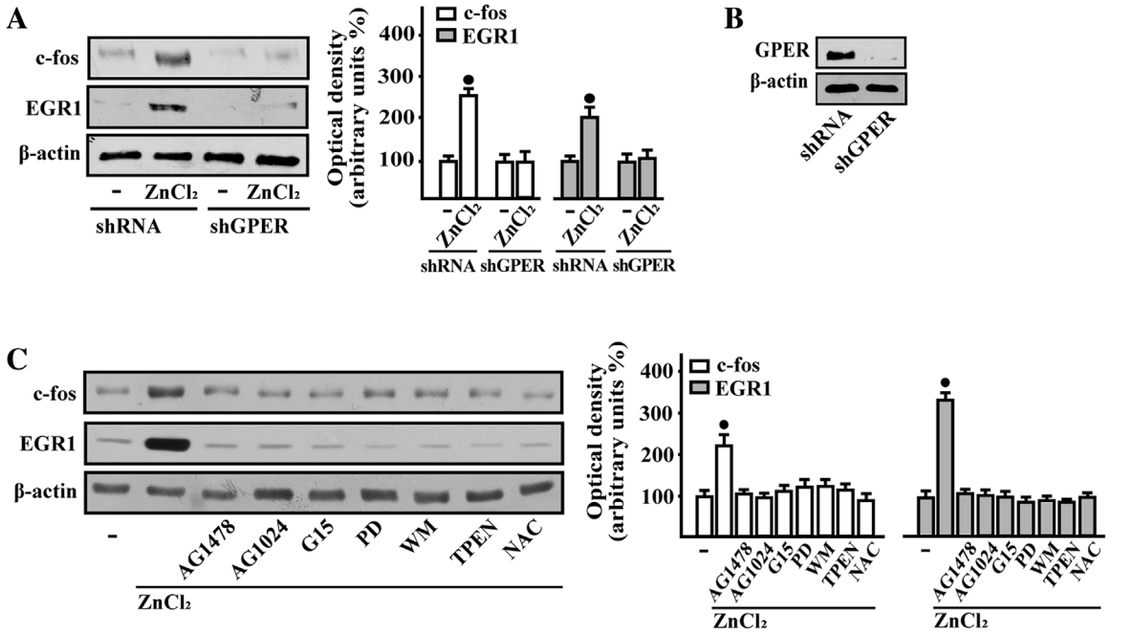

Figure 4. GPER is involved in c-fos and EGR1 protein increase induced by $ZnCl_2$ in breast cancer cells. (A and B) Protein levels of c-fos and EGR1 in SkBr3 cells transfected with shRNA or shGPER, and treated with vehicle (–) or 10 μM $ZnCl_2$ for 4 h. (B) Efficacy of GPER silencing. (C) Immunoblots showing c-fos and EGR1 protein expression in SkBr3 cells treated for 4 h with vehicle (–), and 10 μM $ZnCl_2$ alone or in combination with 10 μM EGFR inhibitor AG1478, 10 μM IGF-IR inhibitor tyrphostin AG1024, 100 nM GPER antagonist G15, 10 μM MEK inhibitor PD98089 (PD), 1 μM PI3K inhibitor wortmannin (WM), 20 μM Zn chelator TPEN, and 300 μM free radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to β-actin. Values represent the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

monolayer. Cells were allowed to migrate for 24 h, the gap area was then photographed and migration distances were measured.

Statistical Analysis

Statistical analysis was done using ANOVA followed by Newman–Keuls' testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

RESULTS

GPER Is Involved in the Activation of EGFR and IGF-IR by Zn in Breast Cancer Cells

As a dysregulated Zn homeostasis may contribute to breast carcinogenesis through different mechanisms [12], including the activation of growth factors transduction pathways [18–20], we began our study by ascertaining that Zn chloride ($ZnCl_2$) triggers the rapid phosphorylation of EGFR and IGF-IR (Figure 1A), as well as the activation of downstream kinases such as ERK and AKT (Figure 1B) in a dose-dependent manner. Similar results were obtained using Zn sulfate ($ZnSO_4$) (data not shown). On the basis of these findings, and considering that Zn serum concentration is approximately 15 μM [30], in subsequent assays 10 μM $ZnCl_2$ were used. As our previous studies have shown that, in cancer cells, both EGFR and IGF-IR transduction signaling are involved in GPER regulation [29,31–34], we evaluated whether the activation of EGFR and IGF-IR by $ZnCl_2$ may involve GPER. By co-immunoprecipitation studies performed in SkBr3 cells, we ascertained that $ZnCl_2$ increases a direct interaction of GPER with EGFR and IGF-IR, while the Zn chelator TPEN prevented this response (Figure 1C). On the basis of these findings, we asked whether the $ZnCl_2$-dependent phosphorylation of EGFR and IGF-IR as well as ERK and AKT may involve GPER. Of note, the silencing of GPER expression by a specific shRNA abrogated the activation of both EGFR and IGF-IR, and their downstream signaling molecules ERK and AKT induced by $ZnCl_2$ treatment (Figure 2A–D). Next, we investigated the mechanisms through which $ZnCl_2$ may induce the activation of ERK and AKT in breast cancer cells. As shown in Figure 2E, the treatment with the EGFR inhibitor AG1478, the IGF-IR inhibitor AG1024, and the GPER antagonist G15 prevented the phosphorylation of both kinases upon exposure to $ZnCl_2$. Likewise, the activation of ERK and AKT triggered by $ZnCl_2$ was no longer evident in the presence of the Zn chelator TPEN, and the scavenger of reactive oxygen species (ROS) NAC (Figure 2E). Taken together, these data suggest that EGFR, IGF-IR, and GPER are involved in ERK and AKT activation induced by $ZnCl_2$. Moreover, the inhibitory effects elicited by

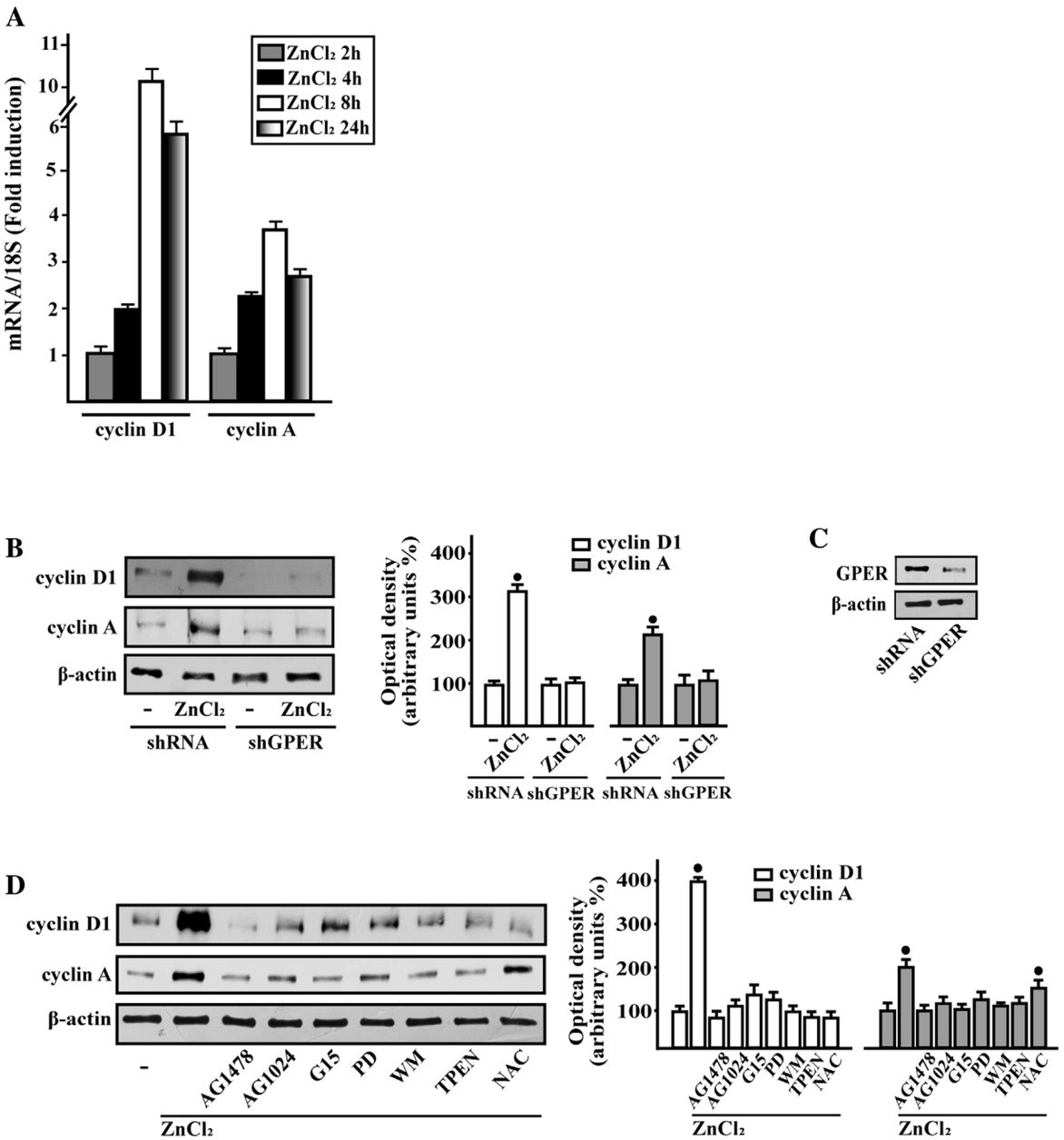

Figure 5. GPER is involved in the up-regulation of cyclins by $ZnCl_2$ in breast cancer cells. (A) The mRNA expression of cyclin D1 and cyclin A was evaluated by real-time PCR in SkBr3 cells treated with vehicle (−) or 10 μM $ZnCl_2$, as indicated. Results obtained from experiments performed in triplicate were normalized for 18 S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (B) Cyclin D1 and cyclin A protein levels in SkBr3 cells transfected with shRNA or shGPER. and treated with vehicle (−) and 10 μM $ZnCl_2$ for 12 h. (C) Efficacy of GPER silencing. (D) Cyclin D1 and cyclin A immunoblots in SkBr3 cells treated for 12 h with vehicle (−), and 10 μM $ZnCl_2$ alone or in combination with 10 μM EGFR inhibitor AG1478, 10 μM IGF-IR inhibitor tyrphostin AG1024, 100 nM GPER antagonist G15, 10 μM MEK inhibitor PD98089 (PD), 1 μM PI3K inhibitor wortmannin (WM), 20 μM zinc chelator TPEN, and 300 μM free radical scavenger NAC. Side panels show densitometric analysis of the blots normalized to β-actin. Values represent the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (−) versus treatments.

TPEN and NAC indicate that the aforementioned responses triggered by $ZnCl_2$ are strictly dependent on the metal and occur through the ROS generation. On the basis of these data and previous results showing that Zn is able to increase ROS levels [19,20], we first confirmed this finding in our experimental model, and thereafter, established that TPEN and NAC inhibit ROS generation triggered by $ZnCl_2$ (Figure 2F). Hence, the production of ROS observed in SkBr3 cells is involved in the rapid activation of

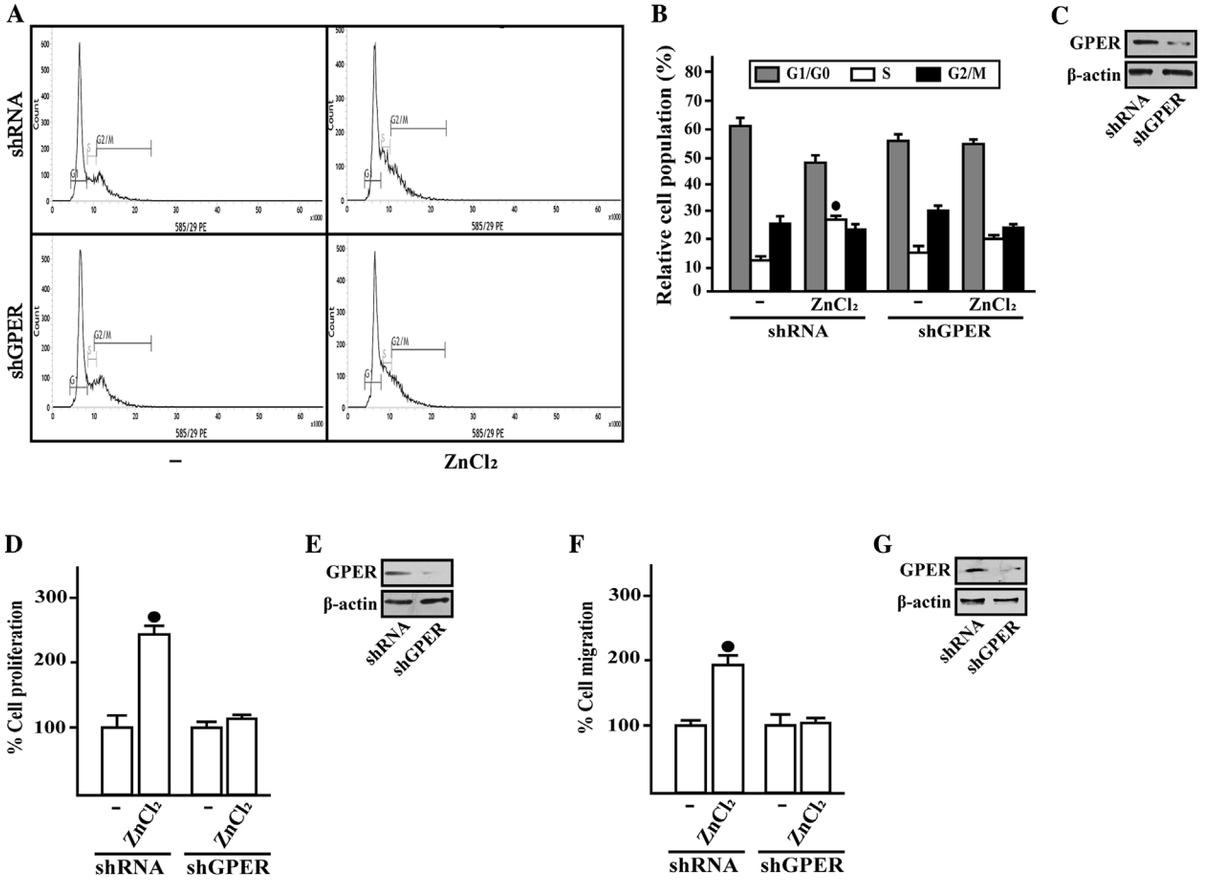

Figure 6. GPER contributes to $ZnCl_2$ induced cell-cycle progression and proliferation of breast cancer cells. (A) Cell-cycle analysis performed in SkBr3 cells transfected with shRNA or shGPER, and treated with vehicle (–) and 10 μM $ZnCl_2$ for 18 h. (B) The histograms show the percentages of cells in G1/G0, S, and G2/M phases of the cell cycle, as determined by flow cytometry analysis. (D) The proliferation of SkBr3 cells upon treatment with 10 μM $ZnCl_2$ is prevented knocking down GPER expression. Cells were transfected with shRNA or shGPER and treated every 2 d with vehicle (–) or $ZnCl_2$ as indicated, and then counted on day 6. Proliferation of cells treated with vehicle was set as 100% upon which cell growth induced by treatments was calculated. (F) The migration of SkBr3 cells upon 6 h treatment with 10 μM $ZnCl_2$ is abrogated knocking down GPER expression, as evaluated by Boyden Chamber assay. (C, E, and G) Efficacy of GPER silencing. Each data point is the mean ± SD of three independent experiments performed in triplicate. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

GPER/EGFR/IGF-IR transduction signaling upon $ZnCl_2$ exposure. Collectively, these observations indicate that $ZnCl_2$ activates a complex transduction signaling that may involve GPER together with EGFR and IGF-IR, and downstream effectors like ERK and AKT, hence, leading to important biological outcomes (see below).

GPER Contributes to Gene Expression Changes Induced by Zn in Breast Cancer Cells

Considering that GPER triggers a specific gene signature [27], we then assessed that $ZnCl_2$ upregulates in SkBr3 cells the mRNA expression of certain GPER target genes like c-fos, CTGF, Cyr61, EGR1, MT1X, and MT2A, without changing GPER levels in our experimental conditions (Figure 3A). Of note, GPER silencing prevented the mRNA induction of two main GPER target genes as c-fos and EGR1 (Figure 3B and C) [27]. Accordingly, the transactivation of c-fos and EGR1 promoter constructs observed upon $ZnCl_2$ exposure was no longer evident knocking down GPER expression (Figure 3D and E). Moreover, the EGFR inhibitor AG1478, the IGF-IR inhibitor AG1024, the GPER antagonist G15, the MEK inhibitor PD, the PI3K inhibitor WM, the zinc chelator TPEN, and the ROS scavenger NAC abolished the luciferase activity of c-fos and EGR1 reporter plasmids induced by $ZnCl_2$ (Figure 3F and G). Next, we sought to determine whether $ZnCl_2$ could regulate c-fos and EGR1 at protein level as well as the transduction pathways involved in this response. According to the results obtained in real-time PCR and luciferase experiments, c-fos and EGR-1 protein expression triggered by $ZnCl_2$ was prevented by GPER silencing (Figure 4A and B) as well as in the presence of the EGFR inhibitor AG1478, the IGF-IR inhibitor AG1024, the GPER antagonist

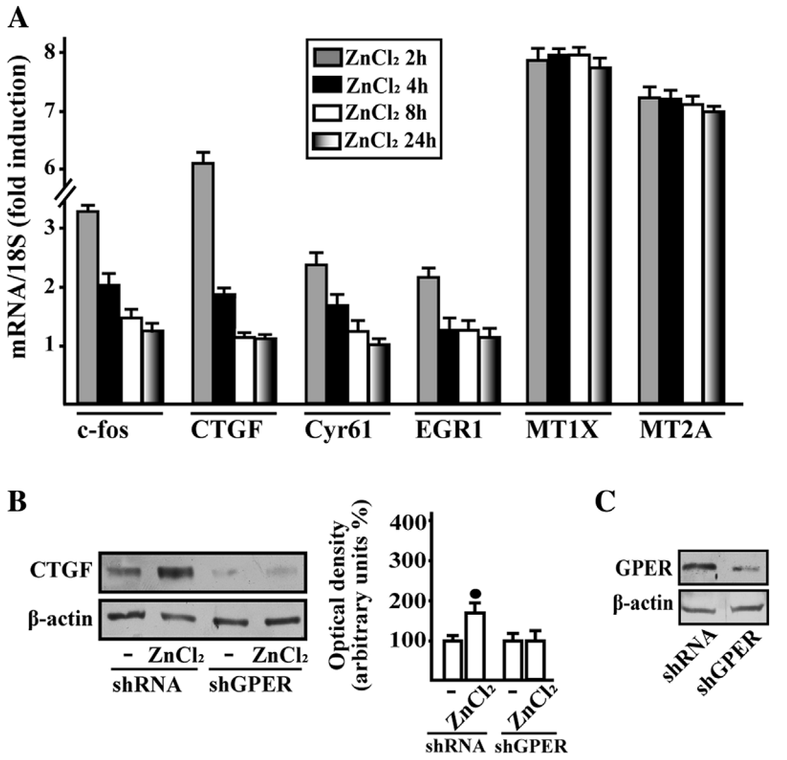

Figure 7. GPER is involved in gene expression changes induced by $ZnCl_2$ in CAFs. (A) The mRNA expression of c-fos, CTGF, Cyr61, EGR1, MT1X, and MT2A was evaluated by real-time PCR in CAFs treated with vehicle (–) and 10 μM $ZnCl_2$, as indicated. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (B) Immunoblots showing CTGF protein expression in CAFs transfected with shRNA or shGPER, and treated for 4 h with vehicle (–) and 10 μM $ZnCl_2$. Side panel shows densitometric analysis of the blot normalized to β-actin. (C) Efficacy of GPER silencing. Values represent the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

G15, the MEK inhibitor PD, the PI3K inhibitor WM, the zinc chelator TPEN, and the ROS scavenger NAC (Figure 4C). Altogether, these data indicate novel transduction mechanisms and gene responses triggered by Zn in breast cancer cells.

GPER Is Involved in the Biological Responses to Zn in Breast Cancer Cells

As cyclin D1 and cyclin A have been implicated in the development of several tumors including breast cancer [35], we next evaluated the potential of $ZnCl_2$ to induce these cell cycle regulators. We found that $ZnCl_2$ stimulates the expression of both cyclins (Figure 5A andB); however, this response was abrogated silencing GPER (Figure 5B and C) as well as in the presence of AG1478, AG1024, G15, PD, WM, TPEN (Figure 5D). As it concerns NAC, its inhibitory action was mainly exerted on cyclin D1 protein increase by $ZnCl_2$ whereas, the up-regulation of cyclin A upon NAC treatment was blunted but still evident (Figure 5D). Indeed, although the chelator TPEN does not act in a selective manner, its ability to prevent the aforementioned responses to Zn may further confirm our findings on the biological properties of this metal. On the basis of the results obtained, it could be therefore, argued that GPER is involved in Zn-dependent gene expression that occurs through both EGFR and IGF-IR transduction pathways. As cyclins are mainly involved in cell cycle progression, we assessed that $ZnCl_2$ increases the percentage of SkBr3 cells in the S phase of the cell cycle (Figure 6A–C). Moreover, we determined that this response to $ZnCl_2$ is abrogated by GPER silencing (Figure 6A–C). In accordance with these findings, the proliferative effects observed in SkBr3 cells treated with $ZnCl_2$ were no longer evident knocking down the expression of GPER (Figure 6D and E). In addition, SkBr3 cell migration induced by $ZnCl_2$ was prevented silencing GPER (Figure 6F and G). Taken together, these data further extend the current knowledge regarding the stimulatory effects exerted by Zn in breast cancer cells.

GPER Contributes to Zn Action in CAFs

In order to further ascertain whether GPER may contribute to the action of Zn, we used CAFs that play an active role toward the growth, expansion, and dissemination of breast cancer cells [36,37]. Remarkably, $ZnCl_2$ increased the mRNA levels of diverse GPER target genes like c-fos, CTGF, Cyr61, EGR1, MT1X, and MT2A in

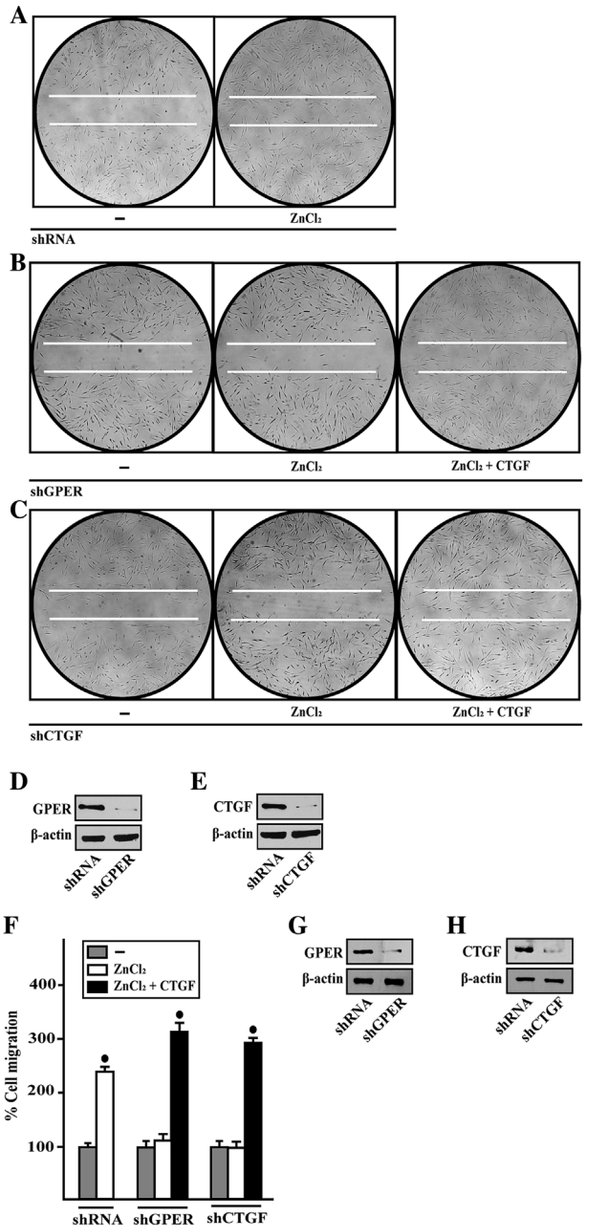

Figure 8. GPER and its target gene CTGF contribute to the migration of CAFs induced by $ZnCl_2$. (A–C) The migration of CAFs upon treatment with 10 μM $ZnCl_2$ for 24 h is prevented knocking down GPER and CTGF expression, as assessed by wound-healing assay. Cell migration is rescued in CAFs transfected with shGPER (B) or shCTGF (C) exposed to 10 μM $ZnCl_2$ for 24 h and treated with 100 ng/ml CTGF. Images shown are representative of three independent experiments. (F) The migration of CAFs induced by a 6 h treatment with 10 μM $ZnCl_2$ is prevented knocking down GPER and CTGF expression, as evaluated by Boyden Chamber assay. Cell migration is rescued in CAFs transfected with shGPER and shCTGF, exposed to 10 μM $ZnCl_2$ for 6 h and treated with 100 ng/ml CTGF. Efficacy of GPER (D and G) and CTGF (E and H) silencing. Values represent the mean ± SD of three independent experiments. (●) indicates $P < 0.05$ for cells treated with vehicle (–) versus treatments.

CAFs obtained from breast cancer specimens (Figure7A). Gene expression profile displayed responses to $ZnCl_2$ similar to those observed in SkBr3 cells (Figure 3A), as the induction of c-fos, CTGF, Cyr61, and EGR1 was rapid (2–4 h) but declined thereafter, whereas, the expression of MT1X and MT2A was still evident up to 24 h. Then, we observed that the up-regulation of CTGF protein levels upon $ZnCl_2$ treatment is prevented knocking down GPER expression in CAFs (Figure 7B and C). As CTGF exerts an acknowledged role in migratory properties of different cell types [27,38], we evaluated whether GPER signaling

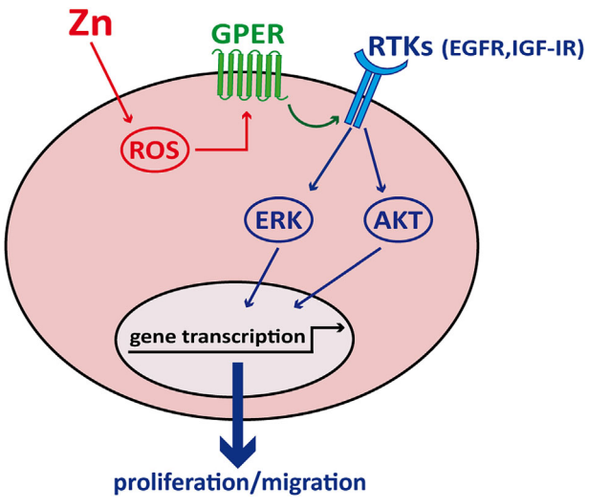

Figure 9. Schematic representation of the functional cooperation of GPER with IGF-IR and EGFR upon zinc exposure.

through CTGF may trigger the migration of CAFs. Scratch experiments and Boyden chamber assays revealed that $ZnCl_2$-stimulated migration of CAFs is abolished silencing GPER or CTGF expression, whereas, adding CTGF the migratory response was rescued (Figure 8). Collectively, the aforementioned results indicate that Zn-activated GPER signaling mediates a similar gene expression profile as well as important biological responses in both breast cancer cells and CAFs. On the basis of these findings, it could be argued that Zn may trigger through GPER a functional interplay between cancer cells and CAFs toward breast tumor progression.

DISCUSSION

Several human activities as well as natural events can lead to heavy metals pollution and therefore, increased incidence of various tumors [39–41]. In the present study, we have demonstrated that one important pollutant such as Zn may trigger a functional interplay of GPER with EGFR and IGF-IR, which leads to the activation of main transduction pathways, gene expression changes, and important biological responses like proliferation and migration in breast cancer cells and CAFs (Figure 9).

Breast cancers have been reported to show an increased Zn uptake and tissue concentration as compared to the normal breast tissue [10,42], while patients with advanced breast tumors show decreased serum Zn levels; hence, the determination of serum Zn levels has been proposed as a prognostic marker in breast cancer patients [9,43,44]. Of note, tamoxifen-resistant breast cancer cells that display an aggressive and invasive phenotype, show increased levels of Zn and its transporter ZIP7, which are involved in the activation of EGFR and IGF-IR transduction signaling toward cell proliferation, and invasion [15]. In accordance with these findings, the growth factors-mediated effects of Zn promoted the activation of kinases, gene expression changes, and growth responses [19,20].

Numerous studies have shown that GPER contributes to the progression of certain tumors including breast cancer [45–50]. In addition, clinical studies have indicated that GPER may be a predictor of aggressive cancer behavior as its expression has been associated with negative clinical outcomes in several cancer histotypes [51–55]. The activation of GPER has been shown to trigger EGFR transactivation, subsequent transduction events such as the activation of MAPK and PI3K cascades, gene expression changes, and relevant biological responses such as proliferation, migration, and angiogenesis in diverse cancer cell types and CAFs [56,57]. In this context, it should be mentioned that the metal cadmium may induce cAMP increase, ERK1/2 activation, and proliferation of breast cancer cells in a GPER-dependent manner [58]. Recently, we also demonstrated that copper activates the HIF-1α/GPER/VEGF signaling in cancer cells leading to angiogenesis and tumor progression [57]. Further extending these findings, in the present study we have demonstrated that in breast cancer cells exposed to Zn the activation of GPER leads to rapid signaling events such as the phosphorylation of EGFR and IGF-IR, and their downstream effectors ERK and AKT, the up-regulation of c-fos and EGR1, two main GPER target genes largely involved in growth responses. It is worth noting that Zn induced also GPER targets namely metallothioneins MT1X and MT2A, whose overexpression correlates with chemoresistance and poor prognosis in breast tumors [59,60]. Moreover, in line with the known capability of GPER to trigger the transcription of genes associated with cell growth [27], we assessed the potential of Zn to regulate the

expression of two members of the cyclin family as cyclin D1 and A. According to their regulatory role of cell-cycle progression, proliferation, and notably migration [61], we detected also that Zn through GPER significantly increases the percentage of SkBr3 cells in the S phase of the cell cycle as well as stimulates cell proliferation and migration.

Several studies have suggested the active role exerted by the cancer microenvironment on the growth and spread of neoplastic cells [62]. For instance, CAFs contribute to breast cancer aggressiveness through the production of secreted factors that promote migration, invasion, and angiogenesis [62]. Further extending these findings, we have ascertained that Zn promotes the migration of CAFs through GPER and the induction of its target gene CTGF, which has been widely involved in cancer cells dissemination and metastasis [27,38]. Moreover, we have assessed that Zn may influence analogous transcriptional and functional responses in both breast cancer cells, and main components of the reactive stroma like CAFs toward more aggressive tumor features.

Altogether, the present data provide novel insights into the molecular mechanisms through which Zn may elicit stimulatory effects in breast cancer cells and tumor microenvironment components such as CAFs. In particular, our findings indicates that GPER may be included together with EGFR and IGF-IR among the transduction mediators of relevant biological responses to Zn in breast cancer cells, and the surrounding stroma.

ACKNOWLEDGMENTS

This work was supported by Associazione Italiana per la Ricerca sul Cancro (MM grant n. 16719/2015 and AB grant n. 14066/13), Programma Operativo Nazionale "Ricerca e Competitività 2007–2013" (PON 01_01078) and Ministero della Salute (grant n. 67/GR-2010-2319511). EMDF was supported by International Cancer Research Fellowship iCARE and European Union (Marie Curie Actions).

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Stimulatory actions of IGF-I are mediated by IGF-IR cross-talk with GPER and DDR1 in mesothelioma and lung cancer cells

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Keywords: DDR1, GPER, IGF-I, IGF-IR, mesothelioma, lung cancer, Pathology Section

Received: *April 06, 2016* ***Accepted***: *June 17, 2016* ***Published***: *June 30, 2016*

ABSTRACT

Insulin-like growth factor-I (IGF-I)/IGF-I receptor (IGF-IR) system has been largely involved in the pathogenesis and development of various tumors. We have previously demonstrated that IGF-IR cooperates with the G-protein estrogen receptor (GPER) and the collagen receptor discoidin domain 1 (DDR1) that are implicated in cancer progression. Here, we provide novel evidence regarding the molecular mechanisms through which IGF-I/IGF-IR signaling triggers a functional cross-talk with GPER and DDR1 in both mesothelioma and lung cancer cells. In particular, we show that IGF-I activates the transduction network mediated by IGF-IR leading to the up-regulation of GPER and its main target genes CTGF and EGR1 as well as the induction of DDR1 target genes like MATN-2, FBN-1, NOTCH 1 and HES-1. Of note, certain DDR1-mediated effects upon IGF-I stimulation required both IGF-IR and GPER as determined knocking-down the expression of these receptors. The aforementioned findings were nicely recapitulated in important biological outcomes like IGF-I promoted chemotaxis and migration of both mesothelioma and lung cancer cells. Overall, our data suggest that IGF-I/IGF-IR system triggers stimulatory actions through both GPER and DDR1 in aggressive tumors as mesothelioma and lung tumors. Hence, this novel signaling pathway may represent a further target in setting innovative anticancer strategies.

INTRODUCTION

Lung cancer is the most frequent cause of cancer incidence and mortality worldwide at least in part due to the increasing number of risk factors in diverse developing countries [1-2]. To date, smoking has been considered the main etiologic factor for lung cancer [3-4], however, several environmental contaminants like asbestos, arsenic, cadmium, nickel and silica, play an important role toward the development of this neoplasia [5]. Among the aforementioned environmental pollutants, asbestos has been particularly acknowledged as prompting factor in malignant mesothelioma (MM), which is an aggressive cancer that arises from mesothelial cells lining lung, pleura or peritoneum [6-7]. Chronic inflammatory processes caused by the deposition of asbestos fibers and the subsequent release of cytokines and growth factors by macrophages and mesothelial cells have been shown to play an active role toward the development of both pleural MM and lung cancer [7-8].

In this vein, the IGF system, the complex system involving the insulin-like growth factors (IGFs) and related receptors as well as IGF-binding proteins, has been established as an important regulator of tumor initiation

and progression in several malignancies, including pleural MM and lung cancer [9-13]. In particular, the IGF-I receptor (IGF-IR), which is often overexpressed in diverse cancer cell types, affects tumor development, progression and resistance to therapies [11, 14-16]. Moreover, a dysregulated IGF system has been shown to be implicated in various chronic diseases, such as pulmonary fibrosis [17-18].

An increasing body of data has demonstrated that the biological responses mediated by IGF-I involve functional interactions of IGF-IR with diverse signal molecules belonging to other members of the receptor tyrosine kinase (RTK) family [19-20]. In this context, we recently discovered a novel functional cross-talk between IGF-IR and the collagen receptor discoidin domain receptor 1 (DDR1), a molecule also overexpressed in diverse malignancies, including lung carcinomas, and implicated in cancer progression [21]. Interestingly, this cross-talk occurs also independently of the collagen binding actions of DDR1 and, in human breast cancer cells, amplifies the stimulatory biological effects of IGF-I toward proliferation, migration and colony formation. Moreover, through a signaling pathway involving Akt/miR-199a-5p, IGF-I is able to upregulate DDR1 [12, 22].

In addition to RTKs, IGF-IR interacts with other important signaling molecules like G protein-coupled receptors (GPCRs) [19, 23]. These functional interactions have also important implications in the development and progression of diverse types of tumors [23-24]. In particular, we found that IGF-IR activation engages the G protein estrogen receptor (GPER/GPR30)-mediated signaling toward the stimulation of proliferation and migration of different cancer cell types [25-26]. Interestingly, high expression levels of GPER were detected in lung cancer cells and involved in growth stimulatory effects [24, 27-28]. To date, other signaling molecules have been implicated in the development of MM including the estrogen receptor (ER)β that may act as a tumor suppressor [29-30]. Therefore, the multifaceted mechanisms and the transduction network of factors involved in the progression of the aforementioned malignancies remain to be fully understood.

In this study, we found that mesothelioma and lung cancer cells show a new complex functional cross-talk involving IGF-IR, GPER and DDR1, which affects gene expression and biological effects in response to IGF-I. Our data, therefore, further extend the molecular mechanisms by which IGF-I may affect tumor progression in mesothelioma and lung cancer, hence providing novel targets in the aforementioned aggressive malignancies.

RESULTS

IGF-I stimulates GPER expression through IGF-IR/ERK/p-38 transduction signaling

On the basis of previous studies showing that IGF-I triggers stimulatory effects in malignant mesothelioma as well as in lung cancer cells [31-32], we began our study evaluating the transduction signaling activated by IGF-I in IST-MES1 mesothelioma and A549 lung cancer cells, which were used as model system. First, we determined that in both cell types IGF-I induces the phosphorylation of IGF-IR (Figure 1A) and both ERK (Figure 1B) and p-38 (Figure 1C). As expected, these responses were no longer observed after IGF-IR silencing (Figure 1 A-1E). The activation of ERK triggered by IGF-I was abolished in the presence of the IGF-IR inhibitor AG and the MEK inhibitor PD, but it still persisted using the p-38 inhibitor SB (Figure 1F). The phosphorylation of p-38 was prevented by AG and SB, but not in the presence of PD (Figure 1G). In addition, we assessed that the phosphorylation of IGF-IR induced by IGF-I is inhibited exclusively by AG, but not in the presence of PD and SB (data not shown), then suggesting that the activation of both ERK and p-38 relies directly on IGF-IR phosphorylation upon IGF-I exposure. On the basis of our previous data showing that IGF-I signaling cooperates with several GPCR family members, including GPER, toward cancer progression [19, 25], we evaluated whether IGF-I regulates GPER expression in IST-MES1 and A549 cells. In this regard, time-course experiments demonstrated that IGF-I up-regulates GPER at both mRNA (Figure 2A) and protein levels (Figure 2B). Moreover, we ascertained that these responses to IGF-I occurred through IGF-IR, as the induction of GPER mRNA (data not shown) and protein levels (Figure 2C-2E) was abolished by knocking-down IGF-IR expression. Recapitulating the aforementioned findings, the transactivation of the GPER promoter by IGF-I was prevented by IGF-IR silencing (Figure 2F), and the IGF-I induced GPER protein up-regulation was abrogated in the presence of AG, PD and SB (Figure 2G). Taken together, these results indicate that the IGF-I/IGF-IR transduction pathway stimulates GPER expression through ERK and p-38 signaling. In order to further investigate this functional cross-talk between IGF-IR and GPER, we performed co-immunoprecipitation studies determining that IGF-I triggers also a direct interaction between these receptors in both IST-MES1 and A549 cells upon either 1 h (data not shown) or 8 h treatment with IGF-I (Figure 2H-2I), thus suggesting that the interaction between IGF-IR and GPER may occur without a newly protein expression of GPER.

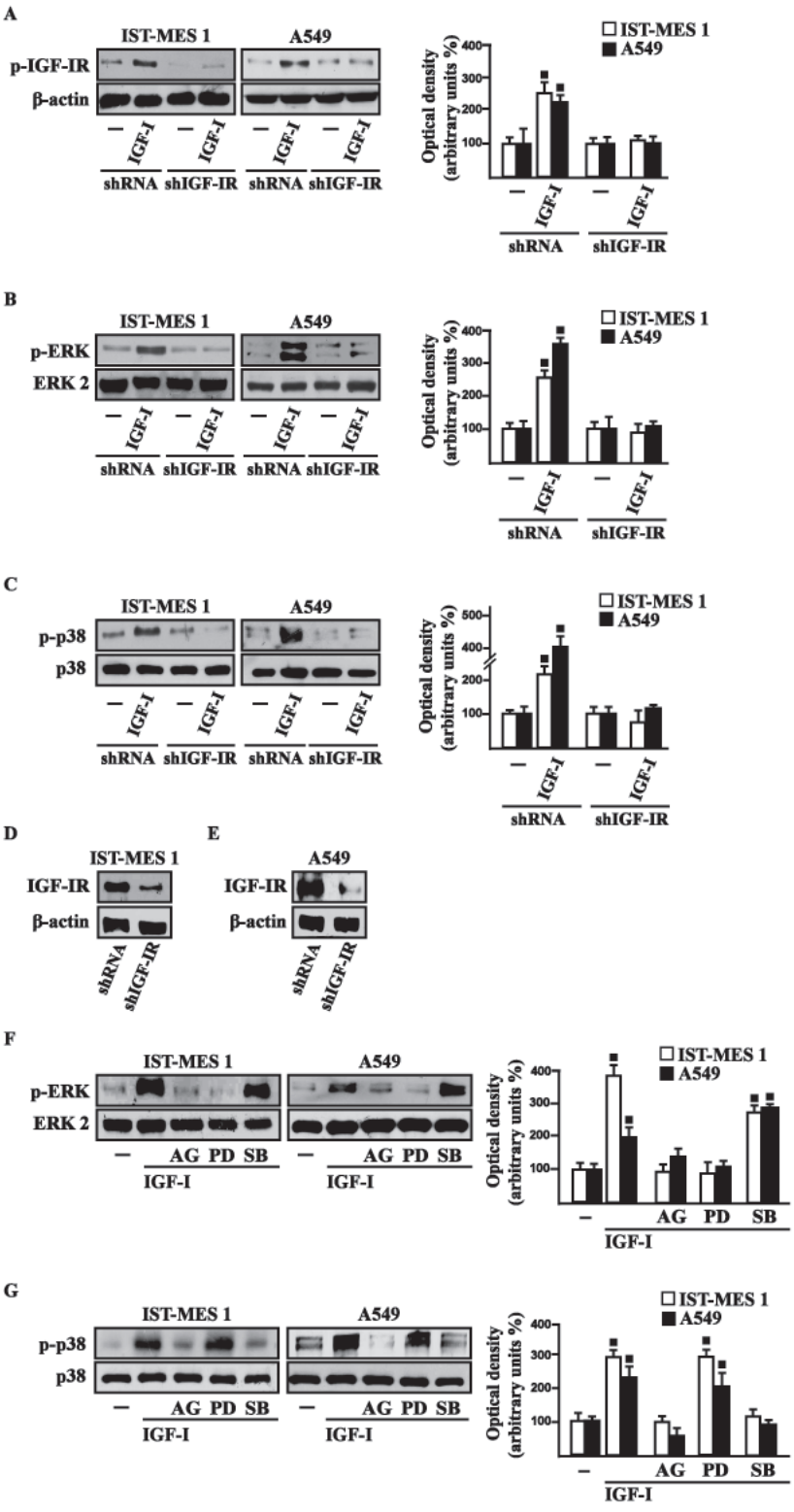

Figure 1: Rapid activation of transduction signaling by IGF-I in IST-MES 1 and A549 cells. IGF-IR **A.**, ERK **B.** and p-38 **C.** phosphorylation in cells transfected for 24 h with shRNA or shIGF-IR treated with vehicle (-) or 100 ng/ml IGF-I for 15 min. **D.-E.** Efficacy of IGF-IR silencing. ERK **F.** and p-38 **G.** activation in cells treated for 15 min with vehicle (-) or 100 ng/ml IGF-I alone and in combination with either 1 μM IGF-IR inhibitor tyrphostin AG1024 (AG), or 1 μM MEK inhibitor PD98059 (PD) or 1 μM p38 inhibitor SB202190 (SB). Side panels show densitometric analysis of the blots normalized to β-actin, ERK2 and p38 that served as loading controls respectively for pIGF-IR, pERK and p-p38. Data shown are the mean ± SD of three independent experiments. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

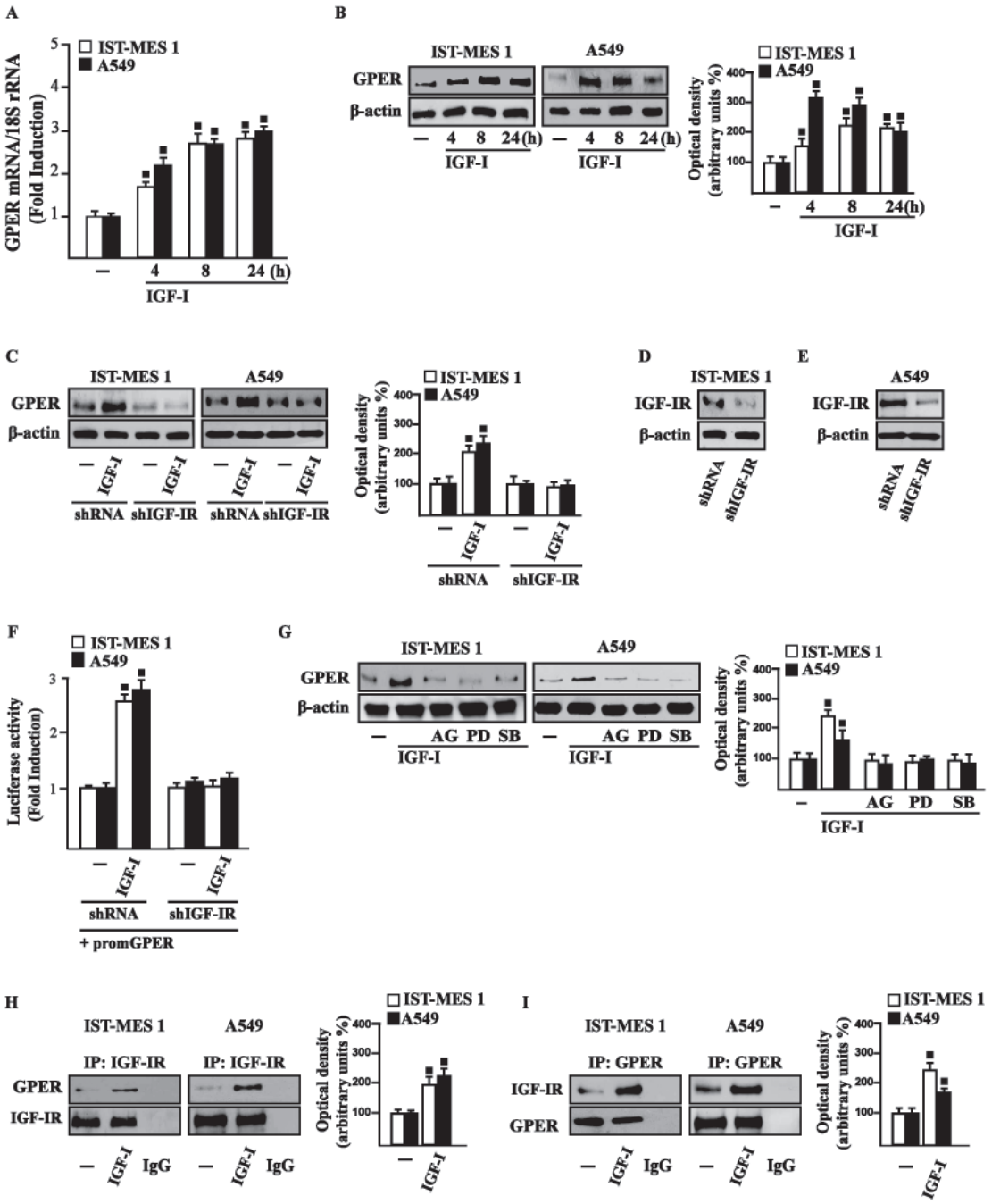

Figure 2: IGF-I up-regulates GPER expression in IST-MES 1 and A549 cells. A. mRNA expression of GPER in cells treated with either vehicle (-) or 100 ng/ml IGF-I, as evaluated by real-time PCR. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. **B.** GPER protein levels were evaluated by immunoblotting in cells treated with either vehicle (-) or 100 ng/ml IGF-I, as indicated. **C.** GPER protein expression in cells transfected for 24 h with either shRNA or shIGF-IR and then treated for 8 h with vehicle (-) or 100 ng/ml IGF-I. **D.-E.** Efficacy of IGF-IR silencing. **F.** Cells were transfected for 24 h with shRNA or shIGF-IR together with the GPER promoter construct. Then, cells were treated for 18 h with vehicle (-) or 100 ng/ml IGF-I. The luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (-) were set as one fold induction upon which the activity induced by treatments was calculated. **G.** GPER protein levels in cells treated for 8 h with vehicle (-) or 100 ng/ml IGF-I alone or in combination with 1 μM IGF-IR inhibitor tyrphostin AG1024 (AG), 1 μM MEK inhibitor PD98059 (PD) and 1 μM p38 inhibitor SB202190 (SB). Side panels show densitometric analysis of the blots normalized to β-actin. **H.-I.** Co-immunoprecipitation studies performed in cells treated for 8 h with vehicle (-) or 100 ng/ml IGF-I, as indicated. In control samples, non-specific IgG was used instead of the primary antibody. **H.** Side panel show densitometric analysis of the blot normalized to IGF-IR. **I.** Side panel show densitometric analysis of the blot normalized to GPER. Data shown are the mean ± SD of three independent experiments. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

IGF-I triggers the expression of GPER target genes

In our previous study [33] we established that GPER mediates a specific gene signature, therefore, we evaluated whether, in IST-MES1 and A549 cells, IGF-I is able to affect the expression of certain GPER target genes like CTGF and EGR1, which have been involved in fibrotic responses in mesothelioma and lung cancer cells [34-36]. Indeed, in time-course experiments we found that

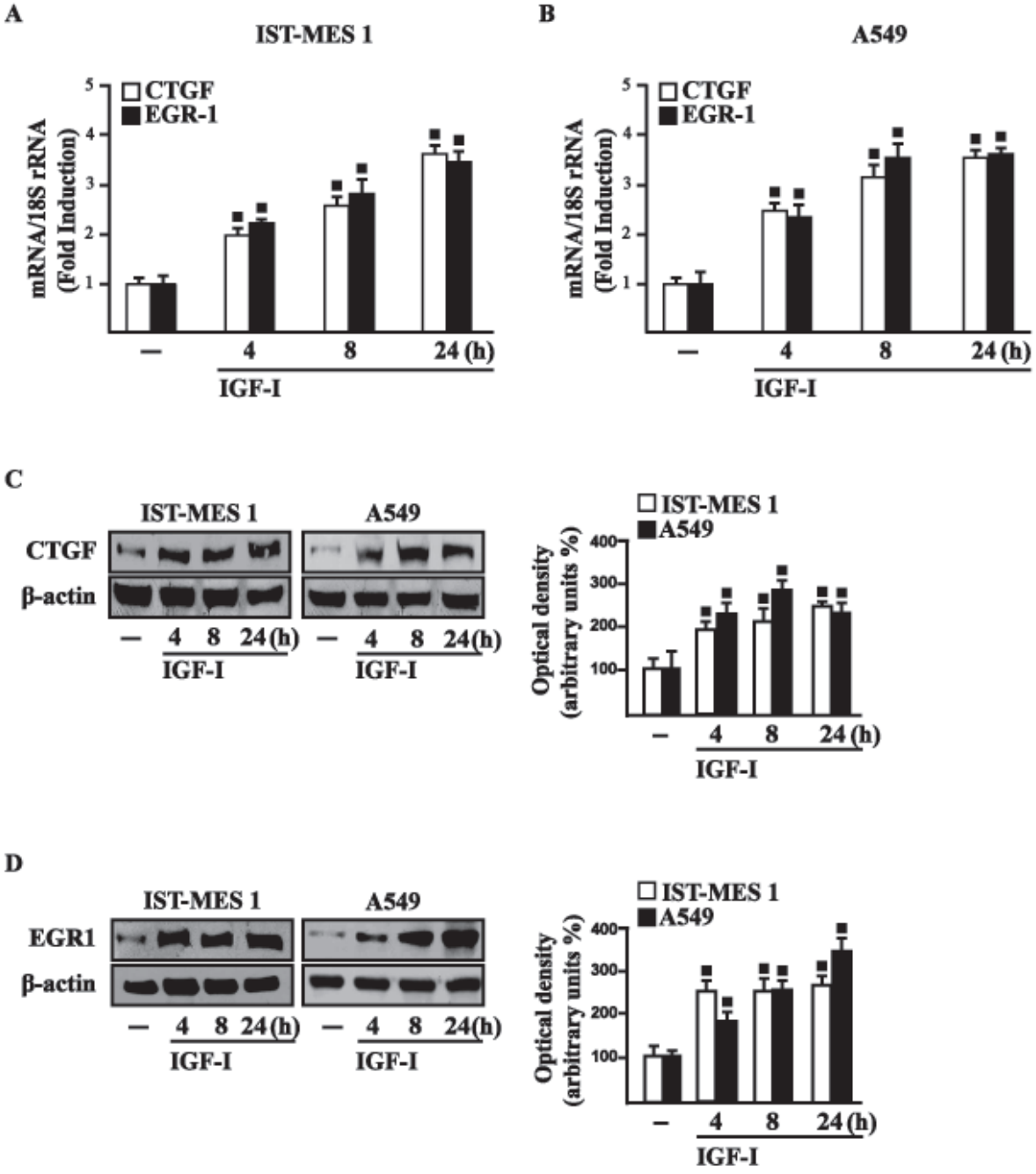

Figure 3: IGF-I up-regulates CTGF and EGR1 expression in IST-MES 1 and A549 cells. (A-B) mRNA expression of CTGF and EGR1 in cells treated with either vehicle (-) or 100 ng/ml IGF-I, as evaluated by real-time PCR. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. CTGF **C.** and EGR1 **D.** protein levels were evaluated by immunoblotting in cells treated with vehicle (-) or 100 ng/ml IGF-I, as indicated. Side panels show densitometric analysis of the blots normalized to β-actin and each data point represents the mean ± SD of three independent experiments. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

IGF-I increases the mRNA (Figure 3A-3B) and protein levels (Figure 3C-3D) of both CTGF and EGR1. Next, we determined that this action of IGF-I involves not only the IGF-IR but also GPER, as the silencing of each of these receptors prevented gene changes (Figure 4A-4H). In accordance with these observations, the IGF-I transactivation of CTGF (Figure 4I) and EGR1 (Figure 4J) promoters required both IGF-IR and GPER, as demonstrated by knocking down the expression of these receptors. As c-fos plays a main role in the up-regulation of GPER target genes [33, 37], we next determined that the promoter transactivation of both CTGF and EGR1 is abrogated by co-transfecting a dominant-negative form of c-fos (DN/c-fos) in IST-MES1 and A549 cells (Figure 4K). Collectively, these findings provide novel mechanisms through which IGF-I/IGF-IR transduction signaling regulates GPER target genes like CTGF and EGR1 in mesothelioma and lung cancer cells.

IGF-IR and GPER are both involved in IGF-I regulation of DDR1 target genes

Considering that in diverse model systems IGF-I stimulates the synthesis of collagen [38-40], we next established that IGF-I regulates in both IST-MES1 and A549 cells the mRNA expression of COL1A1 (Figure 5A) that encodes the major component of type I collagen [41]. We previously reported that IGF-IR functionally interacts with DDR1, which is activated by various collagen types including type I collagen. Therefore, we first ascertained that, in both IST-MES1 and A549 cells, several DDR1 target genes such as matrilin-2 (MATN-2), fibrillin-1 (FBN-1), NOTCH 1 and HES-1, are induced by the DDR1 agonist COL1 (Figure 5B-5C) and abrogated by the DDR1 inhibitor (DDR1 IN) (Figure 5D-5E). Then, we assessed that these DDR1 target genes are also stimulated by IGF-I (Figure 6A-6B) and that this response was inhibited by DDR1 IN (Figure 6C-6D) as well as by silencing IGF-IR (Figure 6E-6F) or GPER (Figure 6G-6H). In accordance with these findings, we determined that the NOTCH 1 protein induction by COL1 and IGF-I is prevented in the presence of the DDR1 IN in IST-MES1 and A549 cells (Figure 7). Accordingly, IGF-I was not able to trigger NOTCH 1 protein expression when IGF-IR (Figure 8A-8C) or GPER (Figure 8D-8F) were silenced. Altogether, these results indicate that, in both mesothelioma and lung cancer cells, IGF-I may up-regulate DDR1 target genes, and this action involves not only IGF-IR but also a cross-talk with GPER.

DDR1, IGF-IR and GPER contribute to the chemotaxis and migration of mesothelioma and lung cancer cells

Previous studies have reported that IGF-I stimulates chemotactic and chemokinetic motility in mesothelioma cells [32]. Moreover, DDR1 also plays an important role in promoting cell-cell interactions and cell migration in various cell contexts [42-45]. Further extending these data, in IST-MES1 cells, we found that both IGF-I and COL1 induce chemotactic motility, which requires DDR1, as these responses were abolished by DDR1 IN (Videos 1-6). Moreover, we ascertained that the chemotactic motility induced by IGF-I requires also IGF-IR and GPER as the aforementioned effect was prevented silencing the expression of these receptors (Videos 7-12). Similar findings occurred in A549 cells (data not shown). Likewise, we determined that IST-MES1 and A549 cell migration induced by both IGF-I and COL1 is abolished using DDR1 IN (Figure 9A), whereas the silencing of IGF-IR or GPER abolished cell migration triggered by IGF-I, as determined by Boyden chamber assay (Figure 9B). Collectively, our data indicate novel cross-talk and biological functions exerted by IGF-I toward tumor progression.

DISCUSSION

In the present study we provide novel evidence regarding the molecular mechanisms by which IGF-I triggers biological responses in mesothelioma and lung cancer cells. In particular, we show a complex functional cooperation involving IGF-IR, GPER and DDR1 through which IGF-I up-regulates first the expression of COL1A1 and certain DDR1 target genes, thereafter stimulating cancer cell motility and chemotactic response (Figure 10).

Lung cancer is a highly heterogeneous tumor that can arise in different sites of the bronchial tree [1-2]. The incidence of lung cancer depends on toxic effects of inhaled substances such as tobacco, asbestos, arsenic, cadmium, nickel and silica [46]. The environmental pollutant asbestos is also considered the main cause of the insurgence of malignant mesothelioma (MM), which is a rare and aggressive tumor that springs from mesothelial cells lining lung, pleura or peritoneum [5-7, 47-48]. The deposition of asbestos fibers has been also related to chronic inflammatory processes as well as to pulmonary fibrosis, which in turn may create a favorable environment for the development of lung and pleura malignancies [6, 49]. As it concerns the multifaceted mechanisms and factors involved in pulmonary fibrosis and neoplasia, an increased expression and activation of DDR1 have been reported [50-53]. To date, DDR1 has been shown to play an important role in cancer progression by regulating the interactions of tumor cells with the surrounding

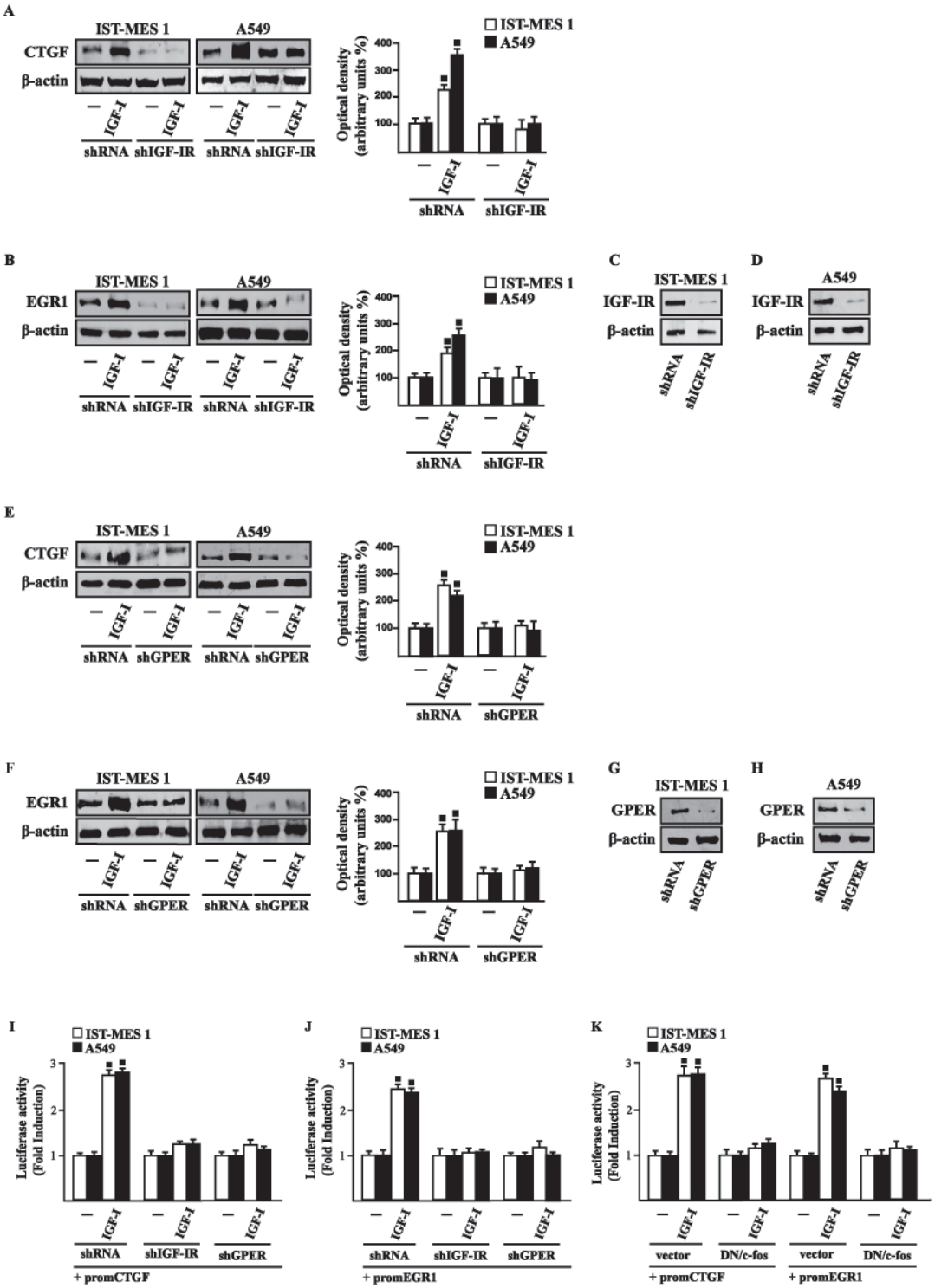

Figure 4: IGF-IR and GPER mediate CTGF and EGR1 stimulation by IGF-I in IST-MES 1 and A549 cells. A.-F. CTGF and EGR1 protein levels in cells transfected for 24 h with shRNA, shIGF-IR or shGPER and then treated for 8 h with either vehicle (-) or 100 ng/ml IGF-I. Efficacy of IGF-IR **C.-D.** and GPER **G.-H.** silencing. Side panels show densitometric analysis of the blots normalized to β-actin. **I.-J.** Cells were transfected for 24 h with shRNA, shIGF-IR or shGPER together with the CTGF or EGR1 promoter construct. Then, cells were treated for 18 h with vehicle (-) or 100 ng/ml IGF-I. **K.** Cells were transfected for 24 h with a dominant negative form of c-fos (DN/c-fos) together with the CTGF or EGR1 promoter construct. Then, cells were treated for 18 h with vehicle (-) or 100 ng/ml IGF-I. The luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (-) were set as one fold induction upon which the activity induced by treatments was calculated. Data shown are the mean ± SD of three independent experiments. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

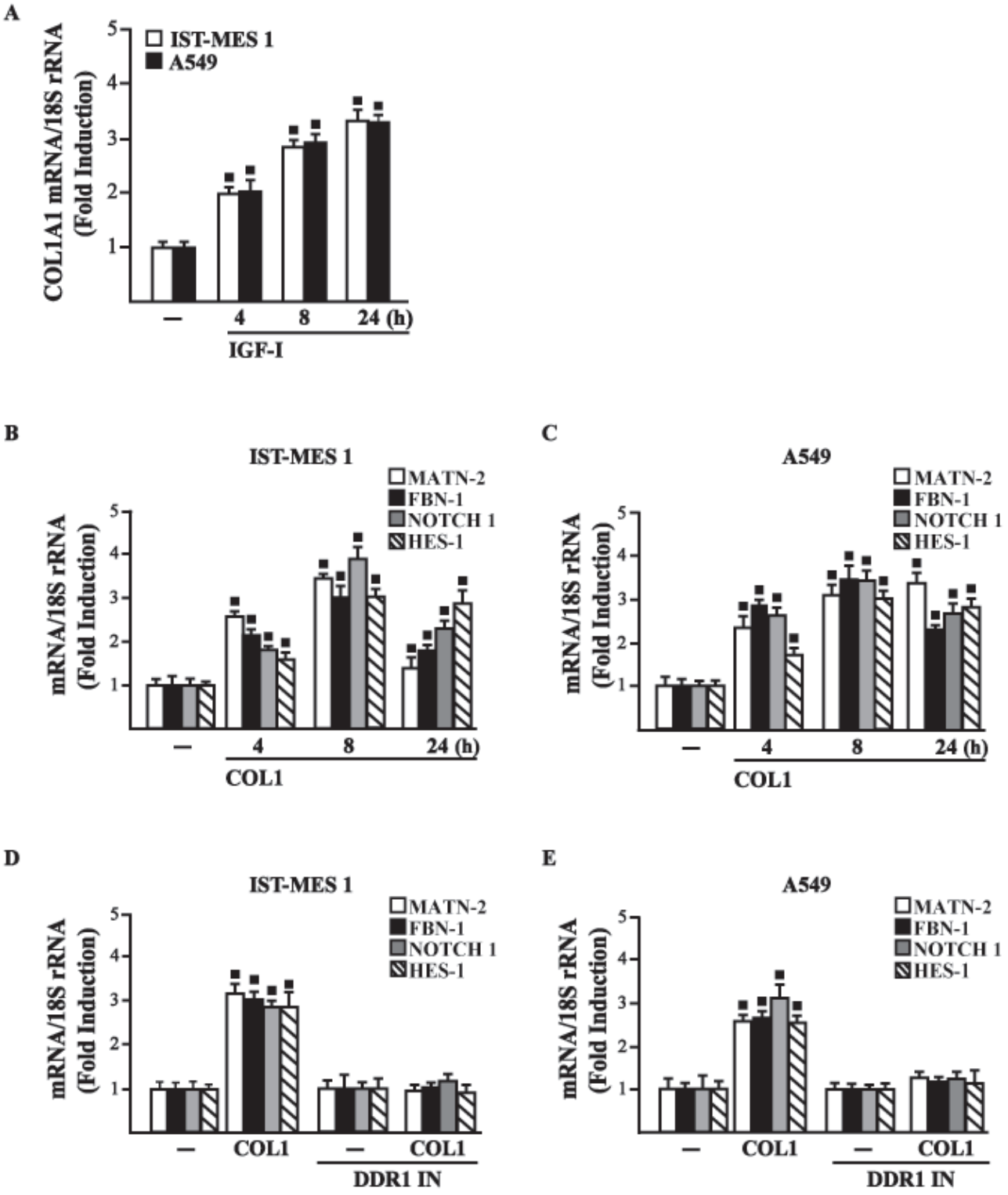

Figure 5: A. mRNA expression of COL1A1 in IST-MES 1 and A549 cells treated with vehicle (-) or 100 ng/ml IGF-I, as evaluated by real-time PCR. mRNA expression of MATN-2, FBN-1, NOTCH 1 and HES-1 in IST-MES 1 **B.**, **D.** and A549 **C.**, **E.** cells treated with vehicle (-) or 10 μg/ml COL1 alone or in combination with 1 μM DDR1 inhibitor (DDR1 IN), as indicated. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

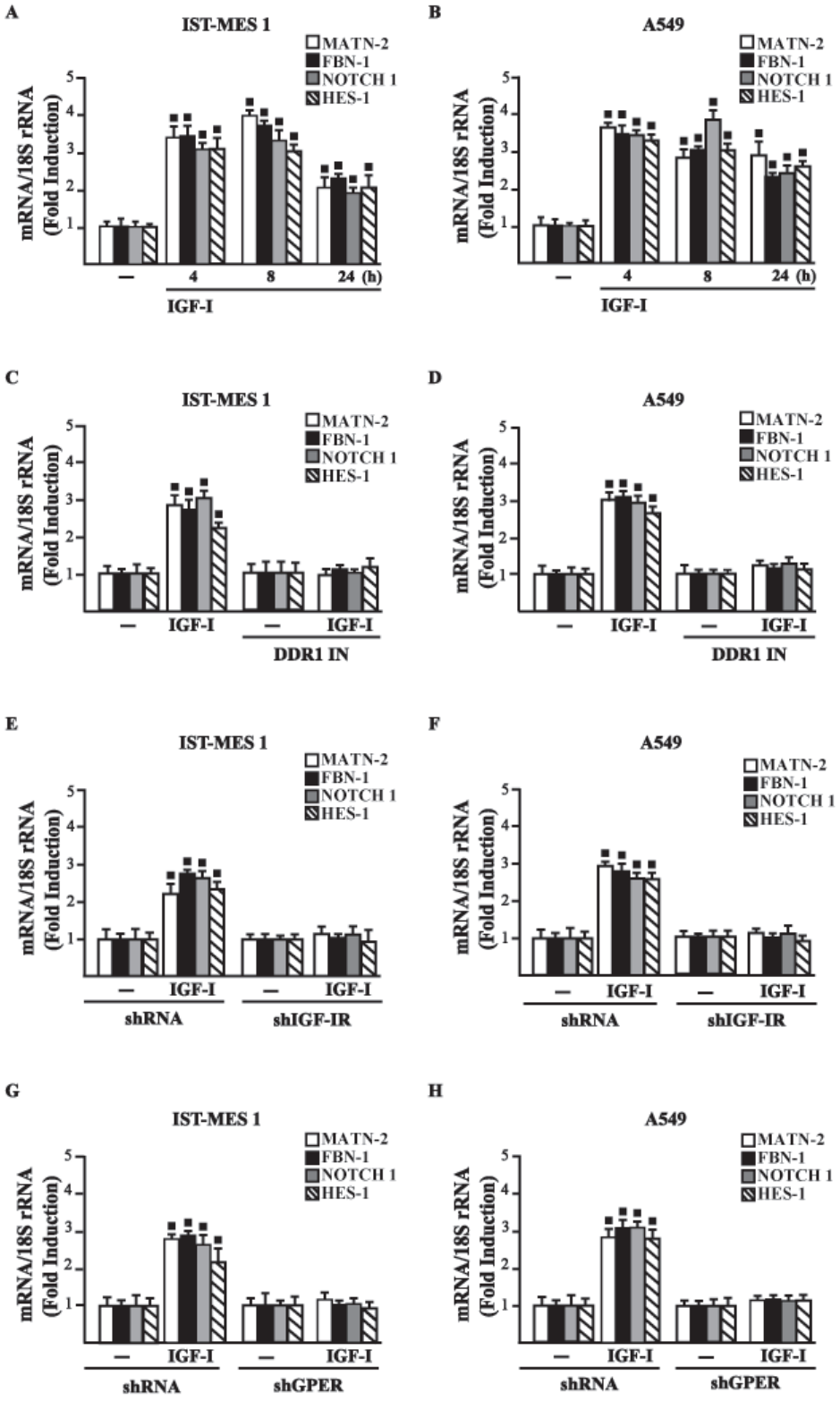

Figure 6: IGF-IR and GPER mediate the IGF-I induced up-regulation of COL1A1/DDR1 target genes in IST-MES 1 and A549 cells. A.-D. mRNA expression of MATN-2, FBN-1, NOTCH 1 and HES-1 in cells treated with vehicle (-) or 100 ng/ml IGF-I alone or in combination with 1 μM DDR1 inhibitor (DDR1 IN), as indicated. **E.-H.** mRNA expression of MATN-2, FBN-1, NOTCH 1 and HES-1 in cells transfected for 24 h with shRNA, shIGF-IR or shGPER and then treated for 8 h with vehicle (-) or 100 ng/ml IGF-I. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

collagen matrix, therefore leading to pro-migratory and pro-invasive responses [21]. Furthermore, collagen activated DDR1 triggers diverse pro-survival pathways toward anti-apoptotic, proliferative and aggressive features in cancer cells [21]. In this regard, it should be noted that several types of collagen are able to bind to and activate DDR1, which then regulates cell and tissue homeostasis acting as a collagen sensor [21, 54]. Of note, an abnormal expression and deposition of collagen has been associated with cancer development [55-56]. As it concerns the synthesis and extracellular accumulation of diverse types of collagen, cytokines and growth factors like IGF-I, the epidermal growth factor (EGF) and the transforming growth factor-βl have been reported to promote these effects [38-40, 57]. Notably, we previously showed that, in breast cancer cells, IGF-I may upregulate DDR1 expression through a signaling pathway involving the DDR1 regulatory miR-199a-5p [12]. Moreover, the activation of one of the main IGF-I transduction signaling, the IGF-IR/PI3K/Akt cascade, inhibits miR-199a-5p expression, thus relieving its inhibition upon DDR1 gene and allowing DDR1 upregulation. In turn, DDR1 increases IGF-IR expression through post-transcriptional mechanisms and amplifies IGF-I downstream signaling and biological effects, such as proliferation, migration and colony formation [12]. Indeed, we previously showed that DDR1 directly interacts with IGF-IR, and that this interaction is enhanced by IGF-I stimulation, which promotes rapid DDR1 tyrosine-phosphorylation and co-internalization of the DDR1 - IGF-IR complex [22]. This interaction was shown to occur in a panel of human breast cancer cells as well as in mouse fibroblasts (R- cells) co-transfected with the human IGF-IR and DDR1, indicating that it is not cell-specific. Notably, the formation of this DDR1 - IGF-IR complex did not require the presence of collagen, the canonical DDR1 ligand. In addition, the critical role of IGF-IR in DDR1 activation and biological actions is supported by the finding that collagen-dependent DDR1 phosphorylation was impaired in the absence of IGF-IR [22].

Extending these previous studies, we now show that IGF-I through the cognate receptor IGF-IR is able to induce COL1A1 expression [54]. Moreover, a panel of DDR1 target genes could be also induced by IGF-I through the previously described functional cross-talk involving IGF-IR and DDR1. Taken together, these findings show that DDR1, besides enhancing the activation of typical IGF-IR downstream cascades, the PI3K/Akt and the ERK1/2 cascades, following cell exposure to IGF-I, modifies significantly these IGF-I effects by allowing the induction of typical DDR1 target genes. These effects confirm the relevance of DDR1 in the amplification and diversification of IGF-I signaling pathways in cancer. We have previously demonstrated that IGF-IR may also functionally interact with the non-canonical estrogen receptor GPER. Indeed, through the IGF-IR/PKCδ/ERK/c-fos/AP1 transduction pathway, IGF-I up-regulates GPER, which plays an important role in sustaining proliferation and migration in response to IGF-I in breast and endometrial human cancer cells [25]. In close accordance with these findings, we now show that the functional cooperation between IGF-IR and DDR1 also requires GPER, and that both DDR1 and GPER are critical to the chemotactic motility stimulated by IGF-I in mesothelioma and lung cancer cells. Notably, we now show that GPER and IGF-IR co-immunoprecipitate in lung and mesothelioma cells (Figure 2), indicating that GPER and IGF-IR also interact. Taken together all these data strongly suggest the possible formation of a ternary functional complex involving IGF-IR - DDR1 - GPER. However, further studies are needed to fully elucidate this aspect. These data may be of a particular interest as GPER expression has been associated with negative clinical features and poor survival rates in diverse types of malignancies [58-61]. In the last years, extensive studies were therefore performed in order to better characterize the role of GPER in cancer development, including the mechanisms and factors involved in its expression. For instance, we determined that EGF and IGF-I, insulin and further tumorigenic factors like hypoxia and endothelin-1 up-regulate GPER expression in diverse cancer cell contexts [25, 62-68].

Our present findings provide significant new insights on the well-established role played by the IGF axis in cancer [9-11, 14-16, 20, 23, 69-71] that involves also the interaction of IGF-IR with other RTKs and GPCRs in diverse tumor histotypes [19, 23, 72-73]. In particular, our findings might be relevant in devising new therapeutical strategies in cancers with a dysregulated IGF system. In the last decade, much effort has been made in targeting the IGF-IR in these malignancies [74]. In particular, both small-molecule IGF-IR tyrosine kinase inhibitors, and humanized monoclonal antibodies with blocking activity to the IGF-IR, have been investigated in Phase III trials of advanced non-small cell lung cancers [13]. Unfortunately, in spite of very promising preclinical studies, clinical trials have clearly indicated that only a small minority of malignancies do respond to target therapies when IGF-IR is the sole target [75], because the frequent occurrence of resistance mechanisms arising by the complex signaling network involving the IGF-IR [76].

Overall, on the basis of our data the multifaceted signaling network between IGF-IR, GPER and DDR1 could be taken into account in setting innovative combined strategies targeting these pathways in mesothelioma and lung cancers.

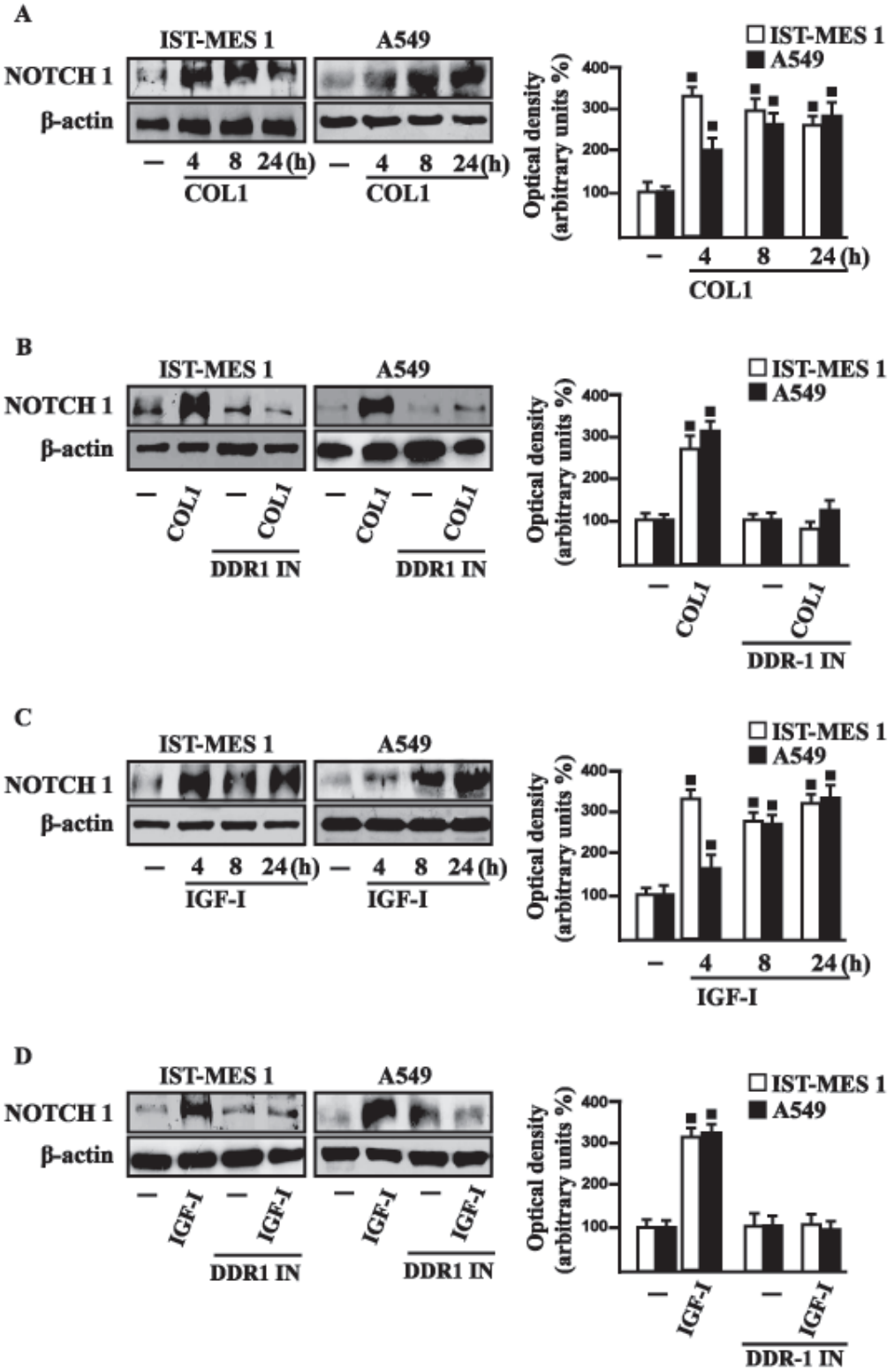

Figure 7: COL1 and IGF-I stimulate NOTCH 1 expression through DDR1 in IST-MES 1 and A549 cells. A. NOTCH 1 protein levels in cells treated with vehicle (-) or 10 μg/ml COL1, as indicated. **B.** NOTCH 1 protein levels in cells treated for 8 h with vehicle (-) or 10 μg/ml COL1 alone and in combination with 1 μM DDR1 inhibitor (DDR1 IN). **C.** NOTCH 1 protein levels in cells treated with vehicle (-) or 100 ng/ml IGF-I, as indicated. **D.** NOTCH 1 protein levels in cells treated for 8 h with vehicle (-) or 100 ng/ml IGF-I alone and in combination with 1 μM DDR1 inhibitor (DDR1 IN). Side panels show densitometric analysis of the blots normalized to β-actin and each data point represents the mean ± SD of three independent experiments. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

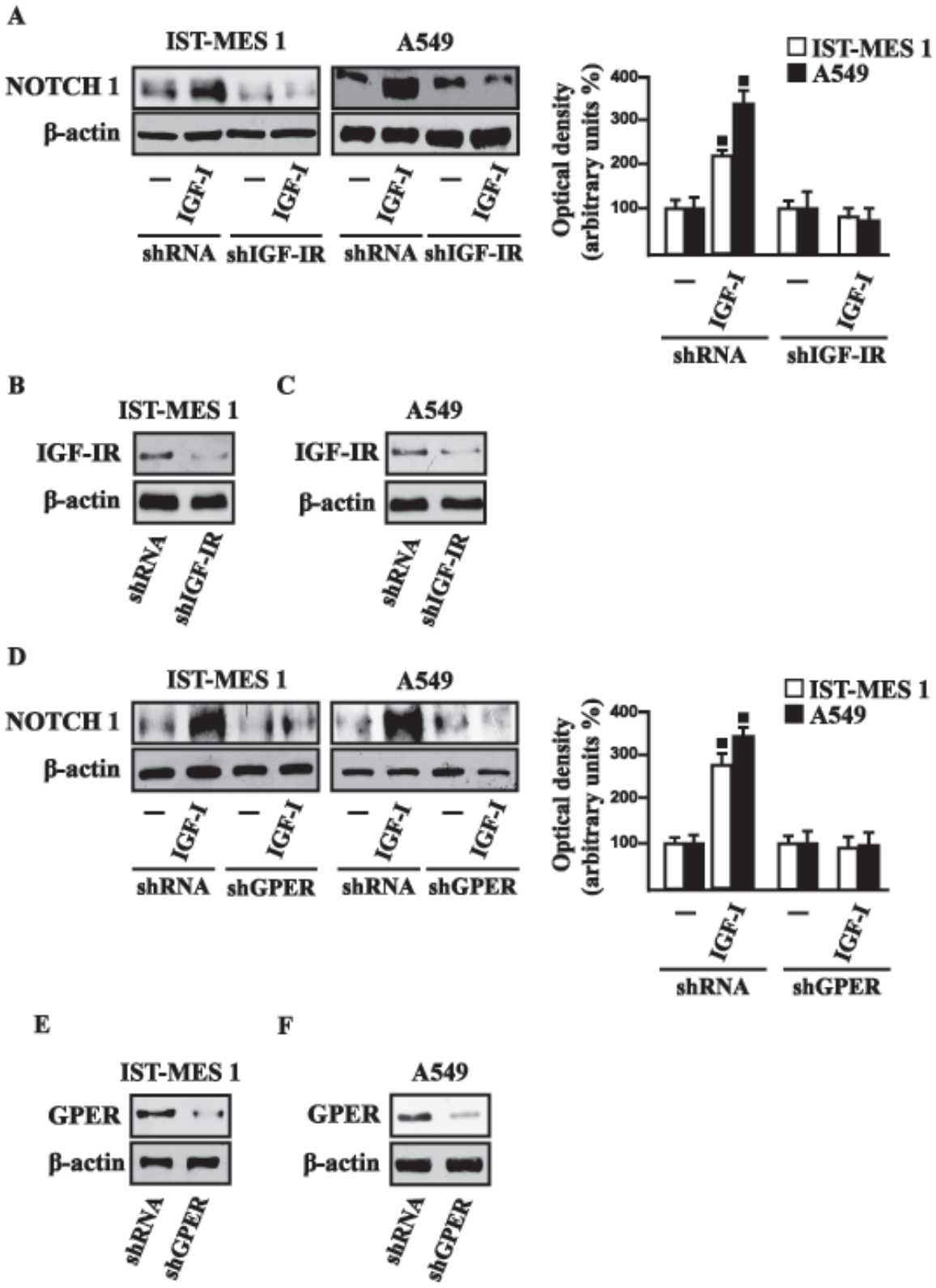

Figure 8: IGF-IR and GPER mediate the IGF-I induced up-regulation of NOTCH 1 in IST-MES 1 and A549 cells. NOTCH 1 protein levels in cells transfected for 24 h with shIGF-IR **A.** or shGPER **D.** and then treated for 8 h with vehicle (-) or 100 ng/ml IGF-I. Efficacy of IGF-IR **B.-C.** and GPER **E.-F.** silencing. Side panels show densitometric analysis of the blots normalized to β-actin. (■) $p < 0.05$ for cells receiving vehicle (-) *versus* treatments.

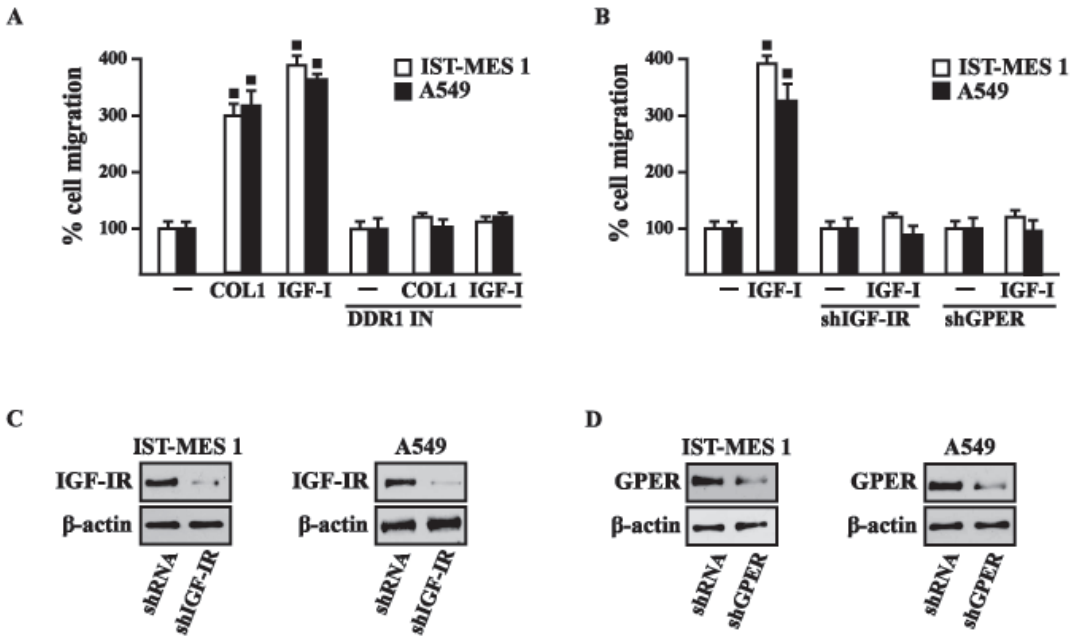

Figure 9: COL1 and IGF-I stimulate IST-MES 1 and A549 cell migration through DDR1, IGF-IR and GPER. A. The migration of IST-MES 1 and A549 cells upon 8 h treatment with vehicle (-), 10 μg/ml COL1 or 100 ng/ml IGF-I alone and in combination with 1 μM DDR1 inhibitor (DDR1 IN), as evaluated by Boyden Chamber assay. **B.** The migration of IST-MES 1 and A549 cells induced by 8 h treatment with 100 ng/ml IGF-I was prevented knocking down IGF-IR and GPER expression, as evaluated by Boyden Chamber assay. Efficacy of IGF-IR **C.-D.** and GPER **E.-F.** silencing. Values represent the mean ± SD of three independent experiments. (●) indicates $p < 0.05$ for cells treated with vehicle (-) *versus* treatments.

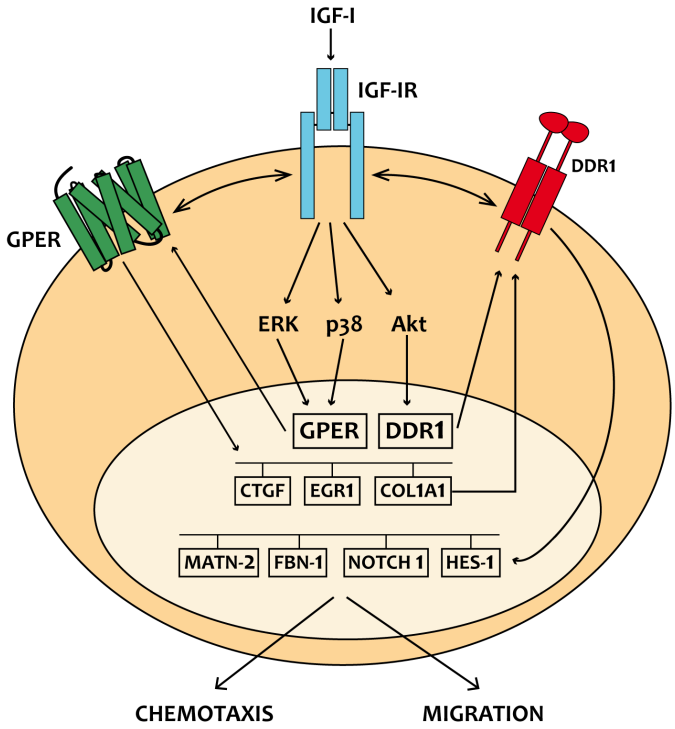

Figure 10: Schematic representation of the signaling network between IGF-IR, GPER and DDR1 activated by IGF-I. IGF-I stimulates the expression of GPER and its target genes, then IGF-IR and GPER trigger the IGF-I regulation of DDR1 target genes. The functional cross-talk of IGF-IR, GPER and DDR1 contributes to the chemotaxis and migration observed in cancer cells.

MATERIALS AND METHODS

Reagents

IGF-I, SB202190 (SB) and collagen I from rat tail were obtained from Sigma-Aldrich Inc. (Milan, Italy). PD98059 (PD) and 3-bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024) were purchased from Calbiochem (DBA, Milan, Italy). All compounds were solubilized in dimethylsulfoxide, except PD and IGF-I, which were dissolved in ethanol and in water, respectively. DDR1IN1 dihydrochloride (DDR-1 in) was purchased from Tocris Bioscience (Space, Milan, Italy).

Cell cultures

IST-MES1 malignant mesothelioma cells were kindly provided by Dr. Orengo (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Cells were previously characterized [77] and were grown in Nutrient Mixture F-10 Ham (Ham's F-10) medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin. A549 lung cancer cells were obtained by ATCC, used < 6 months after resuscitation and maintained in DMEM/F12 (Dulbecco's modified Eagle's medium) supplemented with phenol red 10% FBS and 100 μg/ml penicillin/streptomycin. All cell lines were cultured at 37°C in 5% CO_2 and switched to medium without serum the day before immunoblots and reverse transcription-PCR experiments.

Plasmids and luciferase assays

The GPER luciferase expression vector (promGPER) was previously described [65]. The CTGF luciferase reporter plasmid (promCTGF) (-1999/+ 36)-luc was a gift from Dr. Chaqour. EGR1-luc plasmid, containing the -600 to +12 5'-flanking sequence from the human EGR1 gene, was kindly provided by Dr. Safe (Texas A&M University). The plasmid DN/cfos, which encodes a c-fos mutant that heterodimerizes with c-fos dimerization partners but does not allow DNA biding [78], was a kind gift from Dr C Vinson (NIH, Bethesda, MD, USA). The Renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as internal transfection control. Cells ($1x10^5$) were plated into 24-well dishes with 500 μl/well culture medium containing 10% FBS. Transfection were performed using X-treme GENE 9 DNA transfection reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy), with a mixture containing 0.5 μg of reporter plasmid and 10 ng of pRL-TK. After 24 h, treatments were added and cells were incubated for 18 h. Luciferase activity was measured using the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity. 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 silencing experiments

Cells were plated onto 10-cm dishes and transfected by X-treme GENE 9 DNA Transfection Reagent for 24 h before treatments with a control vector, a specific shRNA sequence for each target gene. The shIGF-IR and the respective control plasmids (shRNA) were purchased from SA Bioscience Corp. (Frederick, MD, USA) and used according to the manufacturer's recommendations. The short hairpin (sh)RNA constructs to knock down the expression of GPER and the unrelated shRNA control construct have been described previously [64].

Gene expression studies

Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described [79-80]. The expression of selected genes was quantified by real-time PCR using Step One sequence detection system (Applied Biosystems, Milan, Italy). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc. Milan, Italy) and are as follows: GPER Fwd 5′- ACACACCTGGGTGGACACAA-3′ and Rev 5′-GGAGCCAGAAGCCACATCTG-3'; HES-1 Fwd 5′-TCAACACGACACCGGATAAA-3′ and Rev 5′-CCGCGAGCTATCTTTCTTCA-3′; NOTCH 1 Fwd 5′-AATGGCGGGAAGTGTGAAGC-3′ and Rev 5′-GCATAGTCTGCCACGCCTCT-3′; MTN-2 Fwd 5′-CTCCGAGTGGGCCAGTAAAG-3′ and Rev 5′- CTGGCTCAGATTCTGTTGGCT-3′; FBN-1 Fwd 5′-GCCGCATATCTCCTGACCTC-3′ and Rev 5′-GTCGATACACGCGGAGATGT-3′; 18S Fwd 5′- GGCGTCCCCCAACTTCTTA-3′ and Rev 5′-GGGCATCACAGACCTGTTATT-3′. Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression.

Western blot analysis

Cells were processed according to a previously described protocol [81] to obtain protein lysate that was electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel, electroblotted onto a nitrocellulose membrane and probed with primary antibodies against antiphosphotyrosine antibody (4G10) (Merck Millipore,

Milan, Italy), IGF-IR (7G11), GPER (N-15), CTGF (L-20), phosphorylated ERK1/2 (E-4), ERK2 (C-14), NOTCH 1 (C-20), EGR1 (588), phosphorylated p-38 (D-8), p-38 (A-12), β-actin (C2), (Santa Cruz Biotechnology, DBA, Milan, Italy). Proteins were detected by horseradish peroxidase-linked secondary antibodies (DBA, Milan, Italy) and revealed using the ECL System (GE Healthcare).

Co-immunoprecipitation

Cells were lysed using 200 µl RIPA buffer with a mixture of protease inhibitors containing 1.7mg/ml aprotinin, 1mg/ml leupeptin, 200mmol/L phenylmethylsulfonyl fluoride, 200mmol/L sodium orthovanadate, and 100mmol/L sodium fluoride. A total of 100 µg proteins were incubated for 2 h with 2 µg of the appropriate antibody (GPER, N-15; IGF-1R, 7G11) and 20 µl of protein A/G agarose immunopreciptation reagent (Santa Cruz Biotechnology, DBA, Milan, Italy). Samples were centrifuged at 13,000 rpm for 5 min at 4°C to pellet beads. After four washes in PBS, samples were resuspended in RIPA buffer with protease inhibitors and SDS sample buffer. Western Blot analysis was performed as described above.

Migration assay

Migration assays were performed using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane, Sigma Aldrich, Milan, Italy). Cells were transfected in regular growth medium. After 8 h, cells were trypsinized and seeded in the upper chambers. Treatments were added to the medium without serum in the bottom wells where applicable, cells on the bottom side of the membrane were fixed and counted 8 hours after seeding.

Time-lapse microscopy

Cells (1×10^5) were seeded in 6-well plates and maintained in regular growth medium for 24 h. For knockdown experiments, cells were transfected for 24 h with shRNA constructs directed against IGF-IR or GPER and with an unrelated shRNA construct. Thereafter, cells were treated and transferred into a time-lapse microscopy platform, equipped with a heated stage chamber (Cytation™3 Cell Imaging Multi-Mode Reader, Biotek, Winooski, VT). Cells were maintained at routine incubation settings (37 °C, 5% CO_2) using temperature and gas controllers. To evaluate chemotaxis the images were recorded using Cytation 3 Cell Imaging Multimode Reader and the software Gen5 (BioTek, Winooski, VT) in 10 min intervals for 8 hours. Then, the images were processed as a movie using the software Adobe Creative Cloud Premier Pro CC. Frames collected every 10 minutes are displayed at a rate of 10 frames s^{-1}.

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.

GRANT SUPPORT

This work was supported by Associazione Italiana per la Ricerca sul Cancro (MM: IG 16719/2015; AB: IG 14066/2013), Ministero della Salute (grant n. 67/GR-2010-2319511); SA was supported by Fellowships INAIL-Regione Calabria; EMDF was supported by an iCARE fellowship from the Associazione Italiana per la Ricerca sul Cancro (AIRC) cofunded by Marie Curie Actions.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Cellular Signalling 28 (2016) 61–71

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The G protein estrogen receptor (GPER) is regulated by endothelin-1 mediated signaling in cancer cells

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ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form 18 November 2015
Accepted 18 November 2015
Available online 19 November 2015

Keywords:
Endothelin-1
GPER
Cancer cells
Migration
Signal transduction

ABSTRACT

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor involved in many diseases, including certain cardiovascular disorders and cancer. As previous studies have shown that the G protein estrogen receptor (GPER) may regulate ET-1 dependent effects on the vascular system, we evaluated whether GPER could contribute to the effects elicited by ET-1 in breast cancer and hepatocarcinoma cells. Here, we demonstrate that ET-1 increases GPER expression through endothelin receptor A (ET_AR) and endothelin receptor B (ET_BR) along with the activation of PI3K/ERK/c-Fos/AP1 transduction pathway. In addition, we show that GPER is involved in important biological responses observed upon ET-1 exposure, as the migration of the aforementioned tumor cells and the formation of tube-like structures in human umbilical vein endothelial cells (HUVECs). Our data suggest that GPER may contribute to ET-1 action toward the progression of some types of tumor.

1. Introduction

The endothelin (ET) family includes three small peptides named ET-1, ET-2 and ET-3 which act in both autocrine and paracrine manner *via* the G protein-coupled receptor namely ET_AR and ET_BR [1]. ETs by binding to the cognate receptors activate diverse transduction pathways like the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), the mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK), the phosphokinase C (PKC) and the epidermal growth factor receptor (EGFR) [2]. ET-1 is the predominant family's isoform and a potent endogenous vasoconstrictor involved in different pathophysiological responses [1]. ET-1 is mainly produced by somatic cells like endothelial cells, vascular smooth muscle cells, macrophages, fibroblasts and various types of cancer cells [1,2]. Numerous studies have shown that ET-1 is not merely a vasoconstrictor but a multifunctional peptide exerting a cytokine-like activity in diverse conditions like inflammation, pain and cancer [1]. As it concerns cancer, ET-1 elicits pleiotropic effects on tumor cells and the host microenvironment toward cell proliferation, apoptosis, migration, epithelial mesenchymal transition, chemo-resistance and neovascularization [3]. ET-1 was found overexpressed in a variety of tumors like prostatic, ovarian, lung, colorectal and hepatic malignancies [2] in particular, its expression was associated with aggressive features of breast tumors [4]. It was also reported that increased levels of ET-1 and cognate receptors positively correlate with vascular endothelial growth factor (VEGF) signaling as well as microvessel density [5]. Indeed, ET-1 can induce tumor angiogenesis up-regulating VEGF and triggering stimulatory effects in endothelial cells [3].

It has been recently reported that ET-1-dependent vasoconstriction is inhibited by G protein estrogen receptor (GPER) agonists as G-1, raloxifene, fulvestrant and genistein [6–8]. Accordingly, G-1 failed to repress ET-1 mediated vasoconstriction in GPER deficient mice [9]. In this context, it should be mentioned that GPER is expressed throughout the cardiovascular system of humans and animals of both sexes and mediates beneficial effects of estrogens on vascular and myocardial function [7]. GPER activates a network of transduction pathways like EGFR, MAPK, PI3K, intracellular cyclic AMP (cAMP) and calcium mobilization, leading to a characteristic gene signature involved in relevant pathophysiological responses including cancer [10]. The function of GPER in tumorigenesis is still a subject of intense debate. Previous studies have revealed that GPER may induce cell-cycle arrest and the inhibition of cancer cell growth [11,12]. Moreover, further studies have shown that high GPER expression is favorable for the survival of breast and ovarian cancer patients [13,14]. On the contrary, GPER has been reported to trigger diverse pathways toward the expression of genes stimulating tumor cell migration and proliferation both *in vitro* and *in vivo* [10,15]. In patients with endometrial and ovarian tumors, the expression of GPER was also associated with aggressive features and lower survival rates [16,17]. Likewise, increased tumor size and metastasis in human breast

Abbreviations: ET-1, endothelin-1; ET_AR, endothelin receptor A; ET_BR, endothelin receptor B; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; CTGF, connective tissue growth factor; Egr-1, early related gene; HIF-1, hypoxia inducible factor-1; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.cellsig.2015.11.010
0898-6568/

malignancies correlated with high levels of GPER expression [18]. In addition, in patients treated with tamoxifen GPER was found increased and negatively correlated with relapse-free survival [19]. The overexpression of GPER and its localization to the plasma membrane were suggested to be critical toward the progression of breast cancer, whereas the absence of GPER in the plasma membrane predicted excellent long-term prognosis in breast cancer patients treated with tamoxifen [20]. On the basis of these observations, an increasing interest is currently addressed toward a better understanding of the mechanisms involved in the regulation of GPER expression and function. In this regard, our previous studies have demonstrated that GPER is upregulated by epidermal growth factor (EGF) [10], insulin growth factor (IGF)-I [21,22], insulin [23] and a main factor contributing to tumor aggressiveness like hypoxia [24]. Moreover, estrogenic GPER signaling was shown to induce hypoxia-inducible factor (HIF)1α-dependent expression of VEGF, which then triggered angiogenesis in different breast cancer models [25,26].

As previous investigations evaluated the functional interaction between GPER and ET-1 signaling in the cardiovascular system, here we have ascertained whether GPER might be involved in the biological responses prompted by ET-1 in cancer cells. We show that ET-1 regulates the expression of GPER which contributes to certain effects mediated by ET-1, like cell migration and angiogenesis. Therefore, our data further extend the current knowledge on the molecular mechanisms by which ET-1 can contribute to worse malignant features, highlighting the potential benefits of combinatorial anti-cancer therapies targeting these transduction pathways.

2. Materials and methods

2.1. Reagents

Endothelin-1 (ET-1), BQ123, BQ788 and 17β-estradiol (E2) were purchased from Sigma-Aldrich Corp. (Milan, Italy). Wortmannin (WM) and PD98059 (PD) were bought from Calbiochem (Milan, Italy). (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). Human VEGF was from Peprotech (Rocky Hill, New Jersey, USA). All compounds were solubilized in dimethylsulfoxide (DMSO), except ET-1, which was dissolved in 5% acetic acid and VEGF which were solubilized in water.

2.2. Cell cultures

SkBr3 breast cancer, HepG2 hepatocarcinoma and HUVEC human umbilical vein endothelial cells, were obtained by ATCC (Manassas, USA) and used less than six months after revival. SkBr3 were maintained in RPMI-1640 without phenol red (Invitrogen, Milan, Italy) and HepG2 cells were maintained in DMEM medium (Invitrogen, Milan, Italy), with a supplement of 10% fetal bovine serum (FBS) and 100 μg/ml of penicillin/streptomycin (Gibco, Life Technologies, Milan, Italy). HUVECs were seeded on collagen coated flasks (Sigma-Aldrich Srl, Milan, Italy) and cultured in Endothelial Growth Medium (EGM) (Lonza, Milan, Italy), supplemented with 5% FBS (Lonza, Milan, Italy). All cell lines were grown in a 37 °C incubator with 5% CO_2. Cells were switched to medium without serum and phenol red the day before the experiments.

2.3. Plasmids and transfections

The plasmid DN/c-Fos, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA biding, was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA). Short hairpin constructs against human GPER (shGPER) and CTGF (shCTGF) were obtained and used as previously described [22]. In brief, they were generated in the lentiviral expression vector pLKO.1 purchased by Euroclone (Milan, Italy). The targeting strand generated from the shGPER construct is 5′-CGCTCCCTGCAAGCAGTC TTT-3′. The targeting strand generated from the shCTGF construct is 5′-TAGTACAGCGATTCAAAGATG-3′. The lentiviral shRNA direct against Egr-1 (shEgr1) and the scramble hairpin (shRNA) were purchased from Open Biosystem (Celbio, Italy). Transfection assays were performed using the X-tremeGene9 Transfection Reagent (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions. Briefly, 50% confluent cells were serum deprived and transfected for 36 h with shGPER and 24 h with shCTGF or shEgr-1 or DN/c-Fos, and then treated as indicated.

2.4. Reverse transcription and real-time PCR

Total RNA was extracted using the TRIzol commercial kit (Invitrogen, Milan, Italy) 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, Milan, Italy) 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., Milano, Italy). Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. The primers used were 5′-ACACACCTGGGTGGACACAA-3′ (GPER forward), 5′-GGAGCCAGAAGCCACATCTG-3′ (GPER reverse), 5′-CGAGCCCTTTGATGACTTCCTG-3′ (c-Fos forward), 5′-GGAGCGGGCTGT CTCAGA-3′ (c-Fos reverse), 5′-ACCTGTGGGATGGGCATCT-3′ (CTGF forward), 5′-CAGGCGGCTCTGCTTCTCTA-3′ (CTGF reverse), 5′-AAGATC CACTTGCGGCAGAA-3′ (Egr-1 forward), 5′-GCCGAAGAGGCCACAACA-3′ (Egr-1 reverse), 5′-GCTGATTTGTGAACCCATTC-3′ (HIF-1α forward), 5′-CTGTACTGTCCTGTGGTGAC-3′ (HIF-1α reverse), 5′-GAGCTTCAGGACAT TGCTGT-3′ (VEGF forward), 5′-AGGAAGGTCAACCACTCACA-3′ (VEGF reverse), 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward) and 5′-GGGCATCA CAGACCTGTTATT-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.

2.5. Western blotting

Cells were grown in 10-cm dishes, transfected and treated as indicated and then lysed in RIPA buffer (Sigma-Aldrich Corp., Milan, Italy) containing a mixture of protease inhibitors (1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Protein concentrations were determinated according to the Bradford method (Sigma-Aldrich Corp., Milan, Italy). Equal amount of whole protein extracts (10–50 μg of protein) was electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences Milan, Italy). Membranes were blocked and probed with primary antibodies for HIF-1α (R&D Systems, Inc. Celbio, Milan, Italy), GPER (N-15), CTGF (L-20), c-Fos (H-125), Egr-1 (C-19), phosphorylated ERK ½ (E-4), ERK2 (C-14), phosphorylated Akt 1/2/3 (ser 473), Akt (H-136) and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA, Milan, Italy). The levels of protein and phosphoproteins 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.

2.6. Chromatin immunoprecipitation (ChIP)

Cells grown in 10-cm plates were shifted for 24 h to medium lacking serum and then treated with vehicle or ET-1 100 nM for 2 h. Chip assay was performed as previously described [22]. The immune cleared

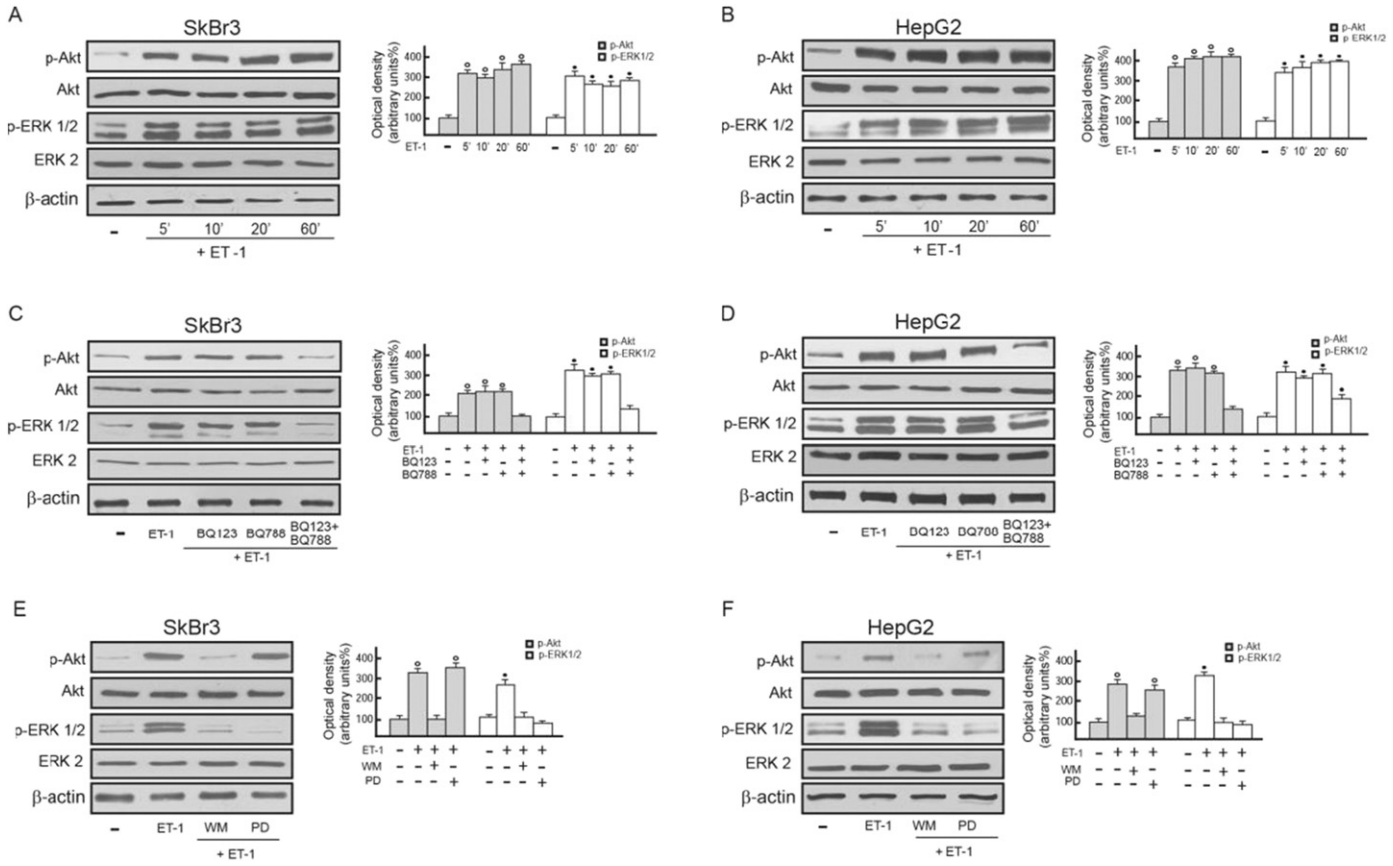

Fig. 1. ET-1 activates the PI3K/ERK1/2 transduction pathways. Akt and ERK1/2 phosphorylation in SkBr3 (A) and HepG2 (B) cells treated with vehicle (−) or 100 nM ET-1 for the indicated times. Akt and ERK1/2 phosphorylation in SkBr3 (C) and HepG2 (D) cells treated for 10 min with vehicle (−) or 100 nM ET-1 alone and in combination with 1 μM ET_AR inhibitor BQ123, 1 μM ET_BR inhibitor BQ788 or both. Akt and ERK1/2 phosphorylation in SkBr3 (E) and HepG2 (F) cells treated for 10 min with vehicle (−) or 100 nM ET-1 alone and in combination with 1 μM PI3K inhibitor wortmannin (WM) or 1 μM MEK inhibitor PD98059 (PD). Results shown are representative of three independent experiments. Densitometric analysis of the blots is normalized to Akt and ERK2. (○), (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

chromatin was immunoprecipitated with anti-c-Fos (H-125) or nonspecific IgG (Santa Cruz Biotechnology, DBA, Milano, Italy). A 4 μl volume of each immunoprecipitated DNA sample was used as template to amplify, by RT-PCR, the region containing an AP-1 site, located into the GPER promoter region. The primers used to amplify this fragment were: 5′-CGTGCCCATACCTTCATTGCTTCC-3′ (forward) and 5′-CCTGGC CGGGTGTCTGTG-3′ (reverse). Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Input DNA quantification was performed by using 4 μl of the template DNA. For knock-down experiments SkBr3 cells were previously transfected in serum-free medium for 24 h with DN/c-Fos or the respective control vector and then treated for 2 h with 100 nM ET-1. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Results were expressed as percent differences with respect to the relative inputs.

2.7. Immunofluorescence microscopy

50% confluent cultured SkBr3 cells grown on coverslips were serum deprived for 24 h and treated for 12 h with 100 nM ET-1 alone or in combination with 100 nM G15. Then cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS and incubated overnight with a mouse primary antibody against VEGF (C-1) (Santa Cruz Biotechnology, DBA, Milan, Italy). After incubation, the slides were extensively washed with PBS and incubated with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), (1:1000), (Sigma-Aldrich Srl, Milan, Italy) and donkey anti-mouse IgG-FITC (1:300; purchased from Alexa Fluor, Invitrogen, Milan, Italy). 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 experiment evaluation.

2.8. Migration assay

Migration assays were performed using Boyden Chambers (Costar Transwell, 8 mm polycarbonate membrane). For knock-down experiments, cells were transfected with shRNA constructs directed against GPER or CTGF or Egr-1 and with an unrelated shRNA construct (500 ng DNA/well transfected with X-tremeGene9 reagent in medium without serum). After 24 h, cells were seeded in the upper chambers. 100 nM ET-1 alone and together with 1 μM BQ123 and 1 μM BQ788 or 1 μM PD or 1 μM WM was added to the medium without serum in the bottom wells. After 6 h, cells on the bottom side of the membrane were fixed and counted.

2.9. Conditioned medium

SkBr3 were cultured in regular growth medium to 80% confluence. Then, cells were washed twice with phosphate-buffered saline (PBS) and treated for 12 h with 100 nM ET-1 alone or in combination with 100 nM G15. Thereafter, the culture supernatant was collected,

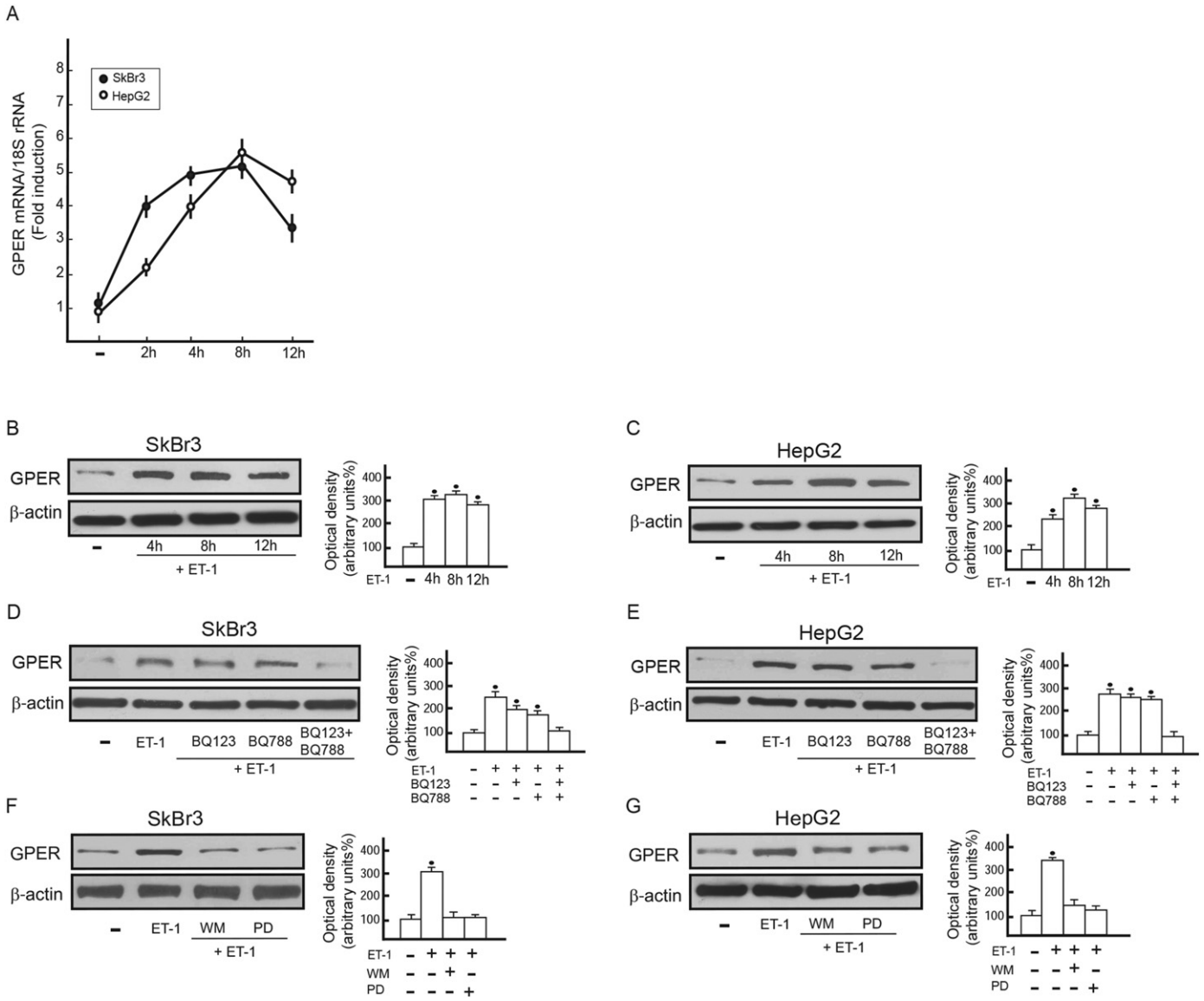

Fig. 2. ET-1 up-regulates GPER expression. (A) Time-dependent mRNA expression of GPER upon exposure to 100 nM ET-1 in SkBr3 and HepG2 cells, as evaluated by real-time PCR. Values are normalized to the 18S expression and shown as fold changes of mRNA expression induced by ET-1 compared to cells treated with vehicle (−). Data shown are the mean ± SD of five independent experiments performed in triplicate. Time-dependent protein expression of GPER in SkBr3 (B) and HepG2 (C) cells treated with vehicle (−) or 100 nM ET-1, as indicated. GPER protein levels in SkBr3 (D) and HepG2 (E) cells treated for 4 h with 100 nM ET-1 alone and in combination with 1 μM ET_AR inhibitor BQ123, 1 μM ET_BR BQ788 or both. GPER protein levels in SkBr3 (F) and HepG2 (G) cells treated for 4 h with 100 nM ET-1 alone and in combination with 1 μM PI3K inhibitor wortmannin (WM) or 1 μM MEK inhibitor PD98059 (PD). Results shown are representative of three independent experiments. Densitometric analysis of the blots is normalized to β-actin. (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

centrifuged at 16,000 g for 5 min to remove cell debris and used as conditioned medium in HUVECs.

2.10. Tube formation assay

The day before the experiment, confluent HUVECs were starved overnight at 37 °C in serum free medium (EBM, Lonza, Milan, Italy). Growth factor-reduced Matrigel® (Cultrex, Trevigen Inc., USA) was thawed overnight at 4 °C on ice, plated on the bottom of prechilled 96 well-plates and left at 37 °C for 1 h for gelification. Starved HUVECs were collected by enzymatic detachment (0.25% trypsin-EDTA solution, Invitrogen, Milan, Italy), counted and resuspended in conditioned medium from SkBr3. Then, 10,000 cells/well were seeded on Matrigel and incubated at 37 °C. Tube formation was observed starting from 2 h after cell seeding and quantified by using the software NIH ImageJ.

2.11. Statistical analysis

Results are presented as means ± standard deviations (SD) from at least 3 independent experiments. Statistical analysis was performed using analysis of variance (ANOVA) followed by Newman–Keuls' testing to determine differences in means. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. ET-1 induces the up-regulation of GPER

ET-1 triggers relevant biological responses through numerous transduction mechanisms, which include the activation of PI3K/Akt and ERK1/2 mediated signaling [3]. Hence, we began our study ascertaining that ET-1 induces the rapid phosphorylation of Akt and ERK1/2 in both

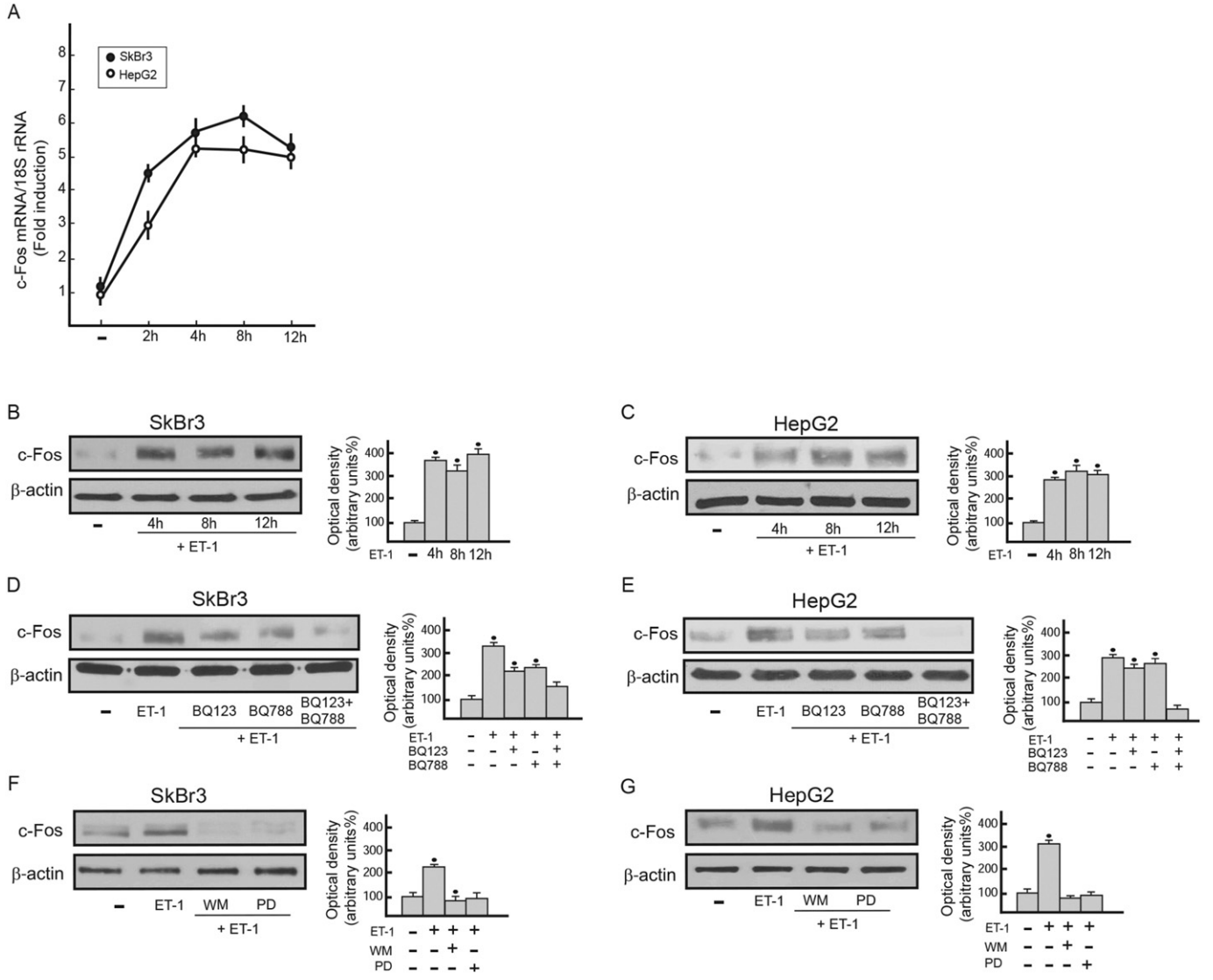

Fig. 3. ET-1 up-regulates c-Fos expression. (A) Time-dependent mRNA expression of c-Fos upon exposure to 100 nM ET-1 in SkBr3 and HepG2 cells, as evaluated by real-time PCR. Values are normalized to the 18S expression and shown as fold changes of mRNA expression induced by ET-1 compared to cells treated with vehicle (−). Data shown are the mean ± SD of five independent experiments performed in triplicate. Time-dependent protein expression of c-Fos in SkBr3 (B) and HepG2 (C) cells treated with vehicle (−) or 100 nM ET-1, as indicated. c-Fos protein levels in SkBr3 (D) and HepG2 (E) cells treated for 4 h with 100 nM ET-1 alone and in combination with 1 μM ET_AR inhibitor BQ123, 1 μM ET_BR BQ788 or both. c-Fos protein levels in SkBr3 (F) and HepG2 (G) cells treated for 4 h with 100 nM ET-1 alone and in combination with 1 μM PI3K inhibitor wortmannin (WM) or 1 μM MEK inhibitor PD98059 (PD). Results shown are representative of three independent experiments. Densitometric analysis of the blots is normalized to β-actin. (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

SkBr3 and HepG2 cells, which were used as a model system (Fig. 1A, B). The aforementioned responses to ET-1 were not abolished in the presence of 10 μM ET_AR antagonist BQ123 or 10 μM ET_BR antagonist BQ788 (data not shown), however using both inhibitors in combination the phosphorylation of Akt and ERK1/2 was no longer evident (Fig. 1C, D). Next, the PI3K/Akt inhibitor wortmannin (WM) but not the MEK/ERK inhibitor PD98059 (PD) abolished the Akt and ERK1/2 phosphorylation by ET-1, suggesting that ERK1/2 signaling relays downstream to PI3K/Akt activation upon ET-1 stimulation in cancer cells used (Fig. 1E, F). As these transduction pathways have been involved in the regulation of many components of the largest family of signal transducers namely GPCRs strongly contributing to cancer development [27], we asked whether certain effects elicited by ET-1 in tumor cells may be prompted through GPER expression and function. Interestingly, ET-1 up-regulated the mRNA and protein levels of GPER in both SkBr3 and HepG2 cells (Fig. 2A–C). The protein induction of GPER was abrogated using in combination the ET_AR and ET_BR antagonists (Fig. 2D, E) as well as in the presence of the PI3K/Akt inhibitor WM and the MEK/ERK inhibitor PD (Fig. 2F, G). Taken together, these data indicate that the induction of GPER by ET-1 occurs through both ETR isoforms along with the activation of the PI3K and ERK1/2 transduction pathways.

3.2. The up-regulation of GPER by ET-1 involves c-Fos/AP1 transcription activation

On the basis of our and other studies demonstrating that the activation of relevant transduction pathways can lead to the induction of c-Fos which is targeted by ET-1 as well [10,28,29], we first ascertained that ET-1 triggers c-Fos expression in both cancer cells used (Fig. 3A–C). Thereafter, we determined that the increase of c-Fos by ET-1 is prevented using in combination the antagonists of ET_AR and ET_BR (Fig. 3D, E) as well as using the PI3K/Akt inhibitor WM and the MEK/ERK inhibitor PD (Fig. 3F, G). Taking into account these observations and considering that an AP-1 site located within the GPER promoter sequence plays a critical role in the regulation of GPER [10,22], we performed ChIP analysis transfecting cells with an expression vector

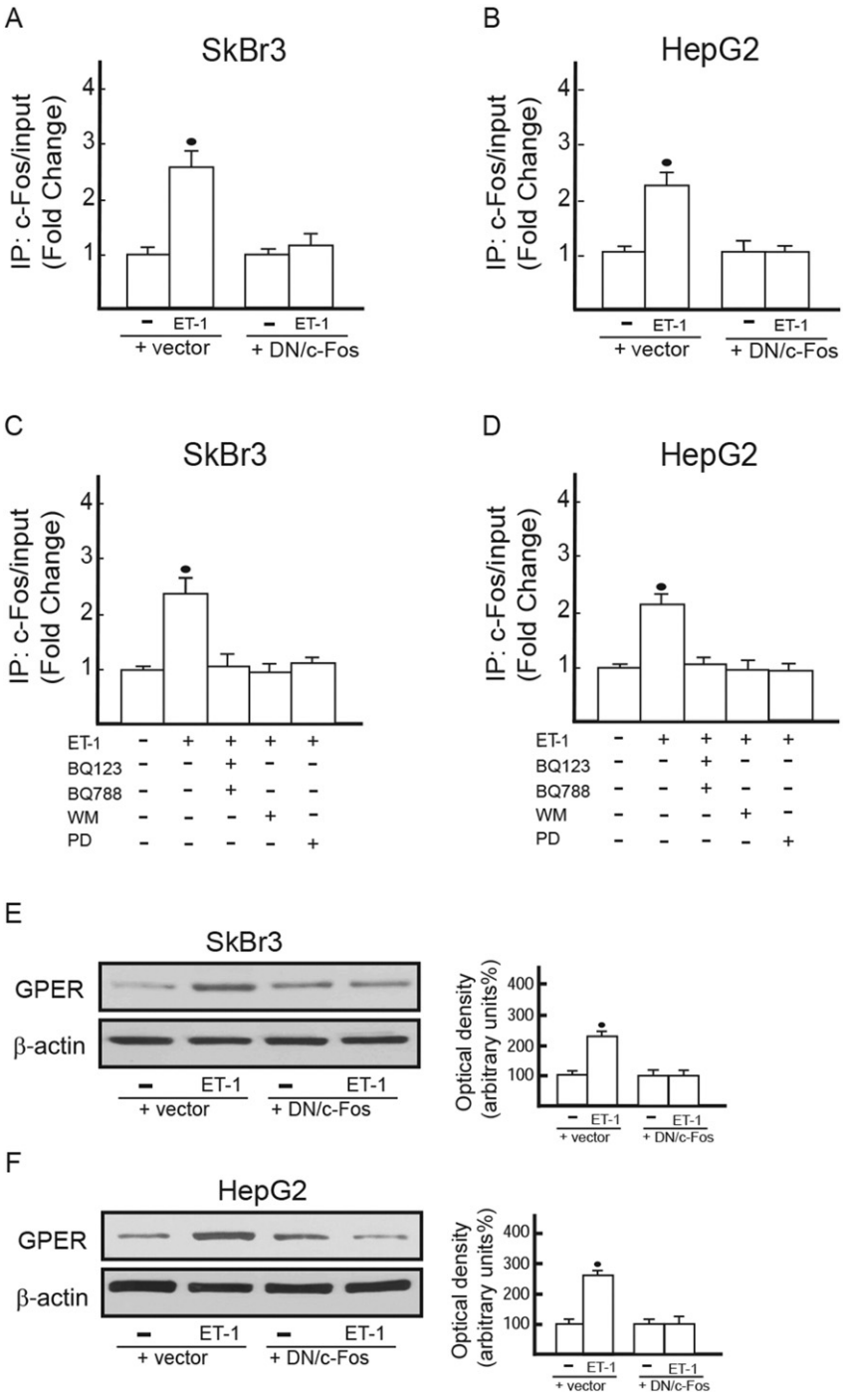

Fig. 4. c-Fos is involved in the up-regulation of GPER by ET-1. Recruitment of c-Fos to the AP1 site located within the promoter sequence of GPER in SkBr3 (A) and HepG2 (B) cells transfected with an empty vector or an expression vector encoding for a dominant negative form of c-Fos (DN/c-Fos) and treated for 2 h with 100 nM ET-1. Recruitment of c-Fos to the AP1 site located within the promoter sequence of GPER in SkBr3 (C) and HepG2 (D) cells treated for 2 h with 100 nM ET-1 alone and in combination with both 1 μM ET_AR inhibitor BQ123 and 1 μM ET_BR inhibitor BQ788, 1 μM PI3K inhibitor wortmannin (WM) or 1 μM MEK inhibitor PD98059 (PD). Each column represents the mean ± SD of three independent experiments performed in triplicate. The up-regulation of GPER protein expression observed upon 100 nM ET-1 treatment is abrogated transfecting SkBr3 (E) and HepG2 (F) cells with an expression vector encoding for a dominant negative form of c-Fos (DN/c-Fos). Results shown are representative of three independent experiments. Densitometric analysis of the blots is normalized to β-actin. (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

encoding a dominant-negative form of c-Fos (DN/c-Fos). In both SkBr3 and HepG2 cells, the ET-1 induced recruitment of c-Fos to the AP-1 site located on the promoter of GPER was abrogated in the presence of the DN/c-Fos (Fig. 4A, B) as well as using the ETR antagonists in combination, the PI3K/Akt inhibitor WM and the MEK/ERK inhibitor PD (Fig. 4C, D). In particular, each ETR inhibitor used alone up to 10 μM concentration did not prevent the aforementioned response (data not shown). Moreover, the ET-1 induced up-regulation of GPER protein levels was abolished transfecting both SkBr3 and HepG2 cells with the DN/c-Fos (Fig. 4E, F). Altogether, these data indicate that ET-1 up-regulates GPER expression through both ET_AR and ET_BR along with the activation of PI3K/ERK/c-Fos/AP1 transduction signaling.

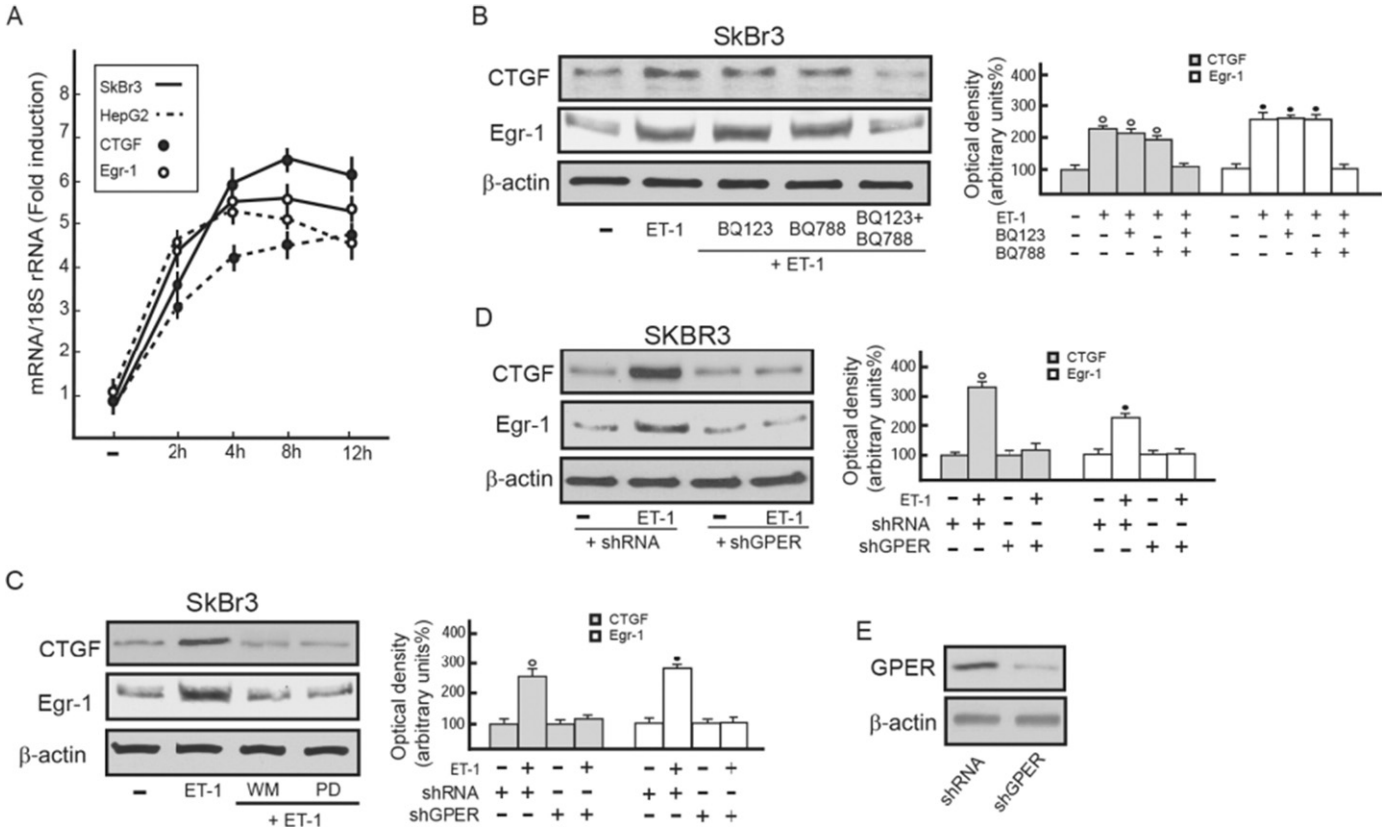

Fig. 5. ET-1 induces CTGF and Egr-1 expression through GPER. (A) The mRNA levels of CTGF and Egr1 are up-regulated in SkBr3 and HepG2 cells treated with 100 nM ET-1, as evaluated by real-time PCR. Values are normalized to the 18S expression and shown as fold changes of mRNA expression induced by ET-1 compared to cells treated with vehicle (−). Data shown are the mean ± SD of five independent experiments performed in triplicate. CTGF and Egr-1 protein levels in SkBr3 cells treated for 4 h with 100 nM ET-1 alone and in combination with both 1 μM ET_AR inhibitor BQ123 and 1 μM ET_BR inhibitor BQ788 (B), 1 μM PI3K inhibitor wortmannin (WM) or 1 μM MEK inhibitor PD98059 (PD) (C). The up-regulation of CTGF and Egr-1 protein expression observed upon treatment with 100 nM ET-1 for 4 h is abrogated by GPER silencing (D). Efficacy of GPER silencing in SkBr3 (E). Results shown are representative of three independent experiments. Side panels show densitometric analysis of the blots normalized to β-actin. (○), (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

3.3. ET-1 induces certain biological effects through GPER

Afterwards, we determined that ET-1 treatment induces the mRNA expression of main GPER target genes, like CTGF and Egr-1 [10] in both cancer cells used (Fig. 5A). The increase of these genes was not further extended using ET-1 (100 nM) in combination with E2 ranging from 1 to 100 nM (data not shown). The increase of CTGF and Egr-1 protein levels upon ET-1 treatment was abolished treating cells with the ETR antagonists, the PI3K/Akt inhibitor WM and the MEK/ERK inhibitor PD (Fig. 5B, C) as well as silencing the expression of GPER (Fig. 5D, E). As biological counterpart, the migration stimulated by ET-1 in SkBr3 and HepG2 cells was prevented in the presence of both ETR antagonists, the PI3K/Akt inhibitor WM and the MEK/ERK inhibitor PD (Fig. 6A, B) as well as transfecting cells with the shGPER, the shCTGF or the shEgr-1 (Fig. 6C, D). Specifically, each ETR antagonist used alone up to 10 μM concentration did not evidence inhibitory effects (data not shown). Collectively, these results suggest that the migratory effects triggered by ET-1 in SkBr3 and HepG2 cells occur through GPER and its target genes CTGF and Egr-1. As ET-1 regulates the expression of critical mediators of hypoxia-dependent tumor angiogenesis like HIF-1α and VEGF [3], we first ascertained that ET-1 induces the mRNA expression of HIF-1 α and VEGF (Fig. 7A). Then, we demonstrated that GPER is required for the protein induction of both HIF-1α and VEGF upon ET-1 treatment as this response was no longer evident silencing GPER or using the specific GPER antagonist G15 (Fig. 7B–F). Additionally, we examined whether conditioned medium from ET-1-stimulated SkBr3 cells could promote in HUVECs the formation of tubule-like structures, which represent a useful model system for the evaluation of the neoangiogenesis process [30]. Worthy, HUVECs cultured in medium from SkBr3 cells, which were previously treated with ET-1, displayed a ramified network of tubules with respect to HUVECs grown in medium from SkBr3 cells treated with vehicle (Fig. 8A). Tube formation was no longer evident in HUVECs cultured with medium collected from SkBr3 cells previously treated with ET-1 in combination with the specific GPER antagonist G15, whereas the addition of VEGF rescued the generation of tubule structures (Fig. 8B). Overall, these data suggest that GPER may be included among the complex transduction network triggered by ET-1 toward angiogenesis and cancer progression.

4. Discussion

The estrogenic GPER signaling has been recently shown to trigger important biological effects like proliferation and migration in diverse cancer cells and CAFs derived from breast tumors [10]. Moreover, GPER has been indicated as a possible predictor of cancer malignancy and aggressiveness as its expression was associated with negative clinical features and poor survival rates in numerous types of tumors [16,17]. Therefore, huge efforts have been addressed toward the discovery of selective GPER ligands in order to better understand the mechanisms involved in its activation and function [10]. Likewise, several studies have been performed to assess the regulation of GPER, in particular in cancer cells. In this regard, it should be mentioned that EGF [10], IGF-I [21], insulin [23] and a main factor contributing to tumor aggressiveness, such as hypoxia, are able to induce GPER expression toward angiogenesis and growth responses [24,25]. Further extending these findings, we have demonstrated that ET-1 up-regulates

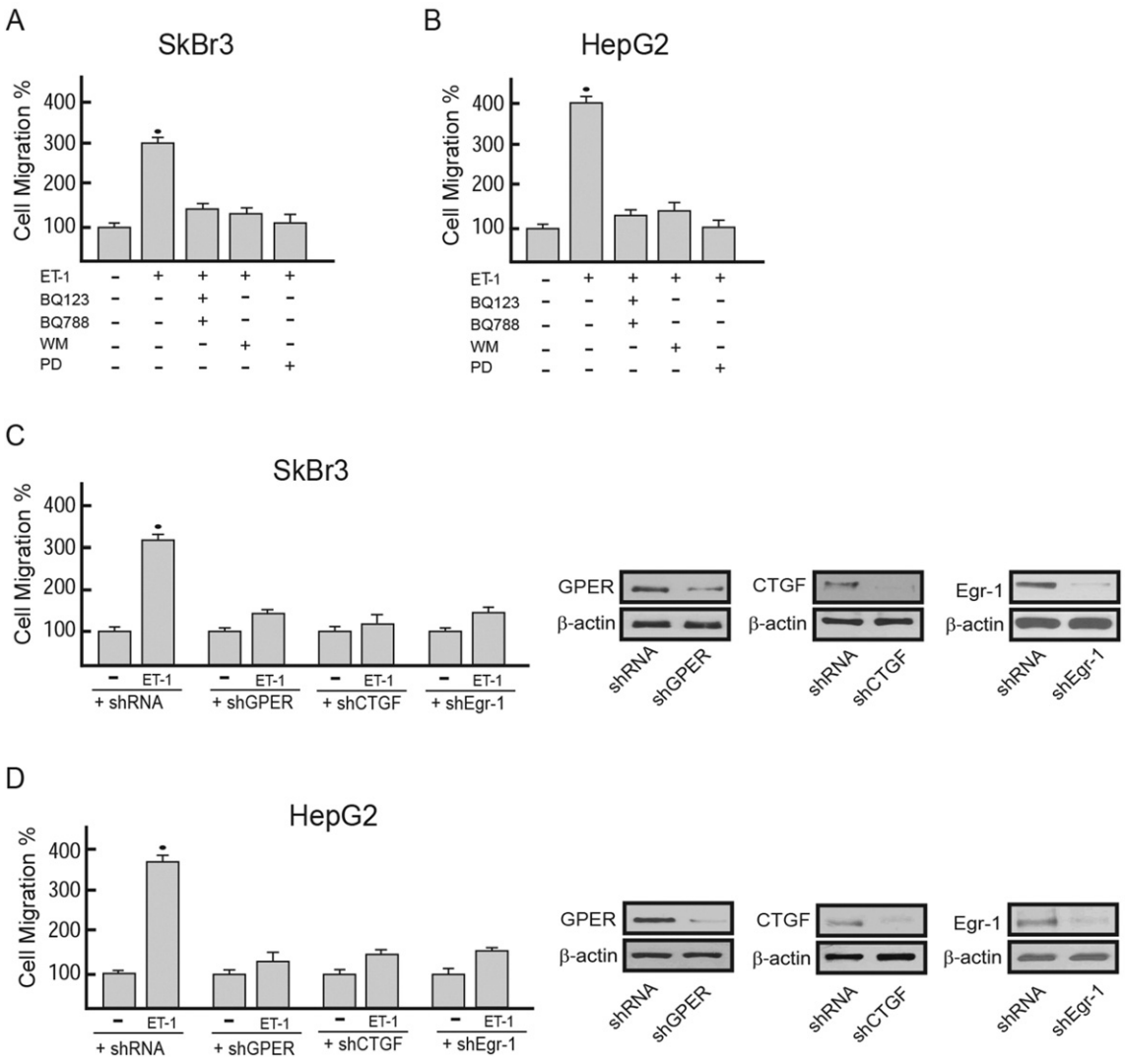

Fig. 6. GPER is involved in cell migration induced by ET-1. The migration of SkBr3 (A) and HepG2 (B) cells induced upon a 6 h treatment with 100 nM ET-1 is abolished in the presence of both 1 μM ET_AR inhibitor BQ123 and 1 μM ET_BR inhibitor BQ788, 1 μM PI3K inhibitor wortmannin (WM) and 1 μM MEK inhibitor PD98059 (PD). The migration of SkBr3 (C) and HepG2 (D) cells induced upon a 6 h treatment with 100 nM ET-1 is prevented knocking down GPER, CTGF or Egr1 expression. Values obtained in cells treated with vehicle (−) were set to 100% upon which results observed in ET-1 treated cells were calculated. Each column represents the mean ± SD of three independent experiments. Side panels in (C) and (D) show the efficacy of gene silencing. (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−).

GPER expression at both mRNA and protein levels in the ETR-positive breast cancer SkBr3 and hepatocarcinoma HepG2 cells, which were used as a model system. The induction of GPER by ET-1 involved both ET_AR and ET_BR and was mediated by the rapid activation of PI3K and ERK1/2 that in turn triggered the up-regulation of c-Fos and its recruitment to the AP1 site located within the GPER promoter sequence. Worthy, CTGF and Egr-1 well acknowledged to be targets of both ET-1 and GPER [10,31,32] as well as involved in cell migration [33,34], were upregulated upon ET-1 treatment through the ETR/PI3K/ERK signaling and GPER. Nicely fitting with these data, the aforementioned transduction mechanisms were also implicated in cell migration observed upon ET-1 stimulation. These results extend the network of factors involved in the action of ET-1 and may recall previous studies demonstrating that ET-1 expression associates with invasive and metastatic properties in many tumors [3]. For instance, an altered ETR expression was assessed in malignant with respect to normal tissues, together with a different ET_AR/ET_BR ratio in accordance with various cancer types [3]. Moreover, it was reported that the exposure of tumor cells to ET-1 triggers relevant biological responses, including invasion and angiogenesis, through both ET_AR and ET_BR [5,35].

Angiogenesis is an intricate process which characterizes the aggressive biological features of diverse types of tumors [36]. VEGF is a potent proangiogenic factor involved in the formation of blood vessels in various tumors [37]. For instance, VEGF was found highly expressed in breast cancer specimens with respect to normal breast tissue and its suppression led to inhibitory effects on tumor growth [36]. Remarkably, increased VEGF expression and vascularity correlated with the expression of ET-1 and its cognate receptors in biopsies derived from invasive cancers [5]. In accordance with these data, previous studies have shown that ET-1 through ETRs may contribute to the production of VEGF in diverse types of tumors [3]. In this regard, our current findings have provided evidence regarding the ability of ET-1 to trigger angiogenesis and cancer progression through GPER along with both HIF-1α and VEGF. Interestingly, the current results recall our previous studies showing that upon hypoxia HIF-1α and GPER regulate VEGF expression as well as endothelial tube formation, thus supporting cancer angiogenesis and progression [25]. Likewise, ligand-activated GPER stimulated the expression of both HIF1α and VEGF triggering tumor growth in a mouse xenograft model of breast cancer [26].

In accordance with these findings, it has been recently reported that low survival rates in patients with endometrial cancer are associated

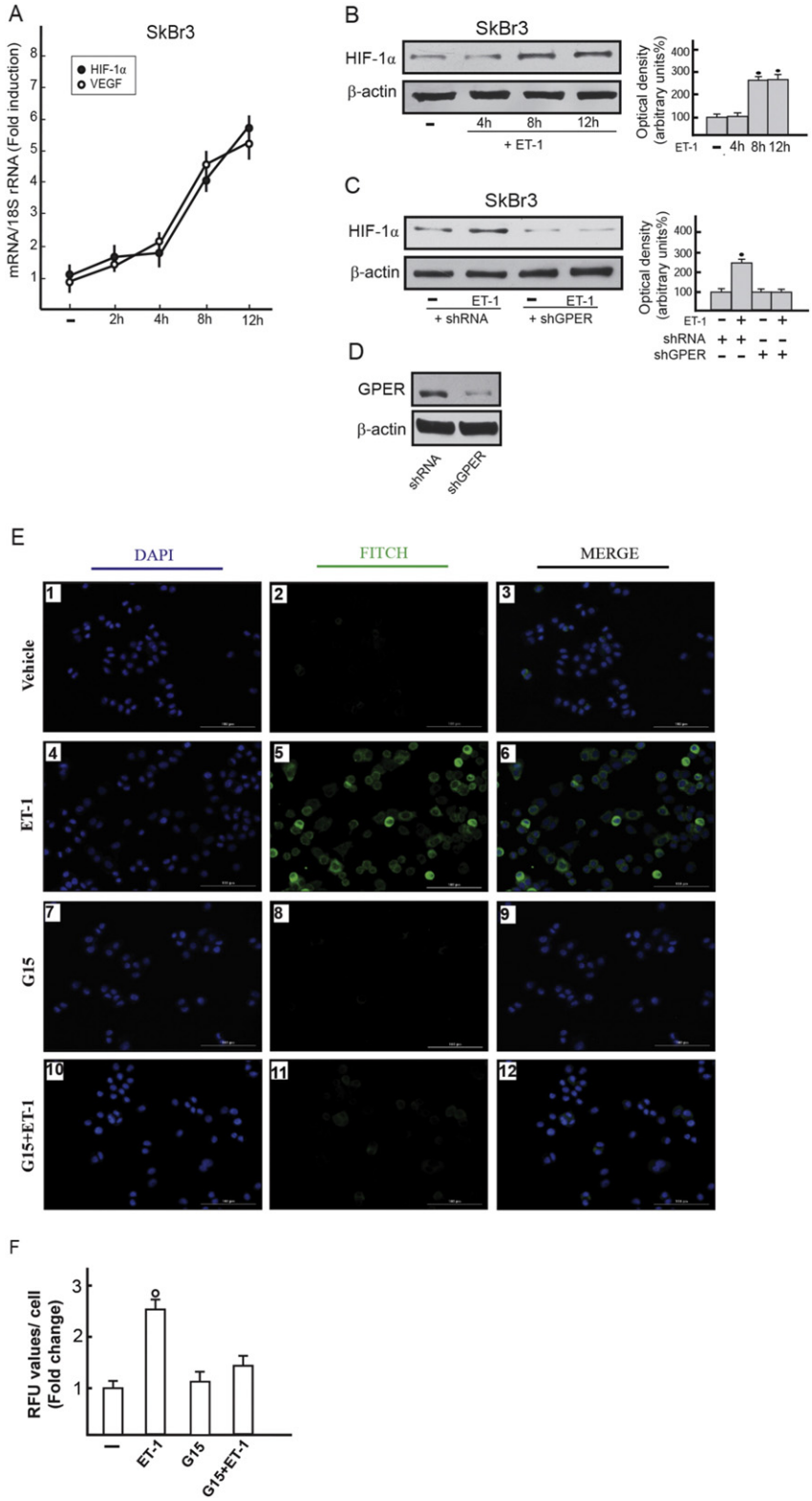

Fig. 7. ET-1 induces HIF-1α and VEGF expression through GPER. (A) HIF-1α and VEGF mRNA levels in SkBr3 cells treated with 100 nM ET-1, as evaluated by real-time PCR. Values are normalized to the 18S expression and shown as fold changes of mRNA expression induced by ET-1 compared to cells treated with vehicle (−). Data shown are the mean ± SD of five independent experiments performed in triplicate. (B) Time-dependent protein expression of HIF-1α in SkBr3 cells treated with vehicle (−) or 100 nM ET-1, as indicated. (C) The HIF-1α protein induction observed treating SkBr3 cells for 8 h with 100 nM ET-1 is abrogated silencing GPER expression. Results shown are representative of three independent experiments. (D) Efficacy of GPER silencing. Densitometric analysis of the blots is normalized to β-actin. (●) $p < 0.05$ for cells receiving treatments *vs* cells treated with vehicle (−). (E) Evaluation of VEGF expression by immunofluorescent microscopy in SkBr3 cells treated for 12 h with vehicle (panels 1–3), 100 nM ET-1 (panels 4–6), 100 nM GPER antagonist G15 (panels 7–9), and 100 nM ET-1 in combination with 100 nM G15 (panels 10–12). VEGF accumulation is evidenced by the green signal; nuclei were stained by DAPI and evidenced by the blue signal. The plates were imaged on the Cytation 3 cell imaging multimode reader (BioTek, Winooski, VT). Images shown are representative of three independent experiments. (F) Fluorescence intensities for the green channel were quantified in 10 random fields for each experimental condition and results are expressed as fold change of relative fluorescence units (RFU) over vehicle-treated cells. Values are mean ± SD of three independent experiments. (○) $p < 0.05$ for cells receiving vehicle (−) *versus* ET-1 treatment.

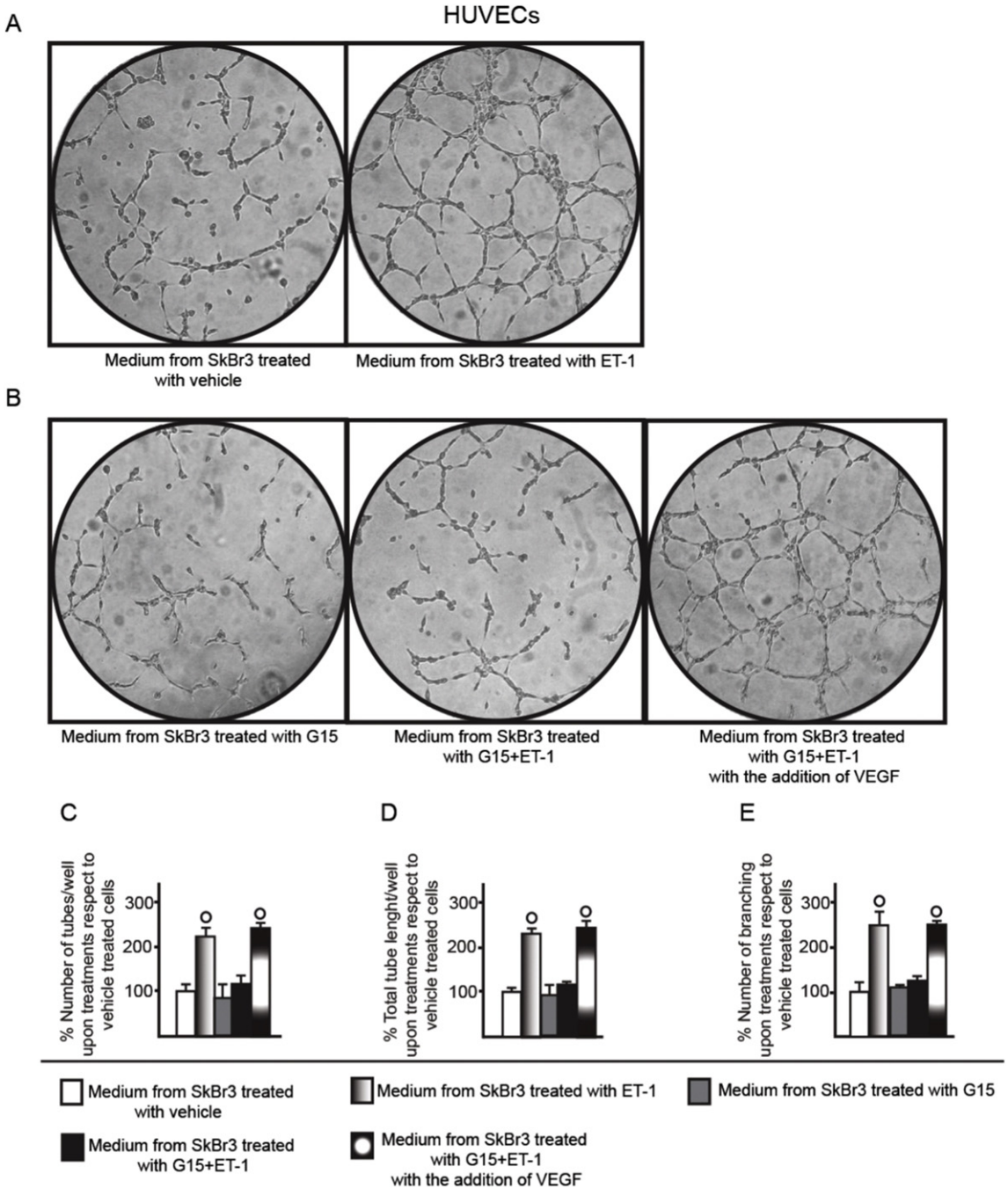

Fig. 8. GPER is involved in tube formation induced by ET-1. (A–B) Evaluation of tube formation in HUVECs cultured for 4 h in medium collected from SkBr3 previously treated for 12 h with 100 nM ET-1 alone and in combination with 100 nM GPER antagonist G15, as indicated. In HUVECs cultured in medium from SkBr3 treated with 100 nM ET-1 in combination with 100 nM G15, tube formation is rescued treating cells with 20 ng/ml VEGF. Data are representative of three independent experiments performed in triplicate. Quantification of number of tubes (C), total tube length (D) and number of branching points (E). Data are representative of three independent experiments performed in triplicate. (○) $p < 0.05$ for HUVECs cultured upon treatments *vs* vehicle-treated cells.

with elevated GPER and VEGF expression [38]. In this context, our present data demonstrate that GPER is involved in the up-regulation of VEGF by ET-1, therefore extending current knowledge regarding the mechanisms by which ET-1 up-regulates VEGF leading to cancer development.

The ET-1 system has been found overexpressed and correlated with advanced stages of various malignancies like breast, ovarian, prostate, colon, lung and hepatic tumors [2]. Hence, a growing interest is currently addressed to define the mechanisms by which ET-1 can trigger cancer cell proliferation, confer apoptosis resistance, stimulate new vessel formation and promote metastatic dissemination [3]. As it concerns breast carcinomas, the expression of ET-1 and ETRs has been detected increased during the malignant progression and associated with aggressive biological features [3,4]. In addition, ET-1 was found to induce an invasive phenotype through the stimulation of both tumor and stromal cells [5] and the modulation of diverse cytokines and matrix metalloproteinases (MMPs) [4]. In ovarian carcinoma cells, ET-1 regulated the expression of MMP-2 and MMP-9 and downregulated tissue inhibitors of matrix metalloproteinases (TIMPs) 1 and 2, whereas in chondrosarcoma cells ET-1 increased COX-2 expression and cell migration in a MAPK and AP-1 dependent manner [3]. An increased immunoreactivity for ET-1 was detected in endothelial cells of colorectal

liver metastases with respect to the surrounding vessels, indicating that ET-1 may be involved in the modulation of tumor blood flow, which is a well known process characterizing liver metastases [39]. The aforementioned observations have suggested that the inhibition of ETRs can be considered as an attractive therapeutic approach in anti-cancer treatments using diverse drugs in combination [40]. In this regard, the signaling network triggered by ET-1 should be further investigated in order to ascertain the benefits of combinatorial therapies aimed to target distinct components of diverse transduction pathways.

5. Conclusions

In the present study, we provide novel insights into the ability of ET-1 to induce GPER expression in cancer cells through both ET_AR and ET_BR, along with the activation of the PI3K/ERK/c-Fos/AP1 transduction signaling. In addition, we show that the migration of cancer cells triggered by ET-1 involves GPER and its target genes CTGF and Egr-1. Next, we evidence that GPER contributes to the HIF-1α/VEGF-dependent tube formation in HUVECs cultured in medium from SkBr3 cells treated with ET-1.

In conclusion, the present data provide new insights into the potential of ET-1 to engage GPER toward angiogenesis and tumor progression. Thus, our findings further extend the molecular mechanisms by which ET-1 may contribute to worse cancer features, although next studies are required to better assess the role elicited by the functional crosstalk between ET-1 system and GPER on the growth stimulation in different types of malignancies.

Author contributions

Conceived and designed the experiments: V. Bartella, A. Vivacqua and M. Maggiolini. Performed the experiments: V. Bartella, E.M. De Francesco, M.G. Perri, R. Curcio and A. Vivacqua. Analyzed the data: V. Bartella, A. Vivacqua, V. Dolce and M. Maggiolini. Wrote the paper: V. Bartella, V. Dolce, A. Vivacqua and M. Maggiolini.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Associazione Italiana per la Ricerca sul Cancro, PON01_01078 and Ministero della Salute grant n. 67/GR-2010-2319511.

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