GPER, IGF-IR, and EGFR transduction signaling are involved in stimulatory effects of zinc in breast cancer cells and...

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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 (ZnCl2) 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, ZnCl2 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 ZnCl2 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. This article is protected by copyright. All rights reserved

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. This article is protected by copyright. All rights reserved 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 (ZnCl2), zinc sulfate (ZnSO4), 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 ZnCl2, ZnSO4, 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 ZnCl2 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% CO2. 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 (1x105) 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, 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

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 (ZnCl2) 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 (ZnSO4) (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 ZnCl2 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 ZnCl2 may involve GPER. By co-immunoprecipitation studies performed in SkBr3 cells, we ascertained that ZnCl2 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 ZnCl2-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 ZnCl2 treatment (Fig. 2A-D). Next, we

investigated the mechanisms through which ZnCl2 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 ZnCl2. Likewise, the activation of ERK and AKT triggered by ZnCl2 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 ZnCl2. Moreover, the inhibitory effects elicited by TPEN and NAC indicate that the aforementioned responses triggered by ZnCl2 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 ZnCl2 (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 ZnCl2 exposure. Collectively, these observations indicate that ZnCl2 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).

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.

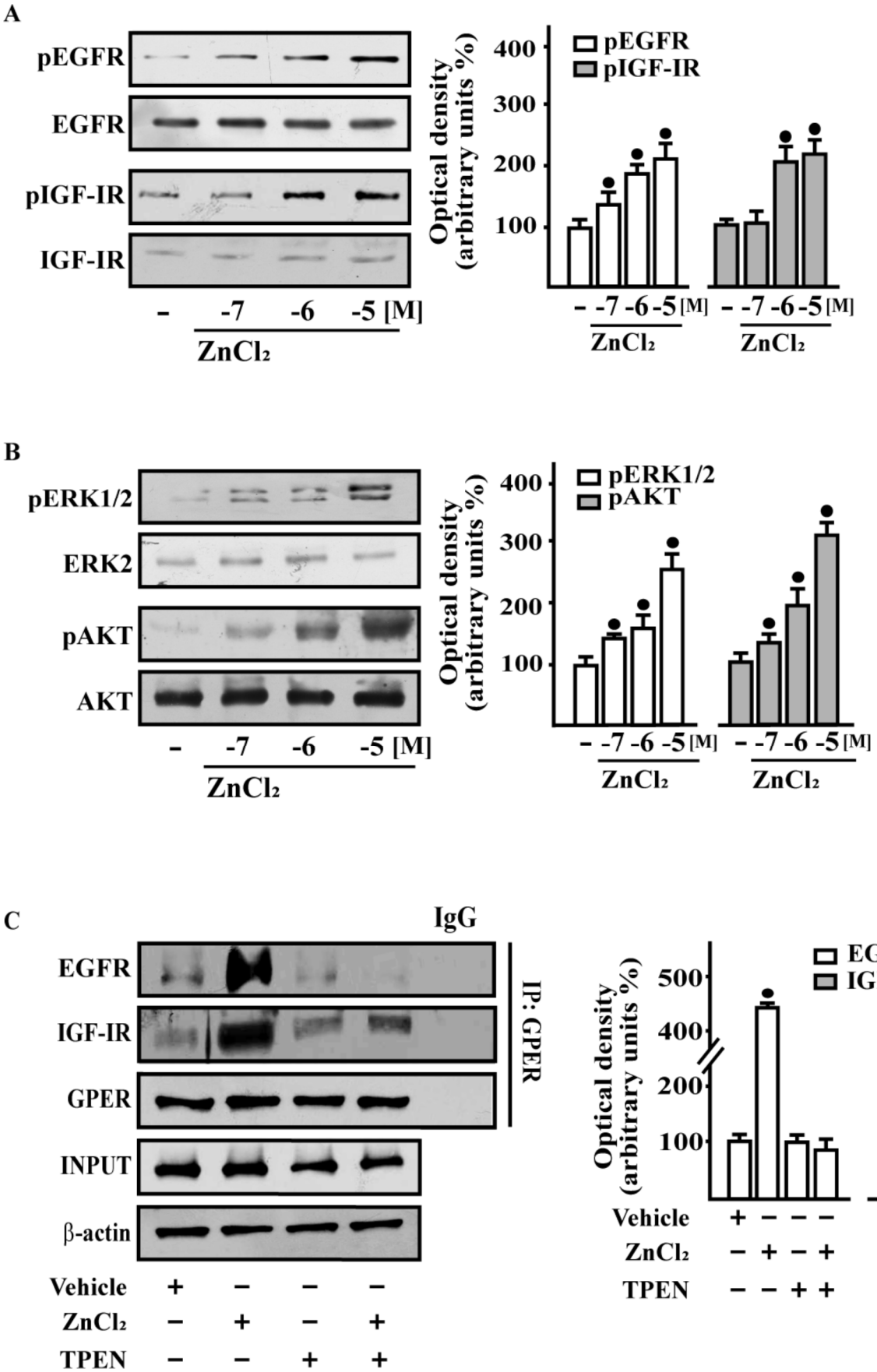
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Fig. 1

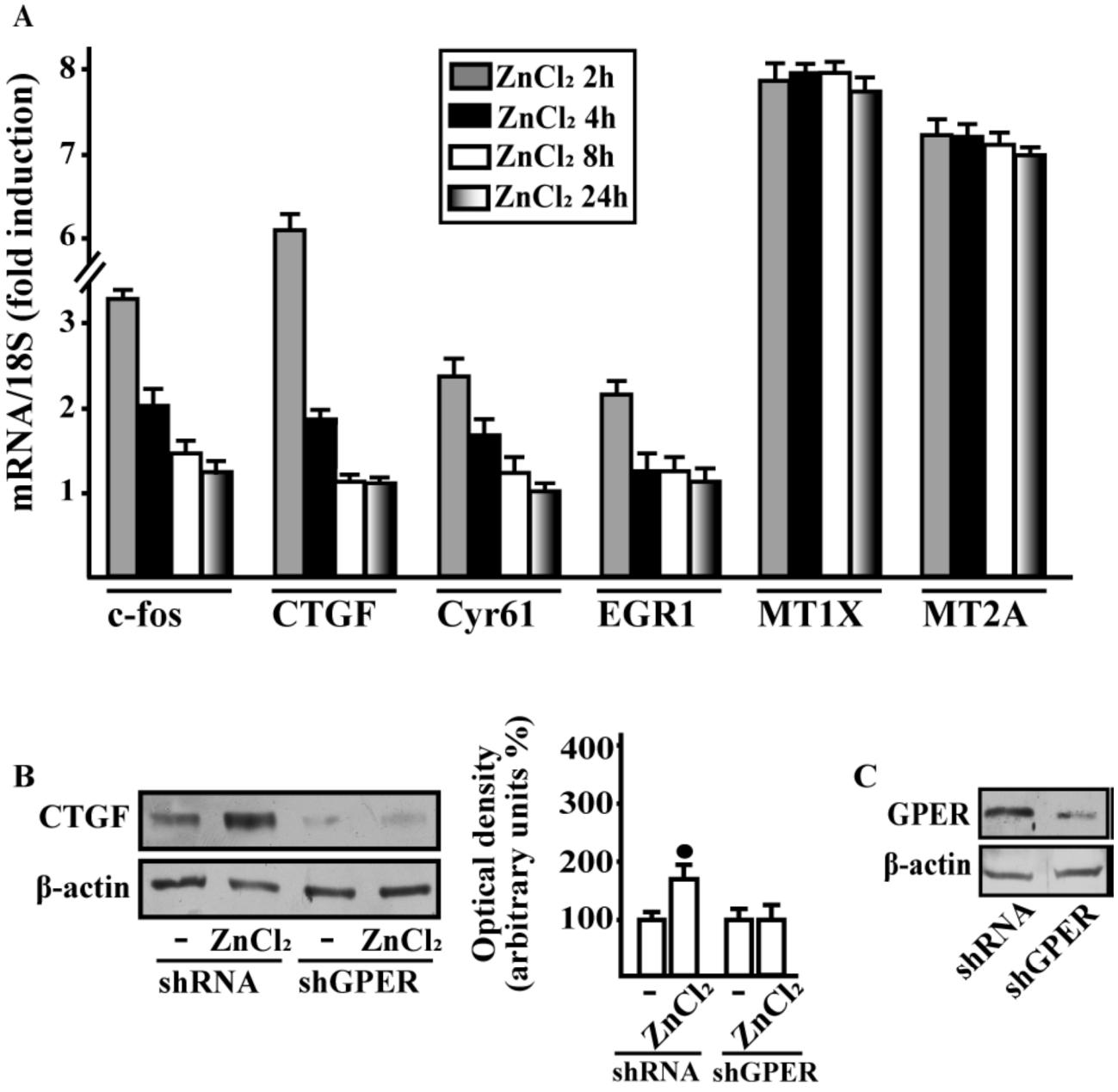
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Fig. 7