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Research Article

GPER, IGF-IR and EGFR transduction signalling are involved in stimulatory effects of zinc in breast cancer cells and cancer-associated fibroblasts†

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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; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; $ZnCl_2$, zinc chloride.

Running title: zinc action in breast cancer

Keywords: zinc, breast cancer cells, cancer-associated fibroblasts, EGFR, GPER, IGF-IR

Abstract

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 signalling, 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.

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].

In the present study, we therefore aimed to evaluate whether Zn might trigger the transduction signalling 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 [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 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. The characterization of primary cells cultures of breast fibroblasts was assessed as described previously [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 ($1x10^5$) 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 1-fold 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 [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, 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.

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 (TM) sequence detection system (Applied Biosystems Inc, Milan, Italy). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc, Milan, Italy). 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 [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), 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 1.7mg/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.

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).

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.

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.

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.

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 (Fig. 1A) as well as the activation of downstream kinases such as ERK and AKT (Fig. 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 signalling 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 (Fig. 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 (Fig. 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 (Fig. 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 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 $ZnCl_2$ (Fig. 2F). Hence, the production of ROS observed in SkBr3 cells is involved in the rapid activation of GPER/EGFR/IGF-IR transduction signaling upon $ZnCl_2$ exposure. 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 (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 $ZnCl_2$ up-regulates 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 (Fig. 3A). Of note, GPER silencing prevented the mRNA induction of two main GPER target genes as c-fos and EGR1 (Fig. 3B-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 (Fig. 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. 3F-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 (Fig. 4A-B) as well as in the presence of 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 (Fig. 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 (Fig. 5A-B), however this response was abrogated silencing GPER (Fig. 5B-C) as well as in the presence of AG1478, AG1024, G15, PD, WM, TPEN (Fig. 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. 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(Fig. 6A-C). Moreover, we determined that this response to $ZnCl_2$ is abrogated by GPER silencing (Fig. 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 (Fig. 6D-E). In addition, SkBr3 cell migration induced by $ZnCl_2$ was prevented silencing GPER (Fig. 6F-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 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. 7B-C). As CTGF exerts an acknowledged role in migratory properties of different cell types [27, 38], 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. 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 (Fig. 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 signalling 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 signalling 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 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 [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.

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Figure legends

FIGURE 1. *$ZnCl_2$ triggers rapid responses and stimulates the co-immunoprecipitation of EGFR and IGF-IR with GPER in breast cancer cells*. (A-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.

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, 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 1-fold 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.

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 and MT2A 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 18S 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-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 PI3K 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-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.

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 18S 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.

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 days 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, 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.

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.

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, G) and CTGF (E, H) silencing. Values represent the mean ± SD of three independent experiments. (●) indicates $p < 0.05$ for cells treated with vehicle (–) versus treatments.

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

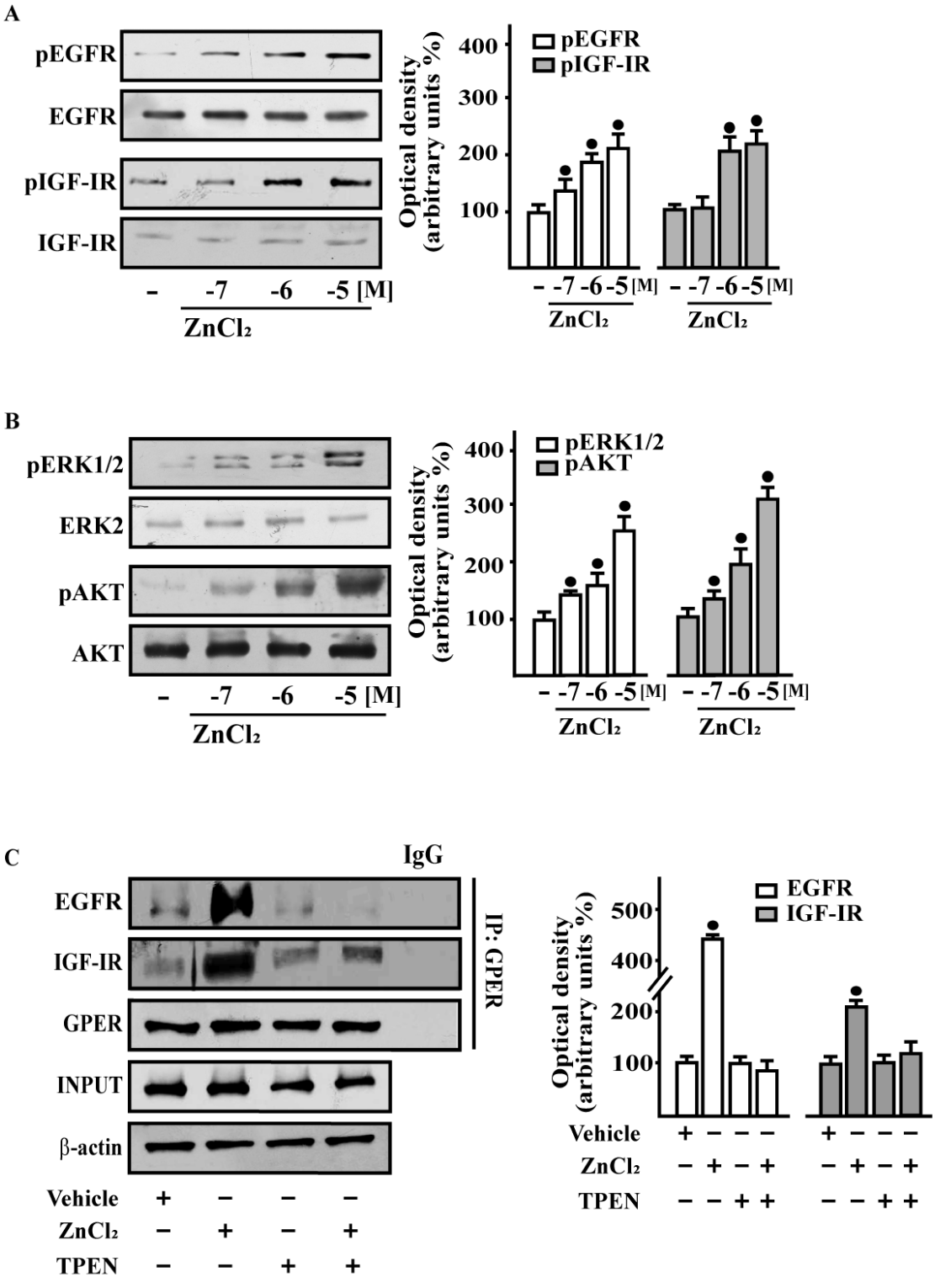


Fig. 1

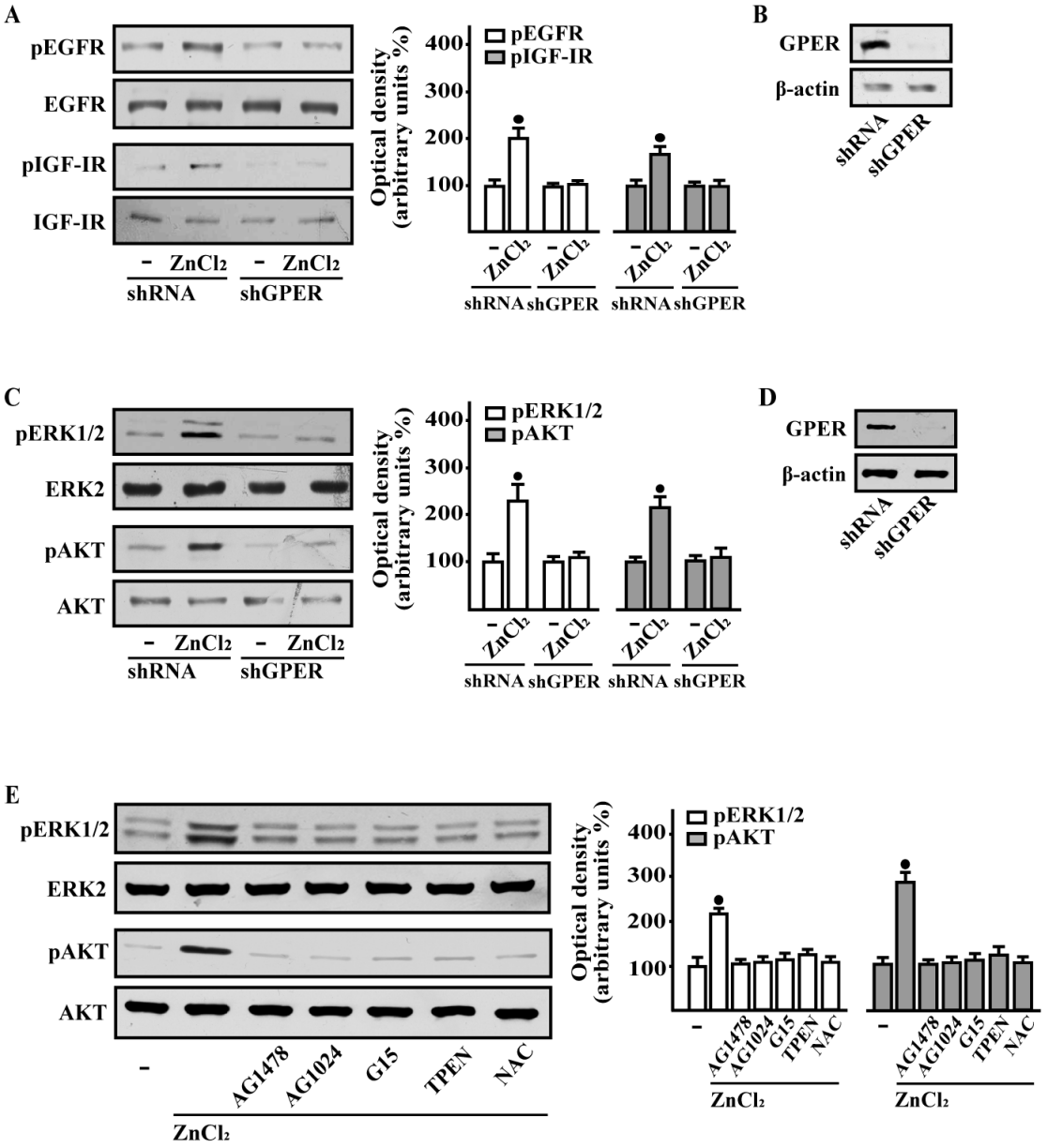


Fig. 2

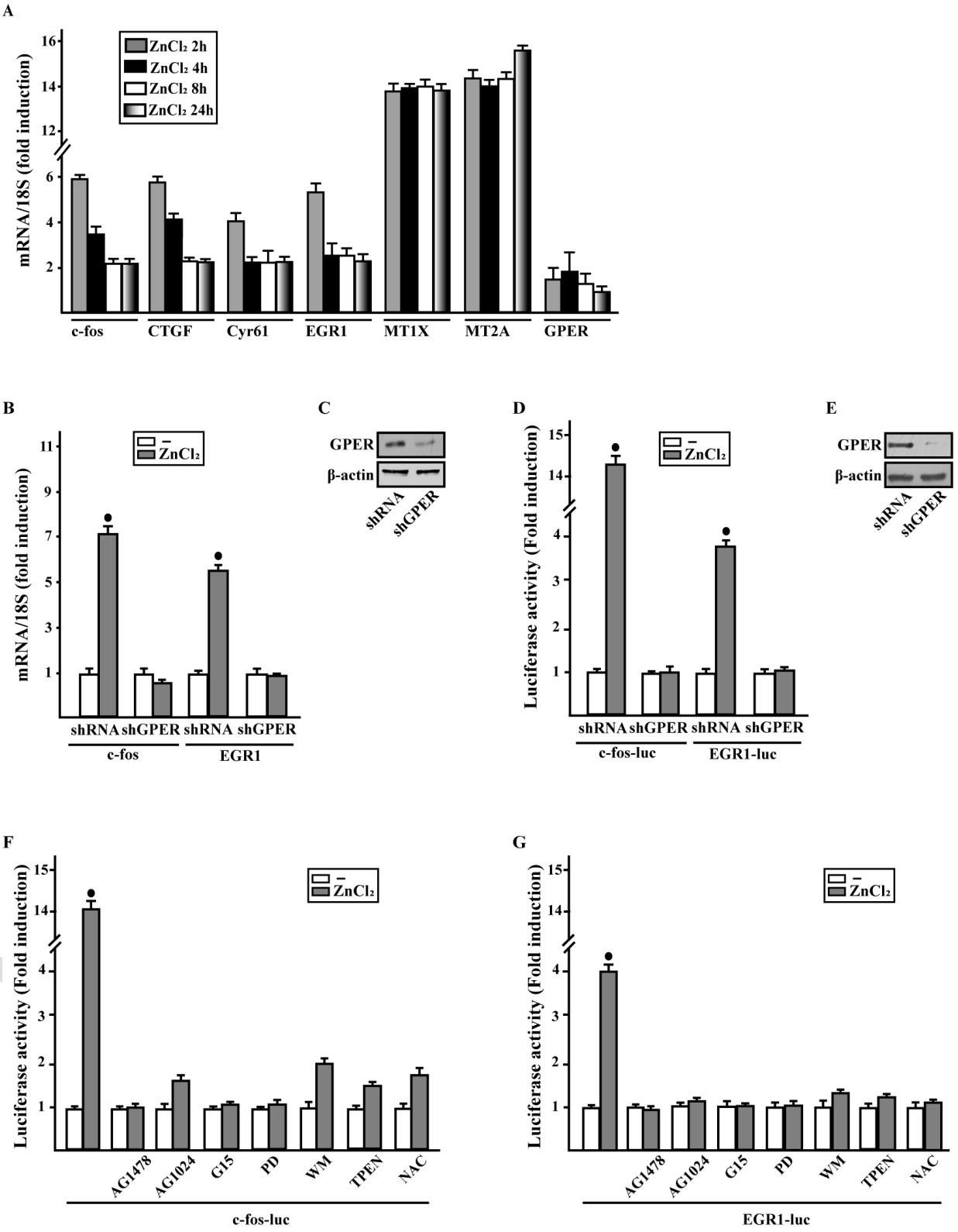


Fig. 3

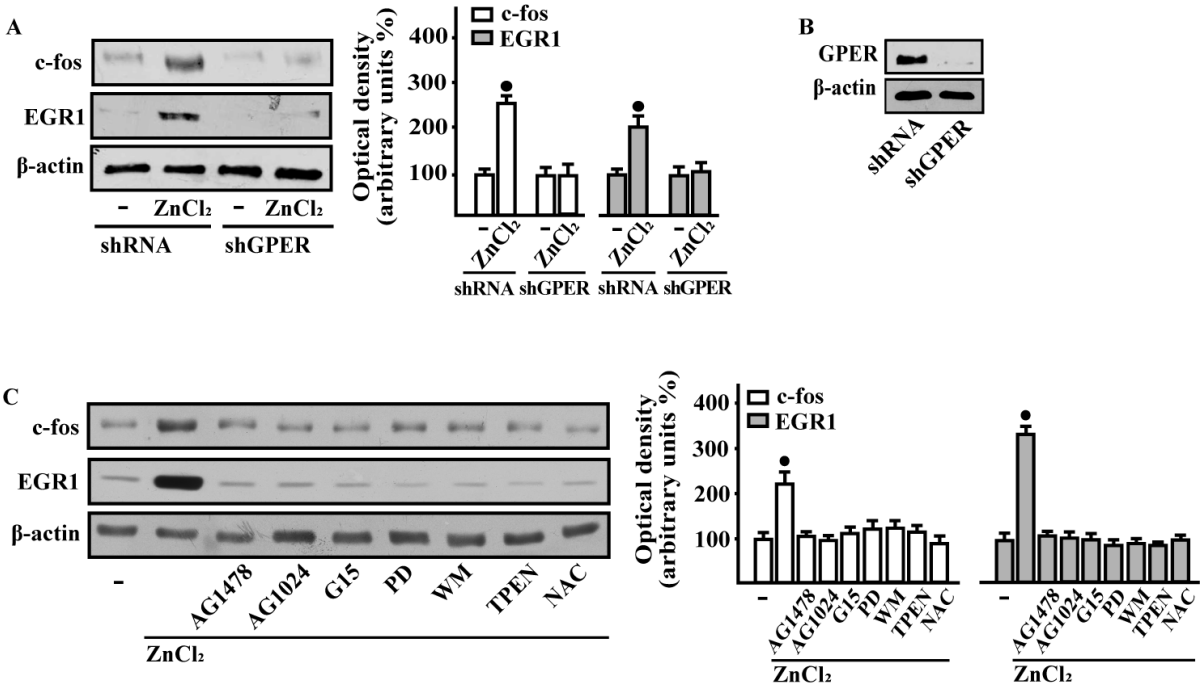


Fig. 4

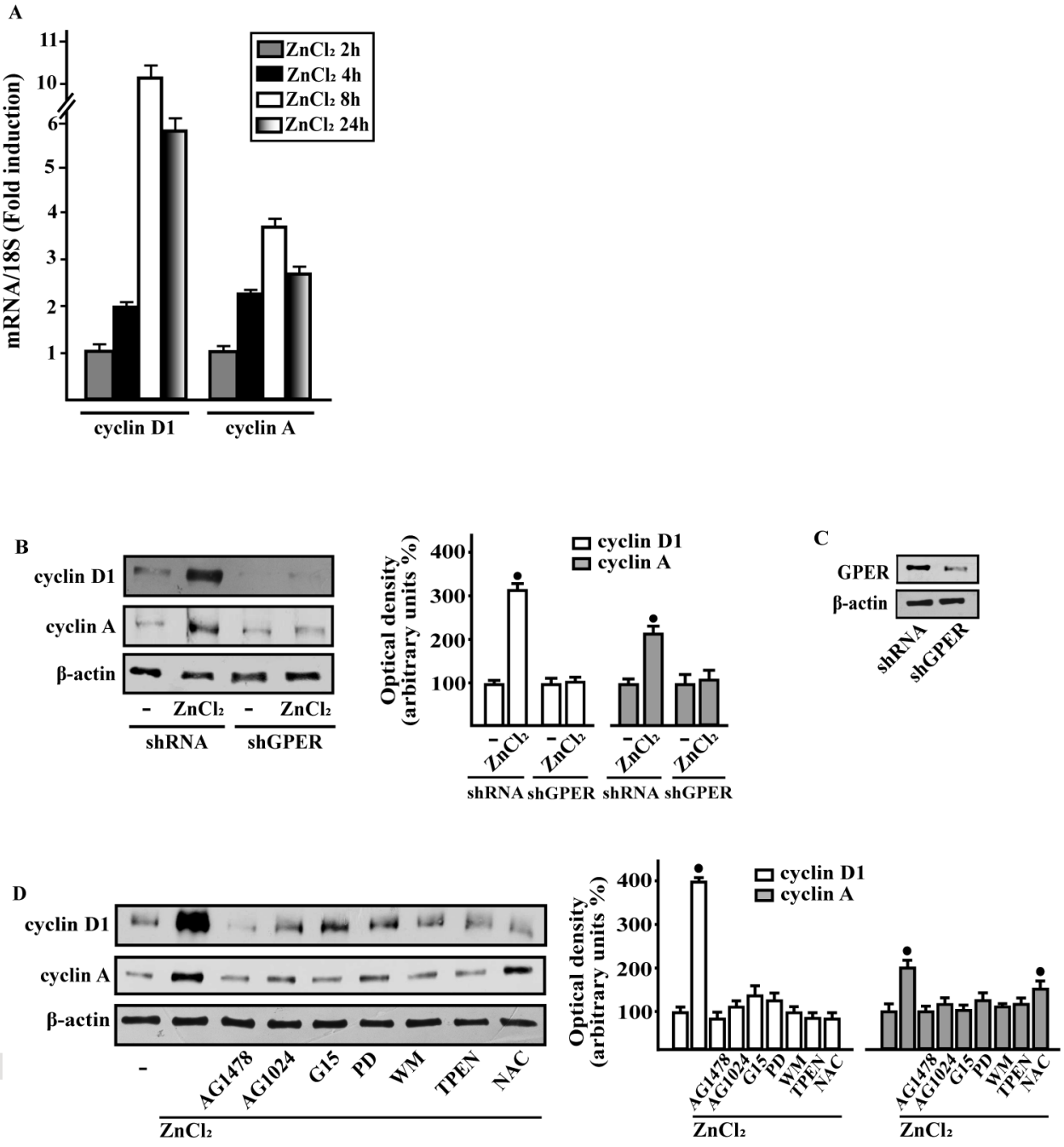
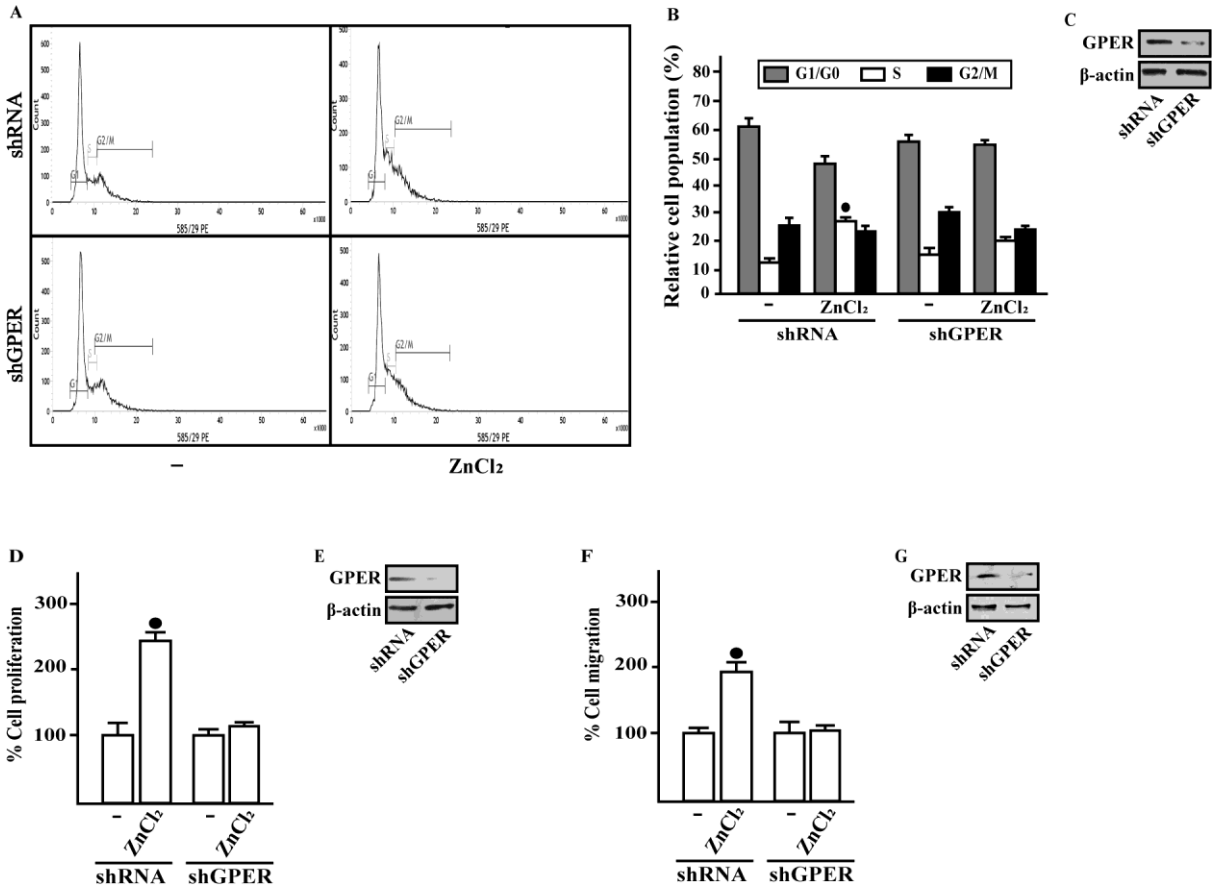


Fig. 5



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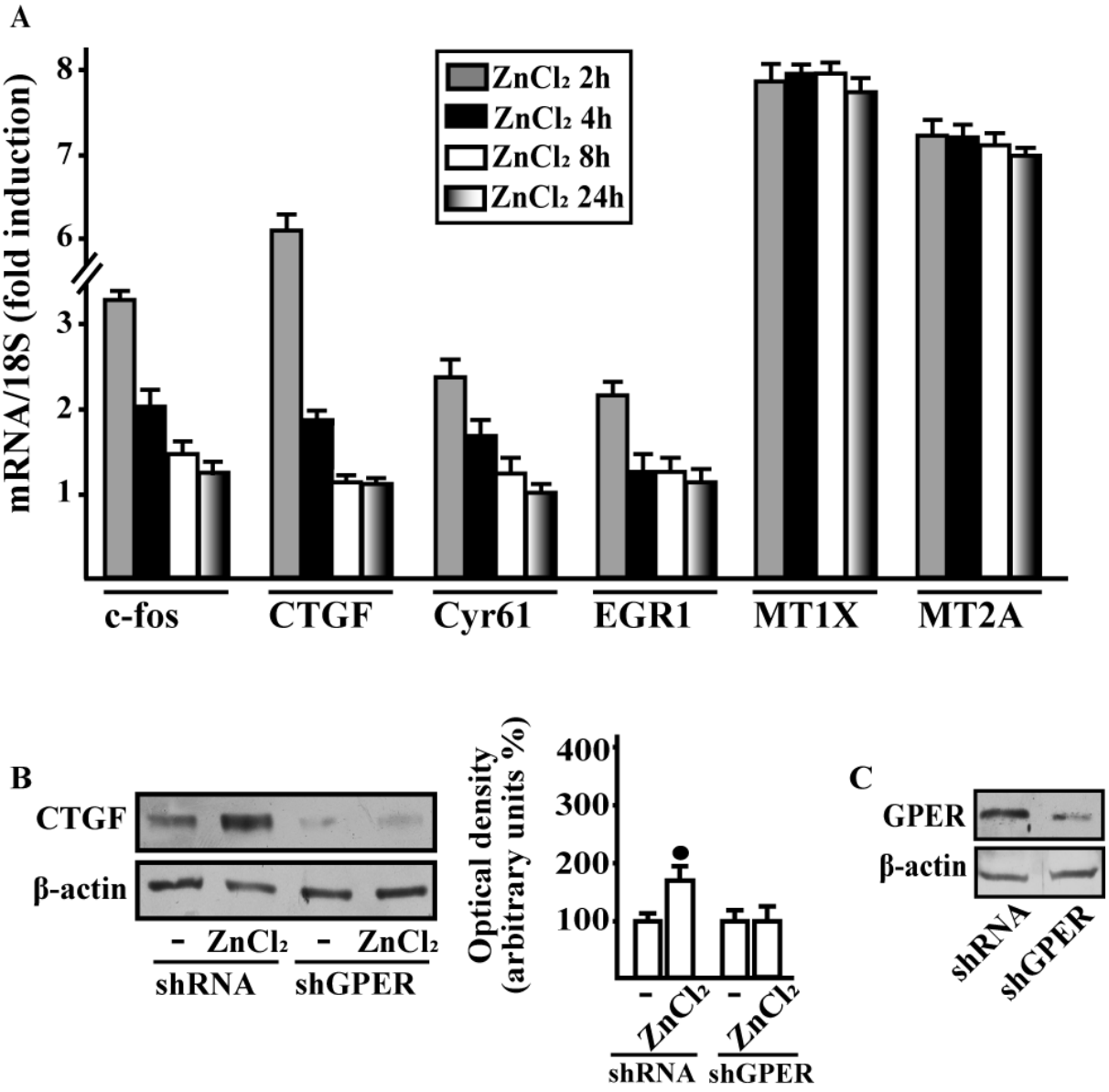

Fig. 7

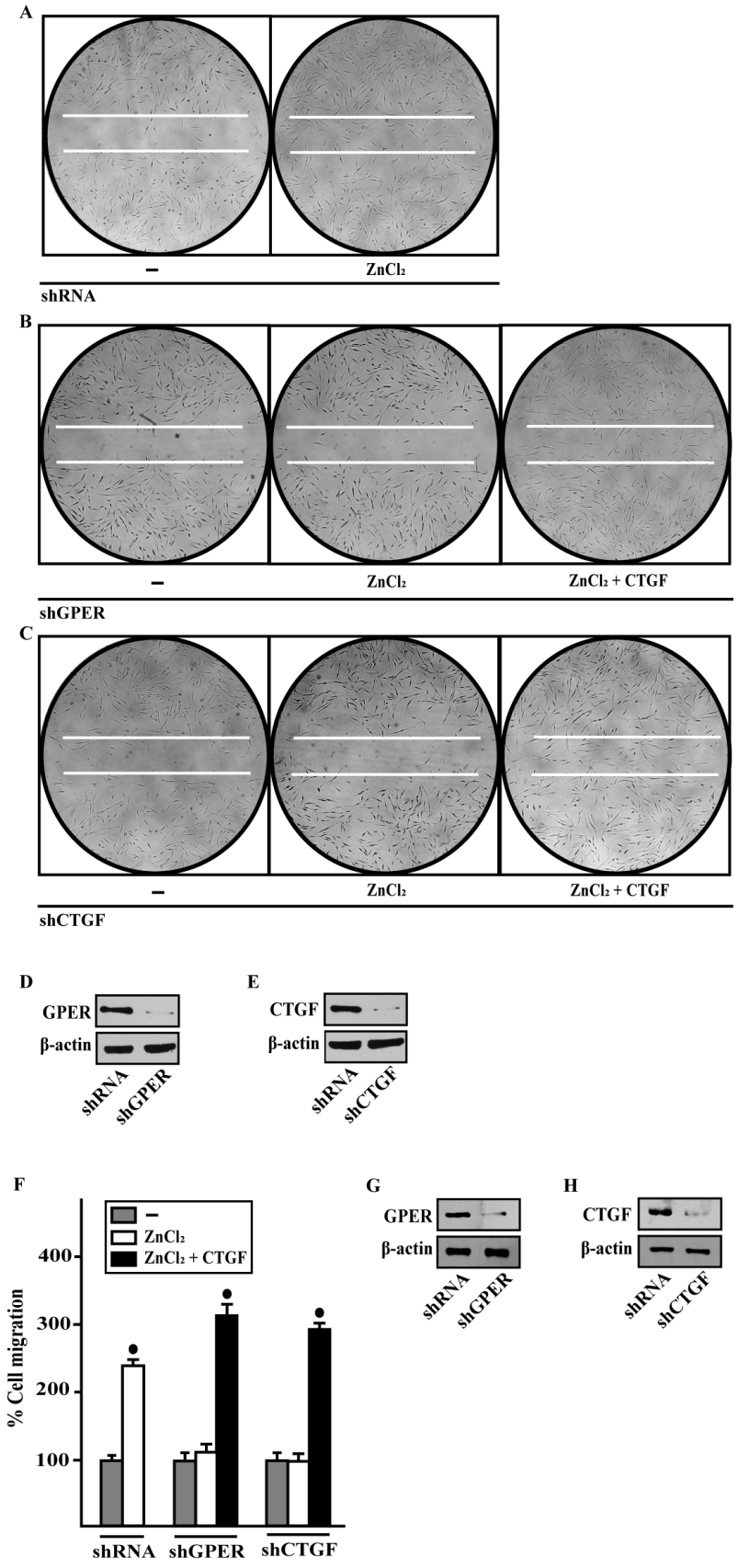

Fig. 8

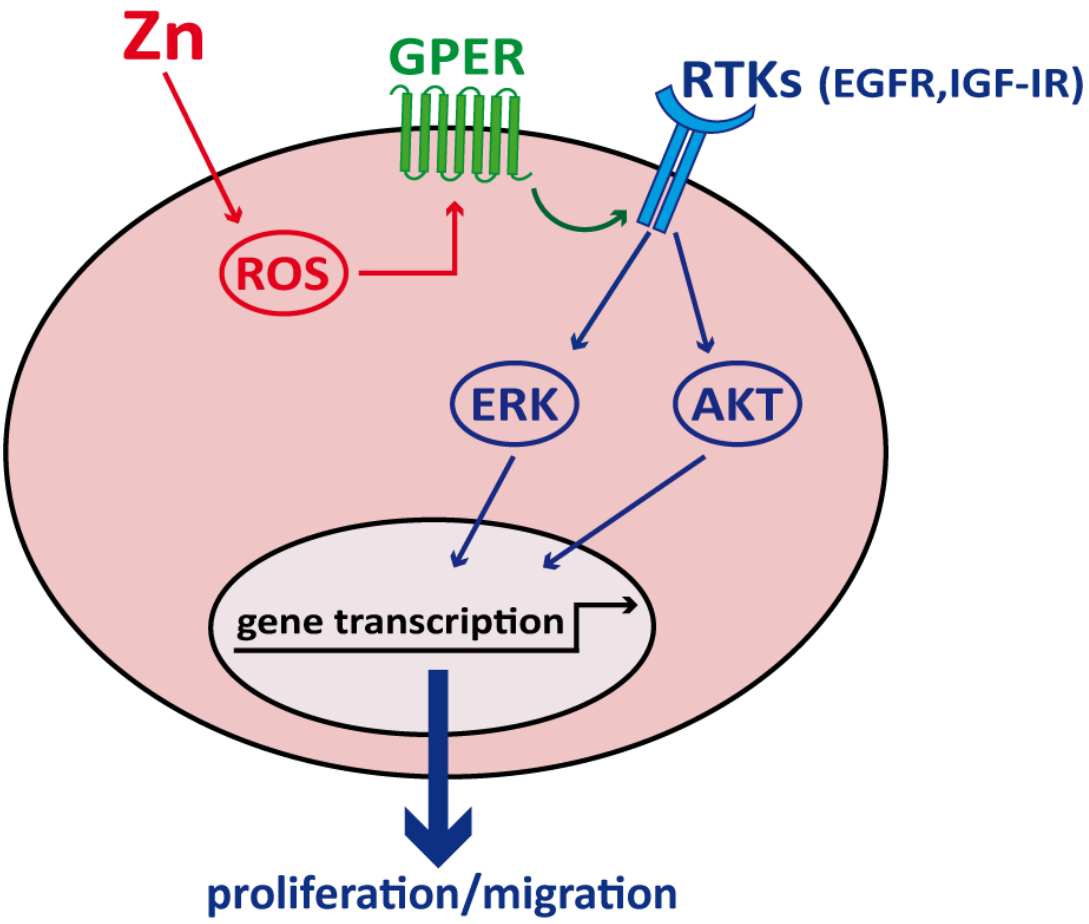

Fig. 9