

# 15d-PGJ<sub>2</sub> inhibits NF- $\kappa$ B and AP-1-mediated MMP-9 expression and invasion of breast cancer cell by means of a heme oxygenase-1-dependent mechanism

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Activation of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) serves as a key factor in the proliferation and invasion of breast cancer cells and is a potential therapeutic target for breast cancer. However, the mechanisms underlying this effect remain largely unknown. Heme oxygenase-1 (HO-1) is induced and over-expressed in various cancers and is associated with features of tumor aggressiveness. Recent studies have shown that HO-1 is a major downstream target of PPAR $\gamma$ . In this study, we investigated the effects of induction of HO-1 by PPAR $\gamma$  on TPA-induced MMP-9 expression and cell invasion using MCF-7 breast cancer cells. TPA treatment increased NF- $\kappa$ B /AP-1 DNA binding as well as MMP-9 expression. These effects were significantly blocked by 15d-PGJ<sub>2</sub>, a natural PPAR $\gamma$  ligand. 15d-PGJ<sub>2</sub> induced HO-1 expression in a dose-dependent manner. Interestingly, HO-1 siRNA significantly attenuated the inhibition of TPA-induced MMP-9 protein expression and cell invasion by 15d-PGJ<sub>2</sub>. These results suggest that 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 expression and invasion of MCF-7 cells by means of a heme oxygenase-1-dependent mechanism. Therefore, PPAR $\gamma$ /HO-1 signaling-pathway inhibition may be beneficial for prevention and treatment of breast cancer. [BMB Reports 2020; 53(4): 212-217]

## INTRODUCTION

Breast cancer is the major cause of cancer death in women worldwide. The high prevalence of breast cancer and the limited options for treatment provide an obvious rationale for discovering new molecular targets that can be pharmacologically modulated. Recent evidence suggests that matrix metalloproteinases (MMPs) may play a role in breast cancer initiation and growth (1-3). Key genes involved in breast cancer metastasis, such as MMP, have been the focus of research into targets for cancer breast treatment.

Phorbol esters bind to protein kinase C (PKC) in a way similar to that of its natural ligand, diacylglycerol, and activate the kinase (4, 5). The phorbol ester is 12-O-tetradecanoylphorbol-13-acetate (TPA), also called phorbol-12-myristate-13-acetate (PMA), which is used as a biomedical tool for research. Recently it has been found that TPA activates integrin signaling pathway (6, 7), which may be activated by some carcinogens.

PPAR $\gamma$  is one of nuclear receptor subfamily that includes receptors for thyroid, steroid, and retinoid hormones. PPAR $\gamma$  from heterodimers with retinoid receptors and these dimers regulate various genes (8). Many recent papers have reported that modulations of PPAR $\gamma$  control the growth of human cancers, such as breast cancer (9-11). One of the earliest events in the metastasis of cancer cells is expression of the  $\gamma$  isoform of PPAR. Thus, PPAR $\gamma$  control may have significant promise for breast cancer prevention. Recently, PPAR $\gamma$  ligands were shown to inhibit the growth of a variety of transformed cells (9, 12, 13); hence signals that modulate PPAR $\gamma$  activity may serve a primary role in regulating breast cancer metastasis and may be major targets for treatment of breast cancer.

Endogenous 15-Deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) has been identified as a ligand of PPAR $\gamma$ . 15d-PGJ<sub>2</sub> inhibited the invasiveness of breast cancer cells by upregulating a tissue inhibitor of MMP-1 (14). A recent study has shown that heme oxygenase-1 (HO-1) overexpression in MCF-7 cells inhibits MMP expression, indicating that HO-1 plays a pivotal role in the

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invasion of breast cancer cells (15). These results suggest that PPAR $\gamma$  ligands control invasion and MMP expression of human breast cancer cells by means of HO-1. In the present study, we examined the role of HO-1 in the action of 15d-PGJ<sub>2</sub> on the invasion and MMP expression of breast cancer cells.

## RESULTS

### Effect of 15d-PGJ<sub>2</sub> on MMP-9 expression in MCF-7 cells

We treated MCF-7 cells with 15d-PGJ<sub>2</sub> (0-5  $\mu$ M) for 24 h, and toxicity was detected using an MTT assay. Treatment with 15d-PGJ<sub>2</sub> did not change MCF-7 cell viability (data not shown). Therefore, we used non-toxic concentrations of 2.5 and 5  $\mu$ M in the experiments. The range of non-toxic concentrations was applied in all subsequent experiments. Gelatin zymography showed that 15d-PGJ<sub>2</sub> suppressed TPA-induced MMP-9 secretion in a dose-dependent manner. Western blotting and real-time PCR revealed that 15d-PGJ<sub>2</sub> suppressed TPA-induced MMP-9 expression at both mRNA and protein levels (Fig. 1A and B). The luciferase assay showed that 15d-PGJ<sub>2</sub>, a known PPAR $\gamma$  agonist, suppressed TPA-induced MMP-9 promoter activity in MCF-7 cells (Fig. 1C). We next examined whether the inhibitory effect of 15d-PGJ<sub>2</sub> on MMP-9 expression depended on PPAR $\gamma$ . In MCF-7 cells treated with 15d-PGJ<sub>2</sub>, inhibition of TPA-induced MMP-9 expression was recovered by the PPAR $\gamma$  anta-

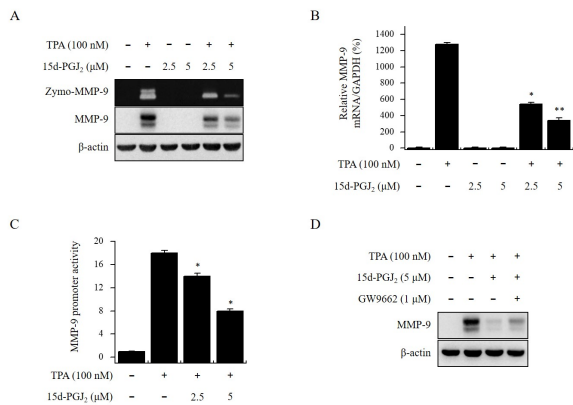
gonist GW9662 (Fig. 1D). These results indicate that the inhibition of TPA-induced MMP-9 expression by 15d-PGJ<sub>2</sub> does depend on PPAR $\gamma$ .

### Effect of 15d-PGJ<sub>2</sub> on TPA-induced activation of NF- $\kappa$ B and AP-1 in MCF-7 cells

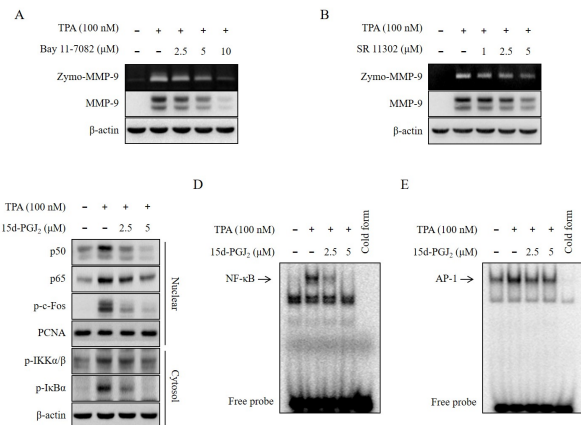
To investigate the signaling and transcription factors by which 15d-PGJ<sub>2</sub> inhibited MMP-9 expression, we used gelatin zymography (zymo) and Western blotting to evaluate the effects of 15d-PGJ<sub>2</sub> on the TPA-induced activation of NF- $\kappa$ B and AP-1. TPA-induced MMP-9 expression was inhibited by NF- $\kappa$ B (Bay 11-7082) and AP-1 (SR 11302) inhibitors in a dose-dependent manner (Fig. 2A and B). Pretreatment with 15d-PGJ<sub>2</sub> inhibited the transfer of p50, p65, and p-c-Fos to the nucleus by TPA, and 15d-PGJ<sub>2</sub> also inhibited phosphorylation of IKK $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$  (Fig. 2C). In addition, an EMSA assay showed that treatment with 15d-PGJ<sub>2</sub> suppressed TPA-induced NF- $\kappa$ B and AP-1 DNA binding activity (Fig. 2D and E). These results indicate that 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 expression by regulating NF- $\kappa$ B/AP-1 activation in MCF-7 cells.

### Role of HO-1 on MMP-9 expression and cell invasion inhibition by 15d-PGJ<sub>2</sub> in MCF-7 cells

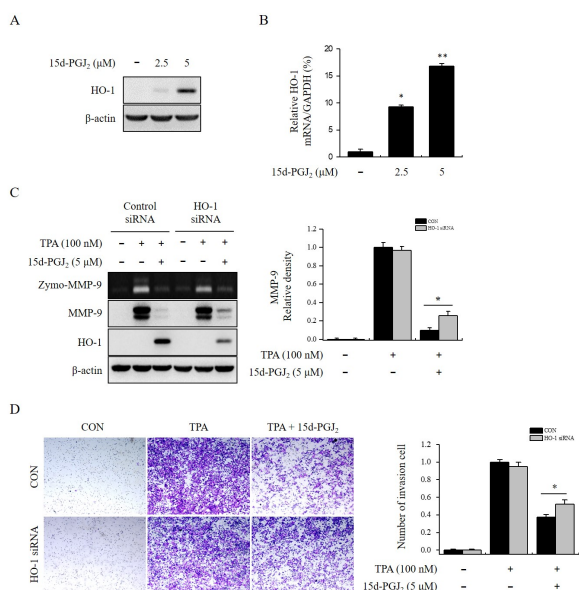
We investigated whether HO-1 plays a role in the inhibition of TPA-induced MMP-9 expression by 15d-PGJ<sub>2</sub>. Western blotting and real-time PCR revealed that 15d-PGJ<sub>2</sub> increased HO-1 expression at both protein and mRNA levels in a dose-depen-



**Fig. 1.** 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 expression in MCF-7 cells. We pretreated MCF-7 cells with 15d-PGJ<sub>2</sub> and then added TPA for 24 h. (A) We analyzed MMP-9 secretion by gelatin zymography (Zymo). MMP-9 protein expression was analyzed by Western blot. (B) We analyzed MMP-9 mRNA levels by real-time PCR using GAPDH mRNA as an internal control. (C) Wild type MMP-9-luc reporters were co-transfected with TK (Renilla) reporter into the MCF-7 cells. We then treated cells with 15d-PGJ<sub>2</sub> in the presence of TPA, and measured the MMP-9 promoter activity with a dual-luciferase reporter assay. (D) PPAR $\gamma$  antagonist GW9662 was added to cells for 1 h before the 15d-PGJ<sub>2</sub> treatment and were analyzed by Western blot. Each value represents the mean  $\pm$  SEM of three independent experiments. Data are presented as means  $\pm$  SE of three independent experiments. \*\*P < 0.01 vs. TPA. \*P < 0.05 vs. TPA.



**Fig. 2.** 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 activation by means of NF- $\kappa$ B/AP-1 signaling pathway in MCF-7 cells. (A, B) We treated MCF-7 cells with inhibitors of NF- $\kappa$ B (Bay 11-7082), AP-1 (SR 11302) for 1 h, and then with TPA for 24 h. The MMP-9 protein expression was analyzed by Western blot. We analyzed MMP-9 secretion by gelatin zymography (Zymo). (C) We treated cells with 15d-PGJ<sub>2</sub> in the presence of TPA for 3 h and then prepared nuclear extracts. PCNA were used as loading controls for nuclear proteins. (D, E) We treated cells with 15d-PGJ<sub>2</sub> in the presence of TPA for 3 h and then prepared nuclear extracts. NF- $\kappa$ B and AP-1 DNA binding was analyzed by EMSA. Data are presented as means  $\pm$  SE of three independent experiments.

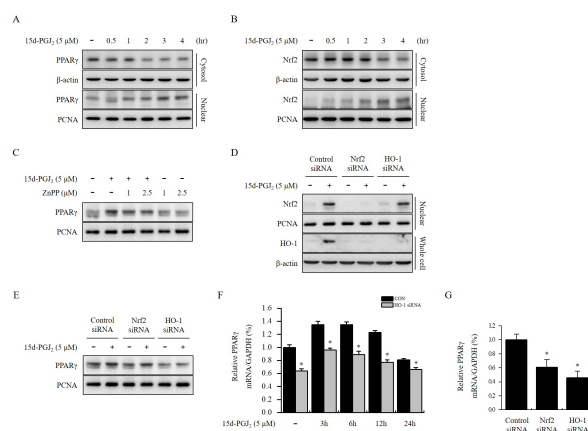


**Fig. 3.** 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 activation and cell invasion by means of the Nrf2/HO-1 signaling pathway in MCF-7 cells. (A, B) We treated MCF-7 cells with 15d-PGJ<sub>2</sub> for 24 h. HO-1 protein expression was analyzed by Western blot. HO-1 mRNA levels were analyzed by RT-PCR using GAPDH mRNA as an internal control. (C) Cells were transfected with HO-1 siRNA, and then treated with 15d-PGJ<sub>2</sub> and TPA for 24 h. HO-1 siRNA attenuated the inhibitory effects of 15d-PGJ<sub>2</sub>. MMP-9 secretion was analyzed by gelatin zymography (Zymo). We analyzed MMP-9 and HO-1 protein expression by Western blot. (D) Cells were transfected with HO-1 siRNA for 24 h. We carried out matrigel invasion assays on cells treated with TPA and 15d-PGJ<sub>2</sub> (5 μM) for 24 h. The data presented as the mean number of migrated cells. Data are presented as means ± SE of three independent experiments. \*\*P < 0.01 vs. control. \*P < 0.05 vs. control.

dent manner (Fig. 3A and B). To verify the involvement of HO-1 in inhibition of MMP-9 expression, we used HO-1 siRNA to knock down HO-1 expression. 15d-PGJ<sub>2</sub> inhibitory effects on TPA-induced MMP-9 expression were significantly decreased in cells transfected with HO-1 siRNA (Fig. 3C). We used a matrigel invasion assay to confirm the inhibition of MCF-7 cell invasion by 15d-PGJ<sub>2</sub>. Treatment with 15d-PGJ<sub>2</sub> (5 μM) effectively inhibited the invasion of MCF-7 cells by approximately 65% compared to untreated control cells. To verify the involvement of HO-1 in inhibition of cell invasion, we used siRNA to knock down HO-1 gene expression. Treatment of cells transfected with HO-1 siRNA with 15d-PGJ<sub>2</sub> showed 50% inhibition, indicating that 15d-PGJ<sub>2</sub> inhibits the invasion of MCF-7 cells by means of HO-1 activation (Fig. 3D). These results suggest that 15d-PGJ<sub>2</sub> inhibits TPA-induced MMP-9 expression and cell invasion by means of the HO-1 pathway in MCF-7 cells.

#### Effect of HO-1 in 15d-PGJ<sub>2</sub>-induced PPAR<sub>γ</sub> expression

15d-PGJ<sub>2</sub> is known as a PPAR<sub>γ</sub> agonist. We investigated whether



**Fig. 4.** HO-1 regulation affected PPAR<sub>γ</sub> expression in MCF-7 cells. (A, B) We treated cells with 15d-PGJ<sub>2</sub> for 0.5, 1, 2, 3, 4 h, followed by cytosol, and then prepared nuclear extracts. Protein expression was analyzed by Western blot. (C) We treated cells with 15d-PGJ<sub>2</sub> in the presence of ZnPP for 4 h and then prepared nuclear extracts. (D, E) Cells were transfected with Nrf2 siRNA and HO-1 siRNA for 24 h, then treated with 15d-PGJ<sub>2</sub> for 4 h and then d nuclear extracts and whole cell lysates were prepared. PCNA was used as loading controls for nuclear and β-actin were used as internal control for the whole cell. (F) Cells were transfected with HO-1 siRNA for 24 h, then treated for 3, 6, 12, and 24 h with 15d-PGJ<sub>2</sub>. (G) Cells were transfected with Nrf2 siRNA and HO-1 siRNA for 24 h. We analyzed PPAR<sub>γ</sub> mRNA levels by real-time PCR using GAPDH mRNA as an internal control. Data are presented as means ± SE of three independent experiments. \*\*P < 0.01 vs. control. \*P < 0.05 vs. control.

HO-1 is involved in PPAR<sub>γ</sub> expression induced by 15d-PGJ<sub>2</sub>. When cells were treated with 15d-PGJ<sub>2</sub> at 0.5, 1, 2, 3, and 4 h, 15d-PGJ<sub>2</sub> induced time-dependent PPAR<sub>γ</sub> nuclear translocation (Fig. 4A). In addition, 15d-PGJ<sub>2</sub> induced Nrf2 nuclear translocation, a major transcription factor for HO-1 expression (Fig. 4B). The pretreatment with ZnPP, an HO-1 inhibitor, inhibited the increased expression of PPAR<sub>γ</sub> by 15d-PGJ<sub>2</sub> (Fig. 4C). To confirm this more clearly, when the cells were transfected with Nrf2 siRNA and HO-1 siRNA for 24 h, Western blotting showed that the increased PPAR<sub>γ</sub> expression by 15d-PGJ<sub>2</sub> was inhibited by Nrf2 and HO-1 knockdown (Fig. 4D and E). When cells were treated with 15d-PGJ<sub>2</sub> for 3, 6, 12, and 24 h, HO-1 siRNA also inhibited 15d-PGJ<sub>2</sub>-induced PPAR<sub>γ</sub> mRNA levels (Fig. 4F). Inhibition of Nrf2 and HO-1 also decreased PPAR<sub>γ</sub> mRNA levels (Fig. 4G). These results suggest that PPAR<sub>γ</sub> expression depends on the activation of Nrf2 and HO-1.

#### DISCUSSION

Activation of peroxisome proliferator activated receptor  $\gamma$  (PPAR<sub>γ</sub>) during carcinogenesis is known to increase as part of normal physiological metabolic factors that affect growth and survival of cancer cells. Furthermore, the potential effect of

PPAR $\gamma$  expression on patient survival has been the focus of breast cancer treatment strategies, suggesting the importance of PPAR $\gamma$  regulation in breast cancer treatment. In this study, we evaluated the relationship between physiologic activation of PPAR $\gamma$  and MMP expression and invasion of breast cancer cells. Thus, we focused on the role of HO-1 in PPAR $\gamma$  activation in the carcinogenesis of breast cancer. Our results showed that 15-Deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), an endogenous PPAR $\gamma$  ligand, blocks TPA-induced MMP-9 expression and cell invasion by means of an HO-1-mediated PPAR $\gamma$  signaling mechanism in MCF-7 cells. This suggests that HO-1 may be a novel member of the PPAR $\gamma$  signaling cascade to target for prevention and treatment of breast cancer.

The promoter region of the matrix metalloproteinase-9 (MMP-9) gene has binding sites for nuclear factor-kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1), and TPA is known to induce MMP-9 by means of NF- $\kappa$ B and AP-1 signaling (16, 17). We confirmed that TPA-induced MMP-9 was inhibited by treatment with NF- $\kappa$ B inhibitor BAY11-7082 and AP-1 inhibitor SR11302, and that 15d-PGJ<sub>2</sub> inhibits TPA-induced NF- $\kappa$ B signal proteins and p-c-fos (AP-1 subunit). 15d-PGJ<sub>2</sub> also inhibited the TPA-induced NF- $\kappa$ B and AP-1 promoter activities. These results indicate that 15d-PGJ<sub>2</sub> inhibits MMP-9 by means of TPA-induced NF- $\kappa$ B/AP-1 signal inhibition.

Heme oxygenase (HO) is a rate-limiting enzyme in the degradation of heme. Three HO isoforms have been identified. HO-2 and HO-3 are constitutively expressed, whereas HO-1 is a potent antioxidant enzyme induced by a variety of factors, such as heme and oxidants (18). HO-1 is associated with pathogenesis in human diseases (19). In breast cancer cells, HO-1 inhibit proliferation and induces apoptosis in human cancer cells (20, 21). Although HO-1 has been studied in various cancer cells, the roles of HO-1 in breast cancer cells are still undefined. Here we found that 15d-PGJ<sub>2</sub> inhibited TPA-induced MMP-9 expression in a PPAR $\gamma$ -dependent mechanism. We confirmed that 15d-PGJ<sub>2</sub> increased HO-1 expression at both protein and mRNA levels in a dose-dependent manner, and that 15d-PGJ<sub>2</sub> inhibitory effects on TPA-induced MMP-9 expression were significantly decreased in cells transfected with HO-1 siRNA. 15d-PGJ<sub>2</sub> also has inhibitory effects on TPA-induced MMP-9 expression that were significantly decreased in cells transfected with HO-1 siRNA. These results indicate that HO-1 plays a role in the inhibition of TPA-induced MMP-9 expression by the 15d-PGJ<sub>2</sub>-PPAR $\gamma$  signaling cascade.

Activation of PPAR $\gamma$  is reported to induce HO-1 expression in cells (22). Conversely, reports that HO-1 influences PPAR $\gamma$  activity are not well known (23, 24). In particular, the effect of HO-1 on PPAR $\gamma$  in breast cancer is still unclear. Our study showed that HO-1 directly induces PPAR $\gamma$  expression in breast cancer cells. We confirmed that 15d-PGJ<sub>2</sub> increased Nrf2 nuclear translocation, a major transcription factor for HO-1 expression, in a time-dependent manner. The pretreatment with ZnPP, a HO-1 inhibitor, inhibited the increased expression of PPAR $\gamma$  by 15d-PGJ<sub>2</sub>. In addition, when the cells were

transfected with Nrf2 siRNA and HO-1 siRNA, the increased PPAR $\gamma$  expression by 15d-PGJ<sub>2</sub> was inhibited by Nrf2 and HO-1 knockdown. Inhibition of Nrf2 and HO-1 also decreased PPAR $\gamma$  mRNA levels. These results indicate that PPAR $\gamma$  expression depends on the activation of Nrf2 and HO-1 in MCF-7 cells. Collectively, these findings suggest that HO-1 is a major regulator of PPAR $\gamma$  expression in the pathogenesis of breast cancer.

In conclusion, this study demonstrates that MMP-9 expression and invasion of ER-positive breast cancer cells may be regulated by means of an HO-1-induced PPAR $\gamma$  signaling mechanism. Our data showed that HO-1 may be a novel target in the PPAR $\gamma$  signaling cascade for prevention and treatment of breast cancer. However, additional experiments are necessary to confirm this relationship between HO-1 and PPAR $\gamma$  at the transcription factor level and to further delineate its molecular basis.

## MATERIALS AND METHODS

### Cells and materials

We purchased:

- MCF-7 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA); these were cultured at 37°C in a 5% CO<sub>2</sub> incubator in DMEM (Gibco, Gaithersburg, MD, USA) with 1% antibiotics and 10% FBS (Gibco).
- 15d-PGJ<sub>2</sub> from Cayman Chemical (Ann Arbor, MI, USA).
- Anti- $\beta$ -actin antibody, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 12-O-tetradecanoylphorbol-13-acetate (TPA) from Sigma-Aldrich (St Louis, MO, USA).
- Primary antibodies to p-c-Fos, p-I $\kappa$ B $\alpha$ , and p-IKK $\alpha$ / $\beta$  from Cell Signaling Technology (Danvers, MA, USA).
- MMP-9, p65, p50, PPAR $\gamma$ , Nrf2, PCNA antibodies, NF- $\kappa$ B inhibitor BAY 11-7082, and AP-1 inhibitor SR 11302 from Santa Cruz Biotechnology (Dallas, TX, USA).
- Heme oxygenase-1 (HO-1) antibody and PPAR $\gamma$  inhibitor GW9662 from Enzo Life Sciences (New York, NY, USA).
- NF- $\kappa$ B and AP-1 consensus oligonucleotides from Promega (Fitchburg, WI, USA).

### Cell viability

We confirmed the effect of 15d-PGJ<sub>2</sub> on cell viability of MCF-7 using an MTT assay. Cells were seeded in each well of 96-well plates at a density of  $3 \times 10^4$  cells/well. After 24 h, cells were stimulated with 2.5, 5, 10, or 20  $\mu$ M 15d-PGJ<sub>2</sub> for 24 h at 37°C. The cells were then washed with PBS, and MTT (0.5 mg/ml of PBS) was added; we then incubated the treated cells at 37°C for 30 min. We dissolved Formazan crystals with DMSO. OD was detected at 570 nm using a microplate reader (Bio-Rad, Richmond, CA, USA).

### Preparation of nuclear extract

We treated MCF-7 cells with 15d-PGJ<sub>2</sub> in the presence or absence of TPA (100 nM) for 3 h. Cells were washed twice, scraped into 1.5 ml of PBS, and pelleted at  $4,000 \times g$  for 4

min. We prepared cytoplasmic and nuclear extracts using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA).

#### Western blot analysis

We pretreated MCF-7 cells with 15d-PGJ<sub>2</sub> for 1 h and then incubated them with TPA for 24 h at 37°C. Cells were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher). We separated samples (10 µg) by SDS-PAGE gels, and the protein loaded-gel were transferred to Hybond-polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 1 h. Each membrane was blocked for 2 h with 5% skim milk or 5% bovine serum albumin and was then incubated with a primary antibody overnight at 4°C. We detected protein levels with an image analyzer (Fuji-Film, Tokyo, Japan). We did densitometry using Image J software (NIH, Bethesda, MD, USA).

#### Gelatin zymography assay

We pretreated cells with 15d-PGJ<sub>2</sub>, BAY 11-7082, and SR 11302 for 1 h and then incubated them with TPA for 24 h at 37°C. After that, we collected conditioned media and separated them in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed with 2.5% Triton X-100, incubated at 37°C for 15 h in developing buffer, subsequently stained with 0.25% (w/v) Coomassie blue buffer and photographed on an image analyzer (Fuji-Film).

#### Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using RNAiso Plus (Takara, Shiga, Japan). We detected the RNA purity and concentration by absorbance at 260/280 nm. We synthesized cDNA using a PrimeScript RT reagent Kit (Takara). GAPDH (NM002046), PPAR $\gamma$  (NM 001330615), HO-1 (NM 002133), and MMP-9 (NM 004994) mRNA expression was detected by real-time PCR using a SYBR Green and ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA).

#### Luciferase assay

MCF-7 cells were seeded on 24-well plates until the cells reached 60-70% confluence and were then transfected with MMP-9 reporter plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). We pretreated the transfected cells with 15d-PGJ<sub>2</sub> for 1 h and then incubated them with TPA for 24 h. We did Luciferase reporter assays using the Dual-Luciferase Reporter Assay System (Promega) and detected them using a luminometer (EG & G Berthold, Gaithersburg, MD, USA).

#### Electrophoretic mobility shift assay (EMSA)

An oligonucleotide containing the AP-1 (5'CGCTTGATGAGT CAGCCGGAA-3') or NF- $\kappa$ B (5'CCGGTTAACAGAGGGGCT TTCCGAG-3') binding sites were synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP

(Amersham, UK). Binding buffer, 10 µg of nuclear extracts, and labeled oligonucleotides (10,000 cpm) were then incubated for 30 min at room temperature. The reaction mixtures underwent electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer, and then the gels were dried and examined by autoradiography.

#### siRNA transfection

We transfected MCF-7 cells with human Nrf2 siRNA (sc-37030, Santa Cruz), human HO-1 siRNA (sc-35554, Santa Cruz) using Opti-MEM medium (Gibco) and Lipofectamine 2000. Negative control siRNA was purchased from Genotech (Daejeon, Korea). We seeded MCF-7 cells on 6-well plates at 40-50% confluence 24 h before transfection. Opti-MEM, Lipofectamine 2000, and siRNA were mixed and incubated at room temperature for 20 min. Lipofectamine 2000-siRNA complexes were added and transfected for 6 h. We used siRNA at a concentration of 10 nM, and knockdown of Nrf2, HO-1 was confirmed by RT-PCR and Western blot.

#### Invasion assay

We did the invasion assay in 24-well chambers (8-µm pore size) coated with Matrigel (Corning Life sciences, Tewksbury, MA, USA). We added cells to the upper chamber; the lower compartment of the invasion chamber contained conditioned medium. After the cells were incubated for 24 h, the migrated cells were fixed with 3.8% formaldehyde solution for 10 min and stained with a crystal violet solution for 30 min. Cells on the upper side of the chamber were removed using a cotton swab, and we counted migrated cells in five random areas of the membrane. Analyzed data are the mean  $\pm$  SE from three independent experiments performed in triplicate.

#### Statistical analysis

We did statistical analysis using ANOVA and Duncan's test. Differences with  $P < 0.05$  were considered statistically significant.

#### ACKNOWLEDGEMENTS

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#### CONFLICTS OF INTEREST

The authors have no conflicting interests.

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