

CDDO, a PPAR- γ ligand, inhibits TPA-induced cell migration and invasion through a PPAR- γ -independent mechanism

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Abstract. Peroxisome proliferator-activated receptor- γ (PPAR- γ) acts as a key factor in breast cancer metastasis. Notably, PPAR- γ can inhibit metalloproteinase (MMP), which is involved in cancer metastasis. Our previous study revealed that PPAR- γ was related to breast cancer metastasis. The present study aimed to investigate whether the PPAR- γ ligand 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) mediated suppression of cell invasion and reduced the expression of MMP-9 in breast cancer cells. The results indicated that CDDO reduced MMP-9 expression, cell migration and invasion of breast cancer cells by inhibiting TPA-induced phosphorylation

of mitogen-activated protein kinases, and downregulating the activities of activator protein-1 and nuclear factor κ B. Notably, knock-out of PPAR- γ by small interfering RNA in MCF-7 cells revealed that TPA-induced MMP-9 expression occurred through a PPAR- γ -independent pathway. These data indicated that the downregulatory effect of CDDO on MMP-9 expression was affected by a mechanism independent of PPAR- γ . In conclusion, the findings of the present study suggested that CDDO may act as a key agent in the regulation of breast cancer metastasis, suggesting CDDO as a new targeted therapy for breast cancer.

Introduction

Although the average human lifespan has been greatly extended because of advances in medical technology, cancer has remained the leading cause of death for the past 30 years. Moreover, the incidence and mortality rate of cancer are expected to continue to increase because of the aging population and lifestyle changes (1). According to the 2019 Breast Cancer White Paper of the Korean Breast Cancer Society (2), breast cancer ranks first and accounts for 24.2% of the global cancer incidence in females and is the leading cause (15%) of cancer mortality. As of 2018, there were approximately 2 million breast cancer cases worldwide, resulting in approximately 600,000 deaths. Metastasis is a major cause of cancer-correlated deaths. Despite the marked developments in current therapies, some patients experience a relapse. Therefore, a more accurate study of metastasis is essential for improving treatment methods for breast cancer (3). Matrix metalloproteinases (MMPs) are endopeptidases secreted by various cell types. The main role of MMPs in the metastatic cascade appears to be the interaction of tumor cells with the extracellular matrix (ECM). Additionally, MMPs release growth factors through their degradation process, resulting in cancer development (4,5). In a previous study, we found that synthetic PPAR- γ ligands reduced MMP-9 expression and cell invasion in breast cancer cells. Therefore, revealing that a novel PPAR- γ ligand modulates MMP-9 and helps prevent breast cancer metastasis could enhance the survival rate of

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Abbreviations: PPAR- γ , peroxisome proliferator-activated receptor- γ ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-9, matrix metalloproteinase-9; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay; PCR, polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, mitogen-activated protein kinase; AP-1, activator protein-1; NF- κ B, nuclear factor κ B

Key words: PPAR- γ , MMP-9, migration, invasion, breast cancer cells

patients (6). Triterpenoids are isopentenyl pyrophosphate oligomers that have been successfully developed for solid cancers (7). Triterpenoids have been studied to determine their cytotoxicity to various cancer cells and their anticancer effects *in vivo* (7). Recent studies have shown that 2-cyano-3,12-dioxooleane-1,9-dien-28-oic acid (CDDO), a PPAR- γ ligand and novel synthetic triterpenoid, targets the NF- κ B pathway, which plays a pivotal role in MMP expression in cancer cells (8). These results indicate CDDO as a hopeful therapeutic agent for inhibiting the development of primary tumors and metastases in solid cancers, such as breast cancer. However, the effect of CDDO on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced breast cancer metastasis has not yet been addressed as a mechanism contributing to MMP suppression.

Materials and methods

Reagents. TPA, T0070709 (a PPAR- γ antagonist), and anti- β -actin (A5441) were obtained from Sigma-Aldrich. CDDO was obtained from Cayman Chemical Co. Antibodies against MMP-9 (ab76003) and p-c-Jun (ab32385) were obtained from Abcam. p-ERK (#4370), ERK (#4695), p-p38 (#4511), p38 (#8690), p-JNK (#4668), JNK (#9258), c-Jun (#9165), p-c-Fos (#5348), c-Fos (#3250), p-I κ Ba (#2859), and p-IKKa/ β (#2697) were obtained from Cell Signaling Technology. PPAR γ (sc-7273), PCNA (sc-56), p50 (sc-7178), p65 (sc-372), I κ Ba (sc-371), and IKKa (sc-71333) and IKK β (sc-56918) were obtained from Santa Cruz Biotechnology. Secondary HRP-conjugated anti-mouse (ADI-SAB-100-J) and anti-rabbit (ADI-SAB-300-J) were obtained from Enzo Life Sciences.

Cell culture. The MCF-7 human breast cancer cell line was purchased from American Type Culture Collection. The cells were cultured in DMEM supplemented with 1% antibiotic and 10% FBS (Thermo Fisher Scientific) in CO₂ at 37°C.

Cytotoxicity assay. Cells were either treated with 0.1, 0.3, 0.5, 1, or 2 μ M CDDO or left untreated at 37°C for 24 h and then washed with PBS. The assay was performed using MTT (0.5 mg/ml; Merck KGaA). After addition of the agent, the cells were incubated at 37°C for 40 min. DMSO was added to dissolve formazan crystals, and absorbance was determined using a microplate reader at 570 nm (Bio-Rad Laboratories).

Cell proliferation assay. Cell proliferation was measured using a BrdU Cell Proliferation ELISA kit (ab126556, Abcam) following the manufacturer's instructions. Briefly, cells (2 \times 10⁶) were incubated in 96-well plates with CDDO at 37°C for 24 h, and then BrdU was added to the cells for 2 h. Cells were fixed and incubated with anti-BrdU monoclonal Detector Antibody for 1 h at room temperature. Peroxidase goat anti-mouse IgG conjugate was added as secondary antibody and incubated for 30 min at room temperature. Color was developed using TMB peroxidase substrate, and BrdU incorporation was measured at 450 nm using a microplate reader (Bio-Rad).

Nuclear and cytoplasmic extracts. Cells were treated with 1 μ M CDDO, treated with TPA at 37°C for 2 h, and washed

with PBS. Nuclear and cytoplasmic extracts were obtained using NE-PER[®] extraction reagents (Thermo Fisher Scientific).

siRNA transfection. Cells were transfected with PPAR γ siRNA (Bioneer) using Opti-MEM (Gibco) and Lipofectamine RNAiMAX (Invitrogen). Negative control siRNA was purchased from Genotech. Experiments were performed according to the Lipofectamine RNAiMAX transfection protocol at an siRNA concentration of 10 nM. The Lipofectamine RNAiMAX-siRNA complex was added to the cells, and after 6 h of transfection, the growth medium was replaced. Cells were treated with TPA and CDDO 24 h after transfection and then used in the experiments.

Zymography assay. Supernatant medium was added with loading buffer and separated using PAGE (0.1% gelatin). The gel was washed for 40 min with Triton X-100 solution (2.5%) at 15–20°C and then incubated for 24 h in a digestion buffer at 37°C. The gel was stained for 40 min with Coomassie brilliant blue (0.25%) and measured with an image analyzer (FUJIFILM Corporation). Densitometric analysis was determined using the Multi-Gauge image analysis software (Multi-Gauge v3.0; FUJIFILM Corporation).

Western blot analysis. Cells were treated with CDDO 1 μ M at 37°C for 1 h and then treated with TPA at 37°C for the time required by each experiment. Proteins were lysed using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) and a proteinase inhibitor. Protein concentration was analyzed using a Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories). Lysates (10 μ g protein) were separated using SDS-PAGE (10%) and transferred to Hybond[™] polyvinylidene fluoride membranes (GE Healthcare Life Sciences). Each membrane was blocked at 4°C for 3 h with 5% skim milk or 5% bovine serum albumin (MP Biomedicals) and then incubated at 4°C for 16 h with primary antibodies (diluted 1:1,000). Secondary antibody of HRP-conjugated anti-IgG (diluted 1:1,000) was applied and incubated at 4°C for 1 h. Immunoreactive signals were visualized using an HRP substrate peroxide solution and luminol reagent (Merck Millipore). Protein density were determined using an imaging system (Las-4000; FUJIFILM Corporation) and image analyzer software (Multi-Gauge v3.0; FUJIFILM Corporation).

Real-time polymerase chain reaction. Cells were treated with CDDO (1 μ M) at 37°C for 1 h and then treated with TPA at 37°C for 24 h. RNA was isolated using the RNAiso Plus Reagent. cDNA was synthesized using a PrimeScript RT Reagent Kit by heating based on the kit protocol. The mRNA levels were analyzed by qPCR using an ABI PRISM[™] 7900 Sequence Detection and Power SYBR[®] Green PCR Master Mix (Thermo Fisher Scientific). The primers used were as follows: GAPDH (NM002046) sense, 5'-ATGGAAATCCCA TCACCATCTT-3' and antisense, 5'-CGCCCCACTTGATTT TGG-3'; MMP-9 (NM 004994) sense, 5'-CCTGGAGACCTG AGAACCAATCT-3' and antisense, 5'-CCACCCGAGTGT AACCATAGC-3'. The qPCR cycle was as follows: 40 cycles at 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. Target genes were normalized to GAPDH. Relative

quantitation was performed using the comparative Cq ($2^{-\Delta\Delta Cq}$) method, following the manufacturer's instructions (9).

Luciferase assay. Luciferase reporter assays was performed using a Dual-Luciferase® Reporter Assay System (E1910, Promega) following the manufacturer's instructions. Cells were transfected with AP-1 or NF- κ B reporter plasmids (provided by Professor Kim Chul Ho, SungKyunKwan University) using the Lipofectamine 3000 Reagent (Thermo Fisher Scientific). Transfected cells were treated with 1 μ M CDDO at 37°C for 1 h and then treated with TPA at 37°C for 2 h. *Renilla* luciferase activity was measured using a luminometer (Lumat LB 9507; Berthold Technologies GmbH & Co.).

Migration assay. The assay was performed using well chambers (8 μ m pore size) without Matrigel. Cells (3×10^5) were added to the upper chamber in DMEM (FBS-free media), and the lower chamber contained supernatant medium (1% antibiotic and 10% FBS). TPA with or without 1 μ M CDDO was added to the upper chamber. After incubation at 37°C for 24 h, upper chamber cells were removed. The migrated cells at the bottom were fixed with formalin (3.5%) for 10 min and stained with crystal violet (0.25%) for 1 h. Migrated cells were counted in four random parts of the layer using a light microscope (40x magnification).

Invasion assay. The assays were conducted using well chambers (8 μ m pore size) in which the upper area of the Transwell insert was coated with Matrigel at 37°C for 40 min (BD Biosciences). Cells (3×10^5) were added to the upper chamber in DMEM (FBS-free media), and the lower chamber contained supernatant medium (1% antibiotic and 10% FBS). TPA with or without 1 μ M CDDO was added to the upper chamber. After incubation at 37°C for 24 h, cells in the upper chamber were removed. The invading cells on the bottom were fixed with formalin (3.5%) for 10 min and stained with crystal violet (0.25%) for 1 h. Invading cells were counted in four random parts of the layer using light microscopy (40x magnification).

Statistical analysis. Data are presented as the mean \pm standard error of the mean of three independent experiments. Statistical analyses were conducted using one-way ANOVA and Tukey test. Differences were statistically significant at $P < 0.05$.

Results

Effects of CDDO on MMP-9. MCF-7 cells were treated with CDDO (0–2 μ M) for 24 h, and cytotoxicity and cell proliferation were confirmed (Fig. 1A and B). Non-toxic concentrations of CDDO were used in subsequent experiments. TPA-mediated MMP-9 secretion was observed, and CDDO suppressed this secretion in a concentration-dependent manner, as determined using zymography (Zymo-MMP-9). Western blot analysis showed that CDDO reduced TPA-mediated MMP-9 protein expression in a concentration-dependent manner (Fig. 1C). Additionally, RT-qPCR indicated that CDDO treatment inhibited TPA-mediated MMP-9 mRNA level (Fig. 1D). Together, these data indicate that CDDO downregulates MMP-9

expression. We next determined whether CDDO inhibited MMP-9 in a PPAR- γ -dependent manner and confirmed that the inhibition of MMP-9 expression after treatment with CDDO was reversed by the PPAR- γ antagonist T0070907 (Fig. 1E). These data showed that CDDO downregulated MMP-9 expression.

CDDO blocks TPA-mediated MAPK and AP-1 activation through a PPAR- γ -independent pathway. The downregulation of MMP-9 by CDDO could be PPAR- γ -dependent or independent. To investigate an associative relationship between downregulation of MMP-9 by CDDO and PPAR- γ , we investigated the change of the expression level of MMP-9 by CDDO in TPA-treated breast cancer cells after knock-out of PPAR- γ by siRNA. In a previous study, we confirmed the expression of PPAR γ in the nuclear fraction of MCF-7 cells (10). In the present study, the transfection efficiency of si-PPAR γ was verified at the protein level in the nuclear fraction of MCF-7 cells (Fig. 2A). As shown in Fig. 2B, CDDO suppressed TPA-mediated expression of MMP-9 regardless of PPAR- γ presence, meaning that CDDO downregulates MMP-9 through a PPAR- γ -independent pathway.

By applying CDDO and TPA to the MCF-7 cells, we further determined the mechanism by which CDDO inhibited MMP-9 expression using western blot analysis. TPA increased the phosphorylation of ERK, p38, and JNK. CDDO suppressed TPA-mediated phosphorylation of MAPKs. However, the total protein levels of ERK, p38, and JNK were unchanged (Fig. 2C). Additionally, the nuclear target of MAPK signaling was the transcription factor AP-1. The results showed that phosphorylation of c-Jun and c-Fos (AP-1 subunit) in the nucleus of TPA-mediated cells was reduced subsequent to treatment with CDDO (Fig. 2D). Luciferase assays were used to analyze the DNA-binding activities of AP-1. TPA treatment significantly reduced these interactions in CDDO-treated cells (Fig. 2E). These data indicate that CDDO blocks MMP-9 by inhibiting the MAPK and AP-1 signaling pathways.

CDDO blocks TPA-mediated NF- κ B activation. We further elucidated the mechanism underlying MMP-9 transcriptional activation by CDDO. The effects of CDDO on NF- κ B regulation were assessed using western blot analysis. MCF-7 cells were treated with CDDO and TPA. CDDO suppressed TPA-mediated nuclear translocation of p50/p65 (NF- κ B subunit) and phosphorylation of cytoplasmic I κ B α and IKK α / β . Levels of total I κ B α , IKK α , and IKK β in the cytosol were unchanged (Fig. 3A). Luciferase assays were used to analyze the DNA-binding activities of NF- κ B. TPA treatment significantly reduced these interactions in CDDO-treated cells (Fig. 3B). These data indicate that CDDO inhibits MMP-9 expression by regulating NF- κ B transcription activity.

CDDO inhibits TPA-induced chamber migration and Matrigel invasion in vitro. CDDO inhibited TPA-induced migration of cells, as determined by chamber migration without Matrigel, compared with the untreated control cells. (Fig. 4A, upper panel). We investigated the effect of CDDO on the invasiveness of MCF-7 breast cancer cells using a Matrigel invasion assay. The results indicated that CDDO effectively inhibited the TPA-mediated invasion of cells relative to non-treated

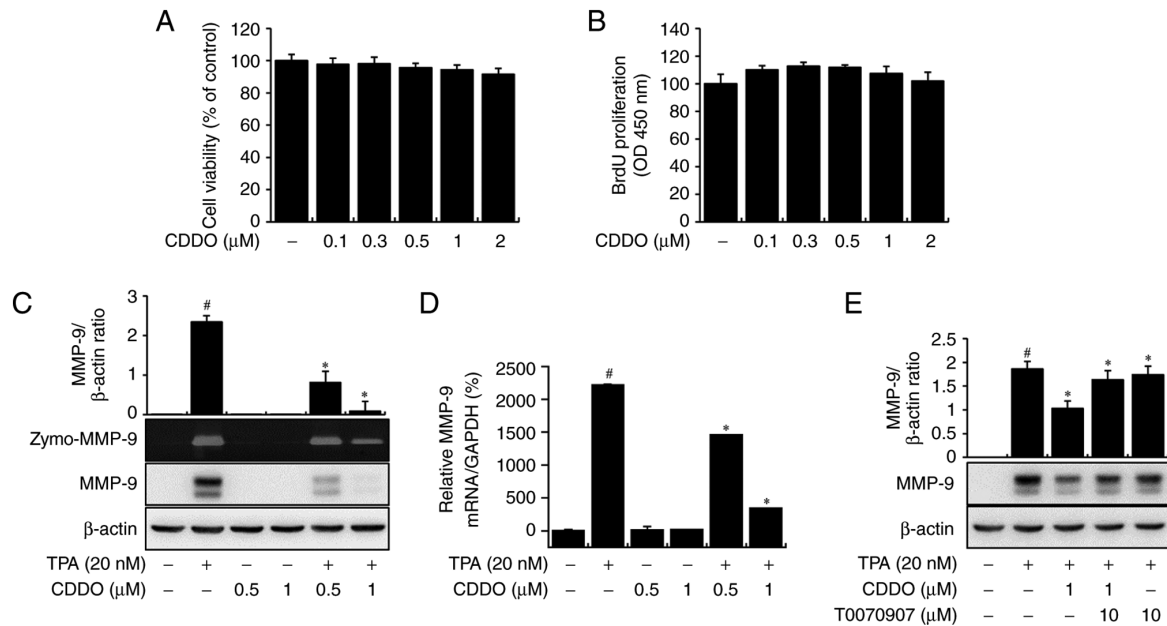


Figure 1. Effects of CDDO on the viability and MMP-9 expression of MCF-7 cells. Cells were treated with CDDO (0–2 μ M) for 24 h. (A) Cytotoxicity was analyzed using MTT assay. (B) Cell proliferation was measured by BrdU assay. (C) MMP-9 secretion was determined by gelatin zymography (top-dark background bands), and MMP-9 protein was determined by western blotting using β -actin as a loading control (bottom bands). (D) MMP-9 mRNA was determined by RT-PCR with GAPDH as a loading control. (E) The PPAR γ antagonist T0070907 was applied to cells for 1 h before CDDO treatment, and cells were determined by western blotting. β -actin was used as the internal control. The ratio of western blots was quantified with ImageJ. Values are the mean \pm standard error of the mean of three independent experiments. # P <0.05 vs. untreated cells, * P <0.05 vs. TPA alone. CDDO, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; BrdU, bromodeoxyuridine; Zymo, zymography.

control cells (Fig. 4A). Fig. 4B and C show the relative numbers of migrating cells and Matrigel-invading cells. These data indicate that CDDO inhibited the migratory and invasive capabilities of breast cancer cells.

Discussion

The target of this study was to determine the effect of CDDO, a novel synthetic triterpenoid and PPAR- γ ligand, on MMP-9 expression and invasiveness of breast cancer cells. Our findings indicate that CDDO inhibits MMP-9 and cancer cell invasion. Furthermore, we described the mechanism of CDDO action using the transcriptional effects on AP-1 and NF- κ B and determined that the CDDO mechanism uses MAPK signaling to repress MMP-9. We showed that the major signaling pathways in the migration and invasion of cancer cells, such as MAPKs, AP-1, and NF- κ B, are targets of CDDO in a PPAR- γ -independent manner, suggesting CDDO as a potential therapeutic phytochemical agent.

MMP-9 is an important molecule that plays a major role in cancer growth and metastasis primarily via degradation of the ECM, which could result in cancer cell migration (11,12). Elevated MMP-9 expression is key in the invasive process of numerous cancers, especially breast cancers (13). The induction of MMP-9 is affected by many factors, such as cytokines, growth factors, and certain oncogenes. TPA, a phorbol ester, activates protein kinase C (PKC), which excites MMP-9 secretion and synthesis during cancer cell invasion (14). MMP is considered a marker of cancer invasion and could be used as a prognostic indicator for various cancers (15).

TPA mediates MMP-9 via regulation of transcription factors, such as AP-1 and NF- κ B, which have binding sites on

the MMP-9 promoter (16). Additionally, the MAPK signaling pathway is important for AP-1 and NF- κ B regulation, which are known modulators of the MMP-9 promoter (17). AP-1 and NF- κ B are nuclear transcription factors involved in several important diseases, including cancer (18). NF- κ B is dominant in breast cancer cells and might play a key role in tumorigenesis, such as cancer cell migration. Studies have shown that NF- κ B activation induces the growth and expansion of breast cancer cells (19). Clinical data have revealed that NF- κ B inhibitors are beneficial in some patients with breast cancer (20).

It has been well known that troglitazone, a PPAR- γ ligand, shows anticancer activity in breast cancer cells *in vitro* (21). Furthermore, some studies have indicated that the PPAR- γ ligand is effective in inhibiting breast cancer *in vitro*, but this drug has a marginal effect *in vivo* (22), suggesting that the role of the PPAR- γ ligand in breast cancer cells should be decided for the development of effective and safe drugs for patients with breast cancer (23).

Recent studies have shown that CDDO, a PPAR- γ ligand and novel synthetic triterpenoid, plays a pivotal role in MMP expression in several cancer lines (24). These results indicate CDDO as a hopeful therapeutic agent for inhibiting the development of primary tumors and metastases in cancers (25). However, the effect of CDDO on TPA-induced breast cancer metastasis has not yet been addressed as a mechanism contributing to MMP repression. Consequently, the role of the PPAR- γ ligand, including CDDO, in cancer growth inhibition has been studied (26), but its dependency on PPAR- γ remain uncertain.

In conclusion, we observed the effects of CDDO on regulation of PPAR- γ signaling and MMP-9 expression of breast cancer cell. CDDO reduced MMP-9 expression, cell migration,

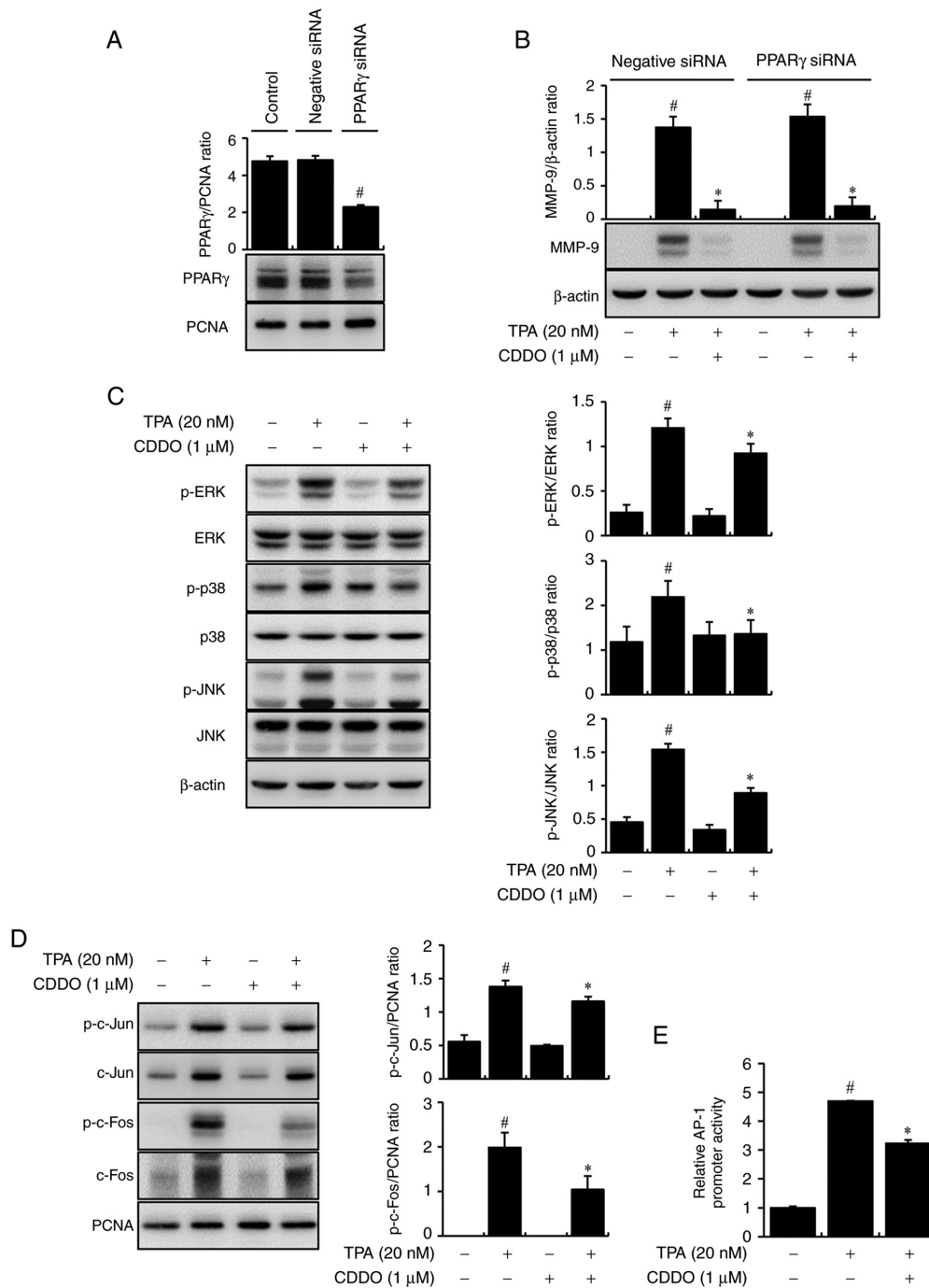


Figure 2. Effects of CDDO on TPA-mediated MAPK and AP-1 activation through a PPAR γ -independent pathway. (A) The transfection efficiency of si-PPAR γ was confirmed in the nuclear fraction of cells. PCNA was used as the internal control. (B) Cells were transfected with PPAR γ siRNA and then treated with TPA and CDDO for 24 h. MMP-9 protein expression was analyzed using western blotting. (C) Cells were treated with CDDO and/or TPA for 30 min. The phosphorylation of ERK, p38, and JNK was determined by western blotting using β -actin as a loading control. (D) Cells were treated with CDDO and/or TPA. After a 2 h incubation, prepare the extracted cytoplasmic and nuclear. The nuclei of p-c-Jun, p-c-Fos, total c-Jun, and c-Fos were quantitated by western blotting. PCNA was the internal control for the nucleus. (E) AP-1-luc reporters were co-transfected with a *Renilla* luciferase reporter. Cells were treated with CDDO, TPA was added for 2 h, and the AP-1 promoter activities were analyzed using a luciferase reporter assay. The ratio of western blots was quantified with ImageJ. Values are the mean \pm standard error of the mean of three independent experiments. [#]P<0.05 vs. untreated cells, ^{*}P<0.05 vs. TPA alone. PPAR γ , peroxisome proliferator-activated receptor γ ; siRNA, small interfering RNA; CDDO, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; p-, phosphorylated; AP-1, activator protein-1.

and invasion of breast cancer by inhibiting TPA-induced phosphorylation of MAPKs and downregulating the activities of AP-1 and NF- κ B. In addition, knock-out of PPAR- γ by siRNA in MCF-7 cells showed that TPA induced MMP-9 expression by a PPAR- γ -independent pathway. These data showed that the downregulation effect of CDDO on MMP-9 expression

occurred through a mechanism independent of PPAR- γ . Our findings support those of several other reports demonstrating that CDDO can regulate cell movement independent of PPAR- γ . These results suggest that the function of CDDO is to act as a key agent in the regulation of breast cancer metastasis in a PPAR- γ -independently.

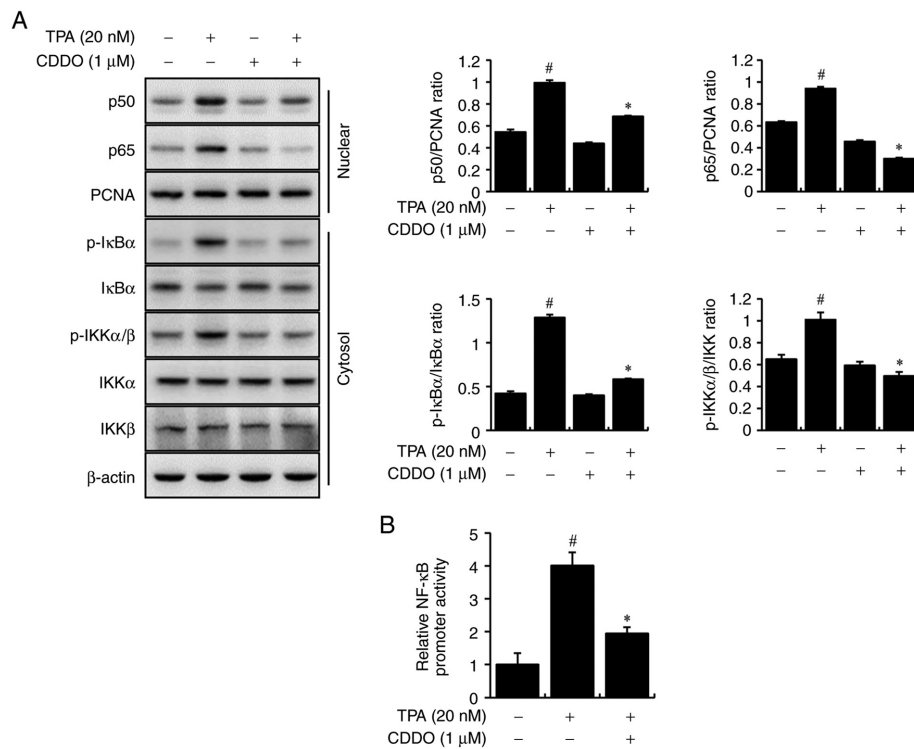


Figure 3. NF- κ B activation of CDDO in MCF-7 cells. (A) Cells were treated with CDDO and/or TPA. After incubation for 2 h, prepare the extracted cytoplasmic and nuclear. The levels of p50 and p65 in the nucleus, of p-I κ B α and p-IKK α / β in the cytoplasm, and of total I κ B α , IKK α , and IKK β were analyzed by western blotting. PCNA was the internal control for the nucleus, and β -actin was the loading control for the cytoplasm. (B) NF- κ B-luc reporters were co-transfected with a *Renilla* luciferase reporter. Cells were treated with CDDO, TPA was added for 2 h, and the NF- κ B promoter activities were analyzed using a luciferase reporter assay. The ratio of western blots was quantified with ImageJ. Values are the mean \pm standard error of the mean of three independent experiments. [#]P<0.05 vs. untreated cells, ^{*}P<0.05 vs. TPA alone. CDDO, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; NF- κ B, nuclear factor κ B.

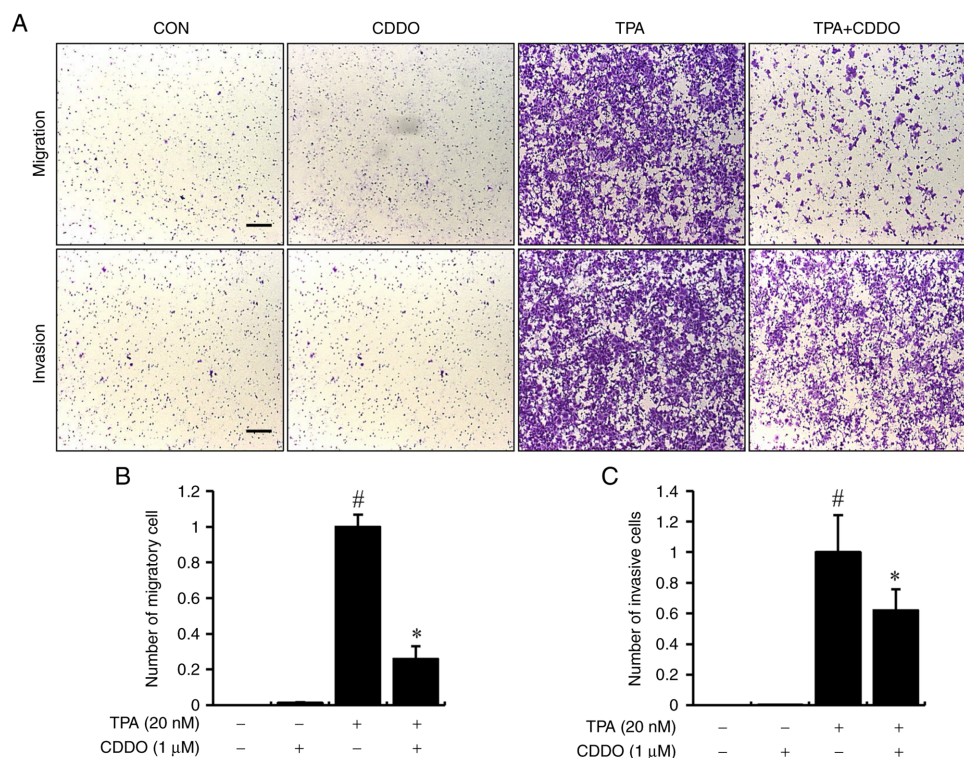


Figure 4. CDDO suppression of migration and invasion of MCF-7 cells. (A) Chamber migration and invasion assays were implemented without or with Matrigel. Cells were treated with CDDO and/or TPA. After 24 h, cells were stained, and microscopic photography was conducted. Bar, 200 μ m. (B) Data were quantified by counting the migrated cells in four randomly selected regions during the migration assay. (C) Data were quantified by counting the invading cells in four randomly selected regions during the invasion assay. Values are the mean \pm standard error of the mean of three independent experiments. [#]P<0.05 vs. untreated cells, ^{*}P<0.05 vs. TPA alone. CDDO, 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JSK and OYH conceived and designed the study and wrote the manuscript. HYJ performed the experiments and analyzed the data. JSK and SHJ contributed to the design and acquisition of funding. HYJ, HJY, JJ and EYC were involved in additional experiments and the revision processes. SHJ and OYH evaluated and revised the manuscript. JSK and SHJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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