Troglitazone inhibits the migration and invasion of PC-3 human prostate cancer cells by upregulating E-cadherin and glutathione peroxidase 3

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Abstract. Troglitazone (TGZ) is a synthetic peroxisome proliferator-activated receptor γ (PPARγ) ligand that exhibits potential antitumor effects on a number of cancer subtypes, including prostate cancer. However, little is known about the effect of TGZ on metastasis in prostate cancer. The aim of the present study was to determine the inhibitory effect and mechanism underlying TGZ on cell growth, migration and invasion using the prostate cancer PC-3 cell line. Cellular migration and invasion were evaluated by performing a wound healing assay and Matrigel assay, respectively. The expression levels of mRNA and protein were determined by reverse transcription-quantitative polymerase chain reaction and western blotting. The results demonstrated that TGZ dose-dependently inhibited cell migration and invasion of PC-3 cells. The present study also revealed that TGZ increased the mRNA and protein levels of E-cadherin and glutathione peroxidase 3 (GPx3) in human prostate cancer PC-3 cells. In addition, GW9662, a PPARγ antagonist, attenuated the increased mRNA and protein levels of E-cadherin and GPx3, suggesting that the PPARγ-dependent signaling pathway was involved. Taken together, these results suggested that the anti-migration and anti-invasion effect of TGZ on PC-3 prostate cancer cells is, at least in part, mediated via upregulation of E-cadherin and GPx3. The present study also concluded that PPARγ may be used as a potential remedial target for the prevention and treatment of prostate cancer cell invasion and metastasis.

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Introduction

Prostate cancer is the most common non-cutaneous malignancy in males. It is also the second leading cause of cancer-associated mortality in the western world amongst males [1]. Androgen-deprivation therapy, a standard-of-care medicine for prostate cancer, is able to proficiently control the development of androgen-dependent tumors [2]. Numerous patients with localized disease have improved long-term survival and elevated cure rates with standard therapies; however, the cancer eventually becomes resistant to hormone deprivation and progresses to castration-resistant prostate cancer [2,3]. The patients with locally advanced and metastatic disease have poor prognoses, leading to high morbidity and mortality rates [2,3]. Therefore, understanding the mechanisms underlying cancer invasion and subsequent metastasis are urgently required to develop therapies for combating metastatic prostate cancer.

Thiazolidinediones (TZDs), including troglitazone and ciglitazone, are synthetic ligands of peroxisome proliferator-activated receptor γ (PPARγ) [4]. They exhibit potential antitumor effects on numerous types of cancers [4-10], including prostate cancer [11-13]. A previous study performed on prostate cancer cell lines has demonstrated that troglitazone (TGZ) decreases cellular proliferation, which is associated with increased expression levels of glutathione peroxidase 3 (GPx3) [11]. TGZ has been investigated in clinical trials against breast, colorectal and prostate cancer [14-16]. Treatment with TGZ in patients with advanced prostate cancer has been associated with long periods of stable disease characterized by the absence of new symptoms without new metastases [14], suggesting that TGZ may have clinical value in suppressing cancer metastasis. However, the role of TGZ in metastasis and the precise molecular mechanisms underlying its action have not been fully elucidated.

During cancer progression, epithelial-mesenchymal transition (EMT) is the main mechanism underlying the invasiveness and metastasis of cancer cells. EMT is a process that converts immotile epithelial cells to motile mesenchymal cells [17-19]. Downregulation of epithelial marker E-cadherin expression is the hallmark of the EMT process. E-cadherin is a tumor suppressor that serves crucial roles in cell-cell adhesion [19].

Loss of E-cadherin expression or function is associated with cancer cell invasion and metastasis (20).

The present study aimed to investigate the anti-invasive and anti-metastatic activities of TGZ and hypothesized that TGZ may act on various stages of the metastatic procedure to prevent cancer cells from metastasizing. The present study therefore determined the effect of TGZ on the reduction of cell invasive activity and also examined the effect of TGZ on the expression levels of E-cadherin and GPx3 in PC-3 human prostate cancer cells.

Materials and methods

Prostate cancer cell line. The human prostate cancer PC-3 cell line was acquired from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 95% air and 5% CO_2. TGZ (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and GW9662 (Cayman Chemical Company, Ann Arbor, MI, USA) were dissolved in 100% ethanol (Merck KGaA) to obtain a concentration of 16 mM. The final ethanol concentration in the solution was 0.25%. Ethanol alone at the same final concentration of 0.25% was used as the control.

Cellular viability assay. The viability of PC-3 cells was analyzed using an MTT (Sigma-Aldrich; Merck KGaA) assay based on the ability of live cells to convert tetrazolium salt into purple formazan. Briefly, PC-3 cells were seeded into 96-well cell culture plates at a density of $8x10^3$ cells/well in 200 μl media. Following 24 h incubation at 37°C, the medium was replaced with 1% FBS-RPMI medium at 37°C for 24 h. Cells were treated with TGZ at concentrations of 1, 10 and 40 μM or vehicle (ethanol) control and cultured at 37°C for 48 h. The medium was then replaced with 100 μl of MTT (diluted to 1 mg/ml in FBS-free medium, from a stock solution of 10 mg/ml) and incubated at 37°C for 3 h. The supernatant was removed and 100 μl of DMSO was supplemented to each well to dissolve the formazan crystals. Plates were agitated at room temperature for 5 min. The absorbance was read at 540 nm on an Epoch BioTek microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All treatments were performed in triplicate.

Cellular migration assay. PC-3 cell motility was analyzed using an *in vitro* wound-healing assay. PC-3 cells were seeded into six-well cell culture plates at a density of $8x10^4$ cells/well and grown until confluent at 37°C. Monolayers of confluent PC-3 cells were then wounded using a pipette tip. Cell repair was monitored using an inverted microscope (Olympus IX70, Tokyo, Japan) following 24 h exposure at 37°C to TGZ at concentrations of 1, 10 and 40 μM or the vehicle (ethanol) control. The widths of the wounded areas were measured by ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA), and the relative wound closure ratios of the distance to that at 0 h were calculated at 6, 9, 12 and 24 h. All treatments were performed in triplicate.

Cellular invasion assay. PC-3 cellular invasion was determined using a Transwell insert. Briefly, 8 μm Transwell inserts (SPL Life Sciences, Pocheon, Korea) were coated with Matrigel (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37°C for 2 h to become gelatinous. Cells were grown to subconfluence, detached by trypsinization, washed twice with PBS and resuspended in serum free RPMI medium. Subsequently, the cells added to the Transwell insert ($2x10^4$ cells/insert) at a final concentration of $1x10^5$ cells/ml together with TGZ at concentrations of 1, 10 and 40 μM or the vehicle (ethanol) control. Cell-free RPMI medium was added to the lower chamber. Assays were incubated at 37°C for 30 h until cells had migrated to the bottom of the membrane. Non-invasive cells in the upper surface of the membrane were removed with a cotton swab. The remaining cells on the membrane were fixed in methanol for 10 min at room temperature, stained with hematoxylin for 10 min at room temperature and washed with PBS. Invaded cells were counted in four fields per insert with a light microscope at x100 magnification. Cells were assessed for their relative invasion ability as percentages of the vehicle (ethanol) controls. All treatments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from PC-3 cells using a Hybrid-R RNA extraction kit (GeneAll Biotechnology, Seoul, South Korea), and then cDNA was synthesized using a M-MLV cDNA Synthesis kit (Enzynomics, Daejeon, South Korea) according to the manufacturer's protocol. RT-qPCR was performed using TOPreal™ qPCR 2X PreMIX (SYBR Green with high ROX; Enzynomics) on a CFX Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction conditions were as follows: Pre-incubation at 94°C for 10 min, followed by 40 cycles (94°C for 10 sec, 58°C for 20 sec, 72°C for 30 sec) ending with a melting curve program with temperature rising from 60 to 95°C. The specificity of the reactions was verified by melting curve analysis. The primers used were: Human GPx3 sense, 5'-ACATGCCTACAGGTATGCGT-3 and antisense, 5'-GAGCAGAACAATTGGACCTA-3'; human E-cadherin sense, 5'-TTGCTACTGGAACAGGGACACT-3' and antisense 5'-GGAGATGTATTGGGAGGAAGGTC-3'; and human β-actin sense, 5'-CATGTACGTTGCTATCCAGGC-3' and antisense, 5'-CTCCTTAATGTCACGCACGAT-3'. The ratio of target gene fold-change was normalized to human β-actin expression level using the comparative $2^{-\Delta\Delta Cq}$ method (21). All treatments were performed in triplicate.

Western blot analysis. Cell lysates were subjected to SDS-PAGE (on 10% acrylamide gel), transferred to nitrocellulose membranes and probed with mouse monoclonal anti-E-cadherin primary antibody (sc-8426, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:1,000 dilution), mouse monoclonal anti-GPx3 primary antibody (ab27325, Abcam, Cambridge, MA, USA; 1:500 dilution) and goat polyclonal anti-actin primary antibody (sc-1616, Santa Cruz Biotechnology, Inc.; 1:1,000 dilution). Blots were incubated with horseradish peroxidase-conjugated anti-mouse Ig secondary antibodies (61-0120, Zymed; Thermo Fisher Scientific, Inc.; 1:2,000 dilution) and anti-goat Ig (81-1620, Zymed; 1:2,000 dilution). Then, the antibody reactive bands were developed

using a chemiluminescent substrate (DoGEN, Seoul, South Korea). Blots were quantified with ImageJ software (version 1.48; National Institutes of Health), and the relative ratio was calculated.

Statistical analysis. All data are presented as the mean ± standard error. Statistical significance was further analyzed by Student's t-test using Excel 2010 (Microsoft Corporation, Redmond, WA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with TGZ decreased cellular viability of PC-3 cells. To examine the effect of TGZ on the cellular viability of PC-3 cells, the present study treated these cells with 1, 10 or 40 μM TGZ for 0, 24 or 48 h, and determined the rate of cell survival using an MTT assay. Results are presented in Fig. 1. TGZ treatment decreased cellular viability in a dose-dependent manner. Compared with the control cells, cells treated with 10 and 40 μM TGZ for 24 h demonstrated decreases in cell growth rates by 20.49 and 34.69%, respectively.

TGZ inhibits cell migration and invasion of PC-3 cells. The present study evaluated cell migration and invasion of PC-3 cells treated with TGZ by *in vitro* wound healing and Matrigel assays. The results are presented in Figs. 2 and 3, respectively. Following incubation with various concentrations of TGZ for 24 h, the migration of PC-3 cells to the denuded area was inhibited in a dose-dependent manner (Fig. 2), indicating that TGZ significantly suppressed the motility of PC-3 cells.

Results of the Matrigel assay revealed that TGZ inhibited the invasion of PC-3 cells through the Matrigel-coated filter pores in a dose-dependent manner (Fig. 3). Treatment with 1, 10 and 40 μM TGZ inhibited 54.27, 65.63, and 73.70% of cell invasion, respectively. These results demonstrated that TGZ significantly inhibited the invasion of PC-3 cells. Taken together, these results revealed that TGZ suppressed cellular migration and invasion of PC-3 cells.

TGZ increases the mRNA levels of E-cadherin and GPx3 in PC-3 human prostate cancer cells. To investigate whether TGZ could enhance the transcriptional levels of E-cadherin and GPx3 genes in PC-3 cells, RT-qPCR was performed. The results are presented in Fig. 4. TGZ treatments of 1 and 10 μM significantly increased the mRNA expression levels of E-cadherin in a dose-dependent manner (Fig. 4A). A TGZ treatment of 10 μM also significantly increased the mRNA expression levels of GPx3 (Fig. 4B).

Although TGZ was able to affect the transcriptional expression levels of E-cadherin and GPx3, the mechanisms underlying these effects remain unclear. To prove whether these results were dependent on the activation of PPARγ, GW9662 (a PPARγ antagonist) was used to inhibit the function of PPARγ in PC-3 cells. TGZ-induced upregulation of E-cadherin and GPx3 mRNAs in PC-3 cells were decreased following treatment with GW9662 (Fig. 4). These results demonstrated that TGZ increased the transcriptional expression levels of E-cadherin and GPx3 in PC-3 cells in a PPARγ-dependent manner.
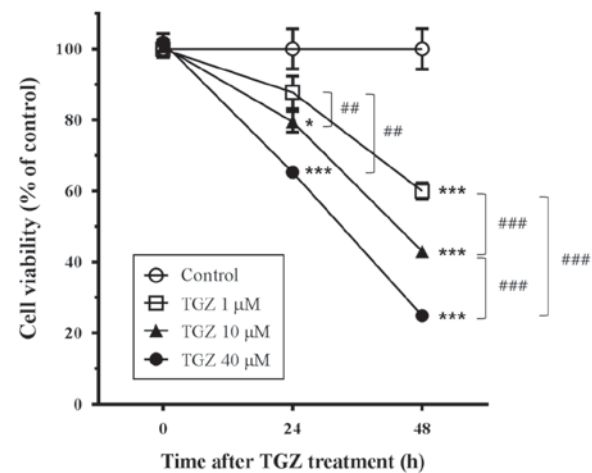

Figure 1. Effect of TGZ on the proliferation of PC-3 cells. TGZ inhibited the proliferation of PC-3 cells. The absorbance values of PC-3 cells treated with 1, 10 and 40 μM TGZ were read at 540 nm using a plate reader. The cell viability of cells was determined based on the proliferation of PC-3 cells compared with the proliferation of the vehicle control group. *$P < 0.05$ and ***$P < 0.001$ vs. the vehicle (ethanol) control. ##$P < 0.01$ and ###$P < 0.001$, comparisons exhibited on graph. Results are presented as the mean ± standard error of the mean. TGZ, troglitazone.

TGZ increases the protein levels of E-cadherin and GPx3 in PC-3 human prostate cancer cells. The protein expression levels of E-cadherin and GPx3 in PC-3 cells were determined by western blot analysis following 48 h treatment with 0, 1 or 10 μM TGZ. Results are presented in Fig. 5. TGZ at 1 and 10 μM significantly increased the protein expression levels of E-cadherin in a dose-dependent manner (Fig. 5A and B). TGZ at 10 μM also significantly increased the protein expression levels of GPx3 (Fig. 5A and C). TGZ-induced upregulation of E-cadherin and GPx3 protein expression levels in PC-3 cells were decreased following the addition of GW9662 (Fig. 5). Thus, TGZ upregulated E-cadherin and GPx3 protein expression levels in a PPARγ-dependent manner, which was consistent with its upregulating effect on E-cadherin and GPx3 mRNA expression levels.

Discussion

TZDs are a novel class of antidiabetics and are specific ligands for PPARγ (22). PPARγ is expressed at various levels depending on the type of tissue and carcinoma (23). PPARγ has been investigated as a therapeutic target for cancer treatment. Certain previous studies have reported that PPARγ is able to induce anti-proliferative, anti-angiogenic and pro-differentiation signaling pathways in specific tissue types, thus serving a role in the pathogenesis and progression of various types of cancer, including prostate cancer (24,25). Using TZDs as PPARγ ligands, previous studies have investigated the effect of PPARγ on the metastatic potential and investigated its underlying mechanisms (26-34). TZDs have been demonstrated to be able to suppress cellular migration, invasion and metastasis of cancer in the colon, liver, breast, lung, bladder and prostate gland (26-34). For example, in colon cancer, TZD inhibited the development and metastasis of HT-29 human colon cancer cells via its differentiation-promoting effects both *in vivo* and *in vitro* by involving the modulation of the E-cadherin/β-catenin
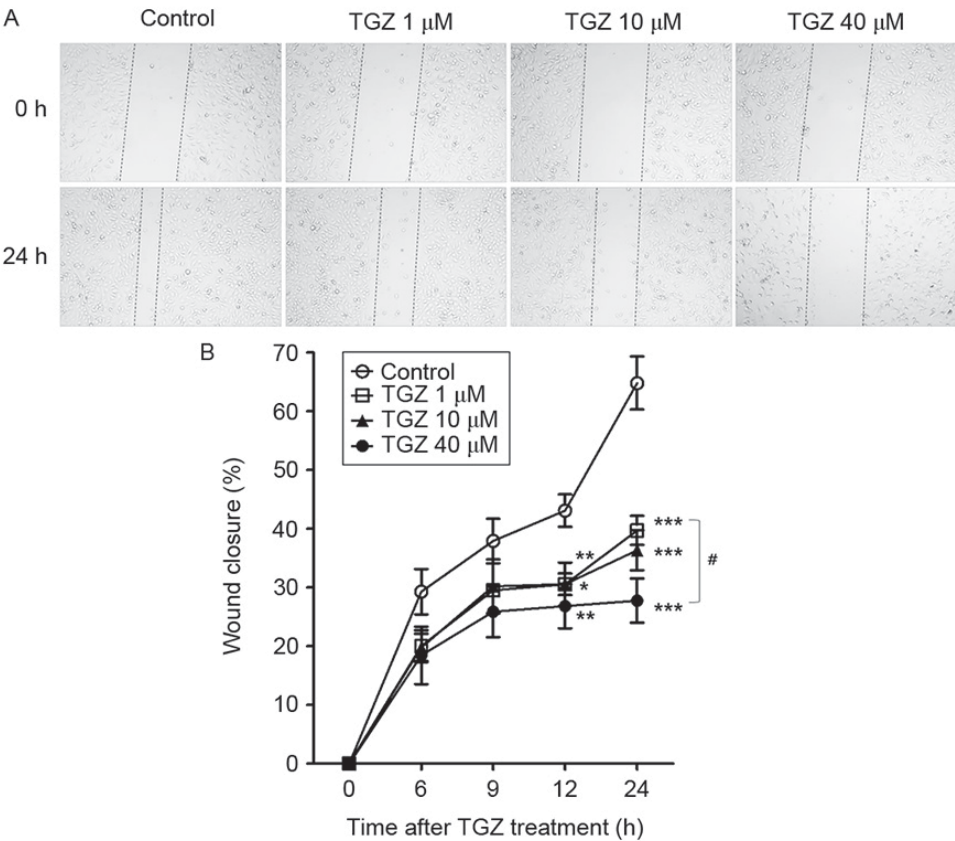
Figure 2. Effect of TGZ on the migration of PC-3 cells. TGZ suppressed the migration of PC-3 human prostate cancer cells. (A) Cells that migrated to the wounded region were imaged (magnification, x40). (B) Migration ability of PC-3 cells treated with 1, 10 and 40 μM TGZ was determined by wound healing assay. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle control at each time point. #P<0.05, comparison exhibited on graph. Results are presented as the mean ± standard error of the mean. TGZ, troglitazone.
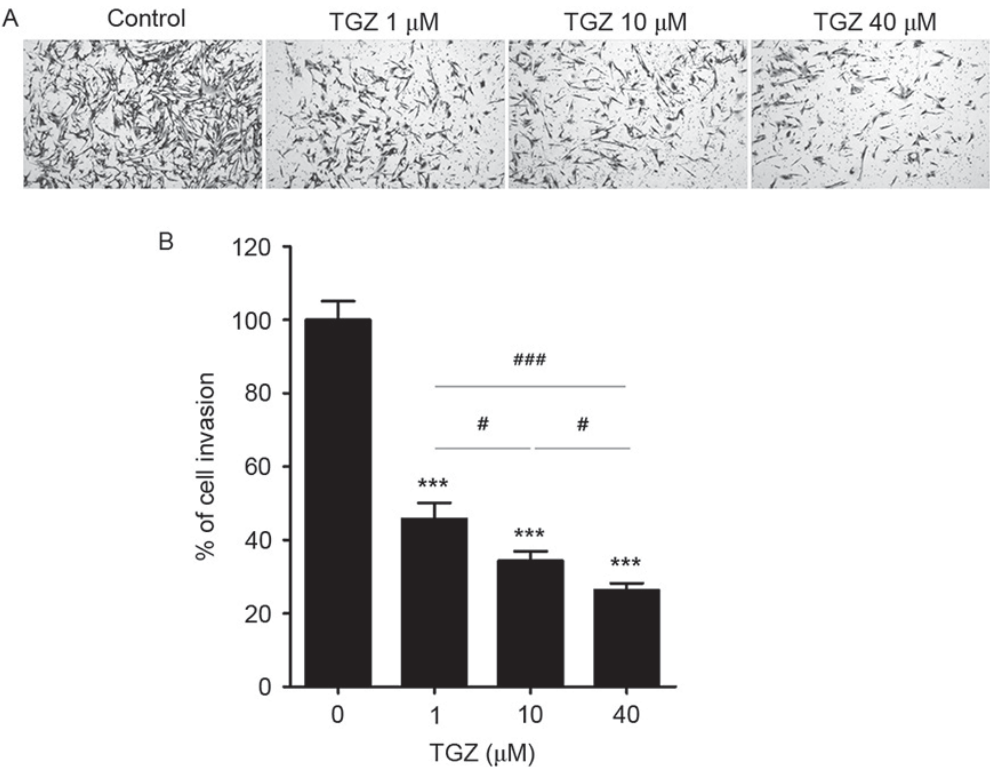

Figure 3. Effect of TGZ on the invasion of PC-3 cells. TGZ suppressed the invasion of PC-3 human prostate cancer cells. (A) Invaded cells were imaged (magnification, x100). (B) Invasion capacity was examined using a Transwell insert coated with Matrigel. ***P<0.001 vs. the vehicle control. #P<0.05 and ###P<0.001, comparisons exhibited on graph. Results are presented as the mean ± standard error of the mean. TGZ, troglitazone.
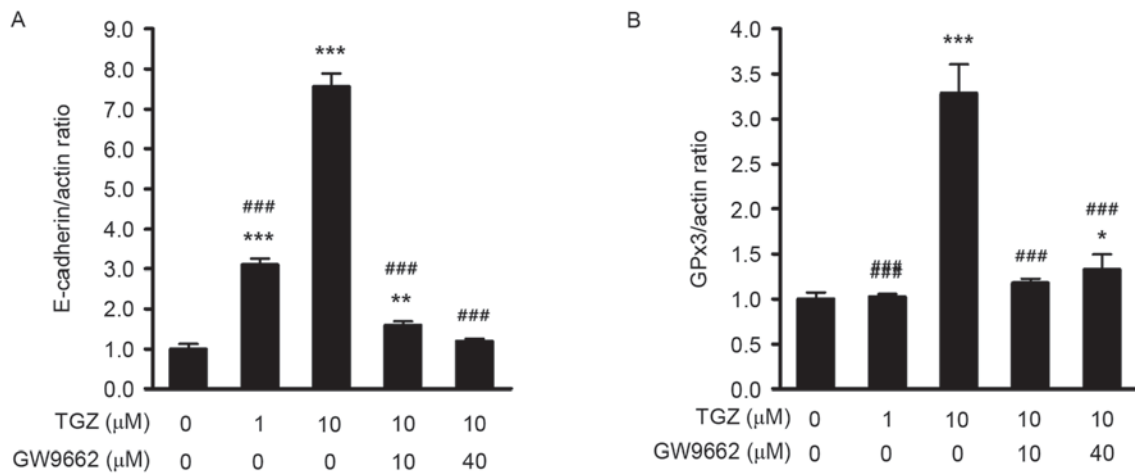
Figure 4. Effect of TGZ on mRNA expression levels of E-cadherin and GPx3 in PC-3 cells. TGZ upregulated the mRNA expression levels of E-cadherin and GPx3 in PC-3 cells. PC-3 cells were treated with various concentrations of TGZ (0, 1 or 10 μM) for 24 h prior to harvesting. (A) Total RNA was extracted and the expression level of E-cadherin was evaluated by RT-qPCR. TGZ at 1 and 10 μM increased the mRNA expression levels of E-cadherin in a dose-dependent manner. (B) GPx3 expression level was evaluated by RT-qPCR. TGZ at 10 μM increased the mRNA expression level of GPx3. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. the vehicle control. ###$P<0.001$ vs. the 10 μM TGZ treatment group. Results are presented as the mean ± standard error of the mean. TMZ, troglitazone; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
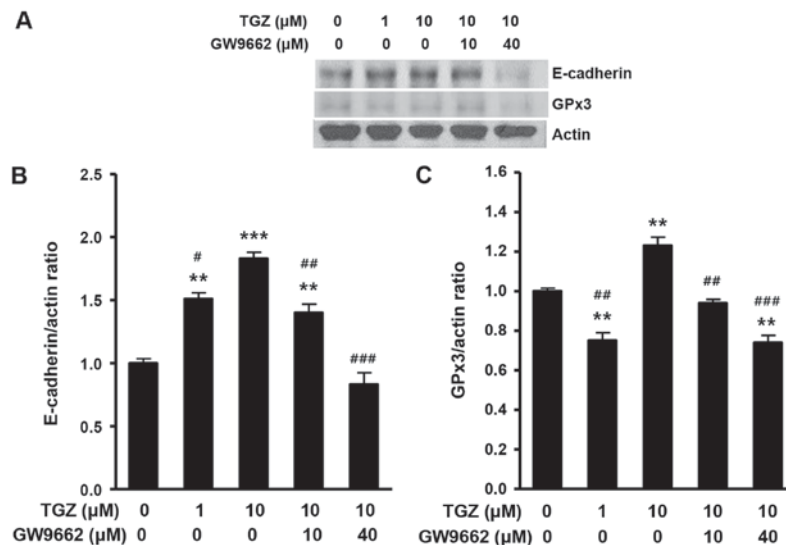

Figure 5. Effect of TGZ on protein expression levels of E-cadherin and GPx3 in PC-3 cells. TGZ increased the protein expression levels of E-cadherin and GPx3 in PC-3 cells. (A) Western blot analysis of the PC3 cell line. Protein expression levels of (B) E-cadherin and (C) GPx3 were quantified using actin as the normalization control. **$P<0.01$ and ***$P<0.001$ vs. the vehicle control. #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ vs. the 10 μM TGZ treatment group. Results are presented as the mean ± standard error of the mean. TMZ, troglitazone.

system (26). In hepatocellular carcinoma, the suppression of cellular invasion and migration mediated by PPARγ has been revealed to be mediated via downregulation of matrix metalloproteinases, by and increased expression levels of TIMP metallopeptidase inhibitor 3 and E-cadherin (28,29).

However, the mechanisms underlying the inhibitory action of TZDs on cell invasion in prostate cancer remain unclear. To better comprehend the molecular mechanisms involved in TGZ-induced inhibition of cell invasion, the present study analyzed the effect of TGZ on the expression levels of E-cadherin and GPx3. E-cadherin and GPx3 may be involved in cancer cell invasion. The present study demonstrated that TGZ reduced cell migration and invasion of PC-3 cells. Consistently, TGZ positively regulated the mRNA and protein expression

levels of E-cadherin and GPx3 in a PPARγ-dependent manner. The results from the present study may provide preliminary evidence for further studies investigating the mechanisms underlying the suppression of metastasis by TZDs.

A number of human solid tumors are carcinomas originating from various epithelial cell types (18,19). Tumor cells change from a highly differentiated epithelial morphology to a migratory and invasive phenotype during the conversion from a benign tumor to a malignant invasive tumor (18). This process of EMT can induce the loss of cell-cell contacts and an increase in cell motility (18,35). It also induces the dissemination of single tumor cells from primary epithelial tumors to distant organs (19). These alterations are necessary for invasion. Throughout the EMT process, numerous genes involved

in cellular adhesion, migration and invasion and mesenchymal differentiation are transcriptionally regulated (19). Amongst these genes, loss of adhesive function of E-cadherin in epithelial cells has been suggested to be a hallmark of EMT and metastatic carcinoma (17,19,20,35).

E-cadherin formation is preserved in the majority of differentiated tumors, including carcinomas of the skin, head and neck, breast, lung, liver, colon and prostate (17-20). However, E-cadherin expression levels demonstrated an inverse correlation with cancer grade or patient survival (17). In a number of human and mouse types of cancer, carcinoma progression and poor prognosis have been revealed to be associated with partial loss of E-cadherin expression (19). Increasing E-cadherin function in cancer cells and mouse cancer models suppresses the ability to invade and metastasize (19). Conversely, disrupted expression of E-cadherin in noninvasive tumor cells induces invasive and metastatic activities (18,19). In the present study, an increase in E-cadherin expression level induced increased cell-cell interactions and decreased motility of PC-3 cells following TGZ treatment. Therefore, changing the functional protein expression levels of E-cadherin may be an alternative approach for cancer therapy.

GPx3 belongs to the family of glutathione peroxidases (36). It is well known that glutathione peroxidases are among the most important reactive oxygen species scavengers that protect cells from oxidative damage (36). Downregulation of GPx3 by hypermethylation has been revealed in numerous types of cancer (37-45), including prostate cancer (11,46,47). In prostate cancer, a negative correlation between GPx3 expression levels and poor clinical outcomes has been demonstrated (47,48). Forced expression of GPx3 in prostate cancer cell lines is able to suppress colony formation and cellular proliferation (11,47,49). Xenografts with higher GPx3 expression levels have resulted in reduced tumor volume, metastasis and animal mortality (47). Our previous study revealed that GPx3 is downregulated in prostates of TRAMP mice and that disruption of GPx3 expression in TRAMP mice increased prostate cancer development and metastasis (50). It has been demonstrated that silencing GPx3 expression promoted cancer metastasis in human thyroid and gastric cancer cells (37,38). In the present study, it was notable that GPx3 expression level was upregulated following treatment with TGZ in human prostate cancer cells. This induction was closely associated with the anti-invasion effect of TGZ. All these results suggested that GPx3 acted as a negative regulator of prostate tumor development and metastasis, producing further evidence for its role as a tumor suppressor.

In conclusion, the present study revealed that TGZ effectively abrogated the migration and invasion of PC-3 cells in vitro by increasing the expression levels of E-cadherin and GPx3. Upregulated expression levels of E-cadherin and GPx3 is a possible mechanism underlying the anti-migration and anti-invasion effects of TGZ. Altered expression levels induced by TGZ may account for the mechanisms underlying the invasion inhibition ability of TZDs and its mode of action.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SC performed all experiments, analyzed the data and wrote the manuscript. JL contributed to the cell culture and RT-qPCR. HO, UK, and BR participated in the MTT assay, wound healing assay and data collection. JP conceived the idea, interpreted the data and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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