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Mycophenolic acid induces adipocyte-like differentiation and reversal of malignancy of breast cancer cells partly through PPARγ

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ABSTRACT

Mycophenolic acid (MPA) has been known for decades to be an anticancer and immunosuppressive agent and has significant anticancer properties, but its underlying molecular mechanisms are poorly characterized. Peroxisome proliferator-activated receptor gamma (PPARγ) has a central role in adipocyte differentiation, and MPA has been shown to be a potent PPARγ agonist. Whether PPARγ activation has a putative role in the anticancer efficacy of MPA via induction of adipocyte-like differentiation has not been elucidated. In the present study, MPA was demonstrated to dose-dependently activate PPARγ transcription in the GAL4-hPPARγ (LBD) chimeric receptor assay and PPRE-luc reporter gene assay with an EC_{50} of 5.2–9.3 μM. Treatment of the breast cancer cell lines MDA-MB-231 and MCF-7 with MPA resulted in differentiation of adipose tissue that was characterized by accumulation of intracellular lipids, enlargement of cell volume, and permanent withdrawal from the cell cycle at the G1/G0 stage. At a molecular level, the expression of three adipocyte differentiation markers (PPARγ, adipsin D, and aP2) was remarkably induced in differentiated breast cancer cells. However, RNA interference experiments showed that PPARγ-knockdown cannot completely reverse the differentiated state of MDA-MB-231 cells after MPA treatment. These data suggest that the effects of MPA on adipocyte-like terminal differentiation of breast cancer cells are (at least in part) due to PPARγ activation, which is a novel anticancer mechanism of MPA.

1. Introduction

Mycophenolic acid (MPA) (Fig. 1A) is an antibiotic produced by *Penicillium*, which has been known for decades to be an anticancer (Suzuki et al., 1969; Tressler et al., 1994) and immunosuppressive agent (Ohsugi et al., 1976; Bardsley-Elliot et al., 1999). Many studies have demonstrated that MPA has significant anticancer properties in leukemias, lymphomas and many types of solid tumor cells (Cohn et al., 1999; Sidi et al., 1988; Floryk and Huberman, 2006; Tressler et al., 1994).

Mycophenolate mofetil (MMF, Cellcept™) (Fig. 1B), a prodrug of MPA, has been approved for use in the prevention of acute allograft rejection after organ transplantation and hematopoietic stem-cell transplantation for a decade. A statistical analysis undertaken in transplant recipients showed that MMF can significantly lower the risk of developing post-transplant malignancy, which has become an important cause of death in transplant recipients (Danovitch, 2005; Buell et al., 2005; Casadio et al., 2005). However, the underlying mechanism is incompletely understood.

It has been shown that MPA as a non-competitive inhibitor selectively inhibits inosine monophosphate dehydrogenase (IMPDH), a critical enzyme for catalyzing the rate-limiting reaction of de-novo biosynthesis of guanosine-5′-triphosphate (GTP) at the IMP metabolic branch point (Franklin and Cook, 1969). Inhibition of IMPDH leads to the reduction of xanthine monophosphate (XMP), GTP, and deoxyguanosine triphosphate (dGTP), which results in inhibition of the proliferation of T/B lympholeukocytes and cancer cells. The investigations undertaken by Engl et al. showed that MPA has suppressive effects on glycosylation and expression of several adhesion molecules relevant to tumor metastasis (Engl et al., 2005). A recent study suggested that MPA exerted its anti-tumor effects via modulation of the tumor microenvironment (Domhan et al., 2008).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor that functions as a transcription factor regulating the expression of genes involved in lipid biosynthesis, glucose metabolism, as well as the proliferation and differentiation of cells (Willson et al., 2000; Kliewer et al., 1992). PPARγ has a key regulatory role in differentiation, and is highly expressed in adipose

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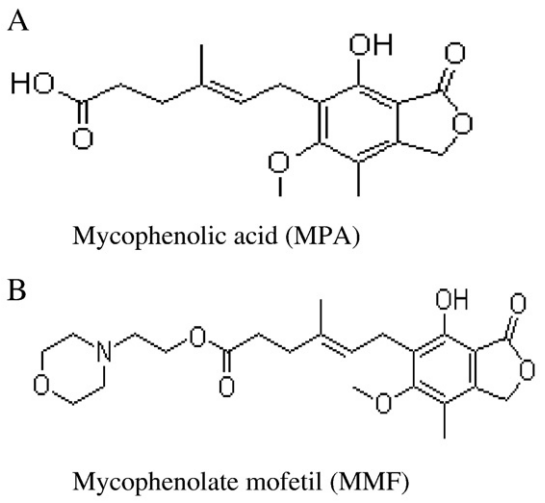


Fig. 1. Chemical structures of mycophenolic acid and mycophenolate mofetil.

tissues. Extensive studies have demonstrated PPARγ to also be expressed in many types of human cancer cells, and activation of PPARγ with its ligands such as thiazolidinediones (TZDs) has a significant inhibitory effect on the proliferation of cancer cells, including leukemia (Saiki et al., 2006) and various human tumor cells (Tontonoz et al., 1997; Demetri et al., 1999; Clay et al., 1999; Suh et al., 1999; Mueller et al., 1998; Chang and Szabo, 2000; Sarraf et al., 1998; U Sarraf et al., 1999; Kubota et al., 1998; Guan et al., 1999; Takahashi et al., 1999). PPARγ has become a novel target for the discovery of anticancer agents (Kopelovich et al., 2002; Panigrahy et al., 2005). In our investigations and those of Nakajima et al. (2007), MPA was identified as a potent PPARγ agonist. However, whether PPARγ activation has a putative role in the anticancer efficacy of MPA has not been elucidated.

In the present study, using the breast cancer cell lines MDA-MB-231 and MCF-7, we provided experimental evidence to show that MPA induces breast cancer cells to undergo adipocyte-like terminal differentiation through (at least in part) PPARγ activation.

2. Materials and Methods

2.1. Reagents

Mycophenolic acid (MPA) was provided by North China Pharmaceutical Group Corporation (Shijiazhuang, China). Rosiglitazone (GlaxoSmithKline, Middlesex, UK) was purchased commercially. Oil red O and hematoxylin were from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were synthesized by Shanghai Sangon Company Limited (Shanghai, China).

2.2. Vector Construction

All the expression and reporter plasmids were constructed according to the scheme shown in Fig. 2A. Briefly, the full-length cDNA of human PPARγ and the cDNA encoding the PPARγ ligand binding domain (LBD) (amino acids 174–475) were amplified from human fat cell total mRNA (Clontech, #7128-1) by reverse transcription-polymerase chain reaction (RT-PCR). The full-length cDNA and PPARγ-LBD cDNA were cloned separately and respectively into vector pTargeT (Promega, Sunnyvale, CA, USA) and vector pBIND (Promega) to construct the full-length PPARγ expression plasmid pTargeT-hPPARγ and the GAL4-hPPAR (LBD) chimeric receptor expression plasmid pBIND-hPPAR (LBD). For the reporter plasmids, three copies of the PPAR response element (PPRE) sequence (5′-AACTAGGTCAAAGGTCA-3′) and five copies of the GAL4 response element (Zheng et al., 2010) were synthesized and inserted into the pGL3-promotor Vector (Promega) to obtain the reporter plasmid pPPRE × 3-SV40-luc and pG5-SV40-Luc (firefly luciferase). The integrity of all constructions was verified by DNA sequencing and restriction enzymatic analysis.

2.3. Cell Lines and Cell Culture

Chinese hamster ovary CHO-K1, and the human breast cancer cell lines MDA-MB-231 and MCF-7 were from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were routinely cultured and passaged in RPMI1640 (Sigma-Aldrich) supplemented with 10% new calf serum and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Transfection and Reporter Gene Assay

Chinese hamster ovary CHO-K1 cells were seeded in 96-well plates at 1×10^4 cells/well 24 h before transfection. For the chimeric receptor reporter assay, 0.07 μg/well pBIND-hPPAR (LBD) and 0.23 μg/well reporter plasmid pG5-SV40-Luc were cotransfected into CHO-K1 cells. For the PPRE × 3-luc reporter assay, 0.07 μg/well full-length expression plasmid pTargeT-hPPARγ, 0.1 μg/well reporter plasmid pPPRE × 3-SV40-luc and 0.01 μg/well internal control plasmid pBIND were cotransfected into CHO-K1 cells. Transfection was carried out with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection for 6 h, cells were treated with MPA, rosiglitazone (positive control) or an equivalent volume of dimethyl sulfoxide (DMSO; negative control) for 24 h. The firefly luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) on a Wallac 1420 Victor2 plate reader (Perkin Elmer, Turku, Finland). The results were expressed as fold induction after normalization by the activity of *Renilla* luciferase. All transfections were carried out in triplicate and repeated at least thrice.

2.5. Proliferation Inhibition Assay

One hundred microliters of MDA-MB-231 or MCF cells was seeded in 96-well plates at 1×10^4 cells/well and incubated with a series of dilutions of MPA for 3–5 days. After treatment, cells were then incubated with a 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche Diagnostics, Indianapolis, IN, USA) solution for 4 h. One hundred microliters of 10% sodium dodecyl sulfate (SDS) solution was added to the culture. The absorbance at 570 nm was measured on the Wallac 1420 Victor2 plate reader (Pekin Elmer). The values of 50% inhibitory concentration (IC_{50}) were obtained from the inhibition curve against various concentrations of MPA.

2.6. Phenotype Analyses of MPA-treated Cells

For the investigation of morphological changes, after incubation with MPA for 7 days, cells were fixed and stained with Oil-red O/hematoxylin. Cell images were taken with a Polaroid digital camera mounted on a light microscope (Nikon, Washington, DC, USA) at 40× magnification.

2.7. Quantification of Lipid Content in Cells

The quantification of lipid content in cells was carried out as previously described (Folch et al., 1957). Briefly, the total triglyceride of cells was extracted with chloroform–methanol–water (2: 1: 1 v/v) and quantified by spectrophotometric means using A Triglyceride Assay Kit (Sigma-Aldrich). Other aliquots of cells were used for measurement of intracellular protein using the Bradford assay (Bio-Rad, Hercules, CA, USA). The lipid content in cells was expressed as total triglyceride per μg protein. Data are mean ± S.D.

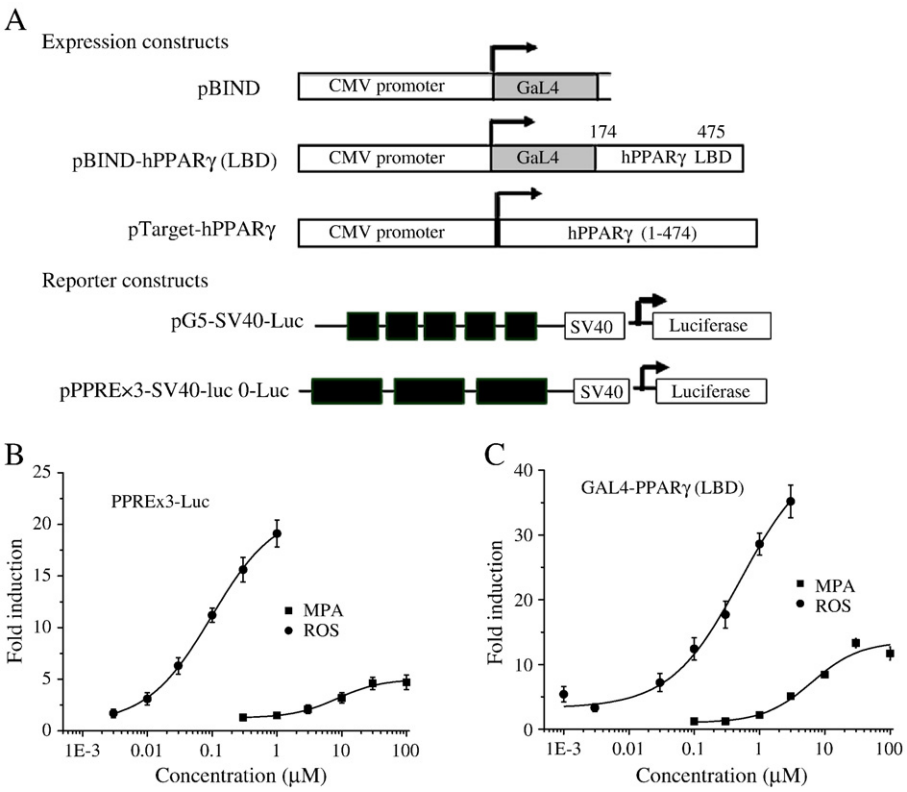


Fig. 2. MPA activates the transcription function of PPARγ. (A) Expression and reporter constructs (schematic). (B) Transactivation of PPARγ in the PPRE×3-luc reporter assay. (C) Transactivation of PPARγ in the GAL4-PPARγ-LBD chimeric receptor assay. For the PPRE×3-luc reporter assay, reporter plasmid pPPRE×3-SV40-luc, expression plasmid pTargeT-hPPARγ and internal standard plasmid pBIND were co-transfected into CHO-K1 cells. For the chimeric receptor assay, reporter plasmid pG5-SV40-Luc and chimeric receptor expression plasmid pBIND-hPPAR (LBD) were co-transfected into CHO-K1 cells. MPA or rosiglitazone (Ros) was added after transfection for 6 h, and the luciferase activity of the cells detected after a 24-h incubation. All experiments were carried out in triplicate wells (n = 3), and results expressed as fold-induction over baseline values after normalization with *Renilla* luciferase activity.

2.8. Flow Cytometric Analyses of the Cell Cycle

For flow cytometric analyses, cells were plated at 1×10^6 cells per T-75 flask and exposed to MPA at the indicated concentration when cell confluence was >80%. Five days later, cells were harvested and fixed with ice-cold 100% ethanol. After incubation at 4 °C for 30 min and centrifugation at 1000 rpm for 5 min, cells were resuspended in 0.5–1 mL of propidium iodide (3 μg/mL; Sigma-Aldrich) solution containing RNase (20 μg/mL, Sigma-Aldrich) and then incubated overnight in the dark. Samples were analyzed on an Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA). The cell cycle was analyzed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

2.9. Analysis for Clonogenic Growth

The capacity for clonogenic formation of cancer cells was investigated as previously described (Mueller et al., 1998). For assessment of the clonogenic capacity of breast cancer cells in response to MPA, MDA-MB-231 or MCF cells were plated in 3.5 cm^2 plates at 10^3 cells/plate, MPA or vehicle (0.2% DMSO) added into the culture, and the mixture maintained for 2 weeks. For assessment of the clonogenic capacity of differentiated cells induced by MPA, MDA-MB-231 and MCF cells were pre-treated with 10 μM MPA for 1 week, re-plated into 3.5 cm^2 plates at 10^3 cells/plate, and cultured in fresh medium without MPA for 2 weeks. Cell clones were fixed with 10% formaldehyde and stained with 0.1% crystal violet.

2.10. Semi-quantitative RT-PCR

For semi-quantitative RT-PCR analyses, cells were harvested after treatment with MPA for the indicated time. The total RNA was extracted using Trizol Reagent (Clontech, Mountain View, CA, USA). Two micrograms of RNA from each sample was reverse-transcribed into a 40-μL reaction volume using an oligo d (T) primer and MMLV reverse transcriptase (Invitrogen Darmstadt, Germany) according to the manufacturer's protocol. One microliter of cDNA was used for further semi-quantitative analyses of the mRNA levels of adipocyte fatty acid binding protein (aP2), PPARγ and adipsin D. Human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) mRNA was used as an internal reference gene. Gene expression was analyzed using the following pairs of primers: PPARγ (forward: 5′-CCCTCATGGCAATTGAATGT-3′, reverse: 5′-GGCATTATGAGACATCCCCAC-3′), aP2 (forward: 5′-TTGTAGGTACC TGGAAACTTGTCT-3′, reverse: 5′-CTTGCTTGCTAAATCAGGGAAAAC-3′), adipsin D (forward 5′-CCTGTGCGGCGGCGTCCTGGTG-3′, reverse: 5′-GGTGATGGCGCCGTCGTGGTG-3′), and hGAPDH (forward: 5′-CAC-CATCTTCCAGGAGCGAG-3′, reverse: 5′-TCACGCCACAGTTTCCCGGA-3′).

2.11. Identification of Efficient siRNA Sequences and Generation of Stable Cell Lines

Three pairs of complementary single-stranded DNA oligonucleotides (Fig. 5A) targeting human PPARγ (NM_015869) were designed and synthesized by Invitrogen. The pre-siRNA expression vector was constructed by inserting the annealed double-stranded (ds) oligo into the linearized pcDNA™6.2-GW/EmGFP-miR vector (Invitrogen/Life Technologies) and named SR70-1, SR70-2 and SR70-3. For identification of the optimal siRNA sequence for PPAR-γ knockdown, 0.03 μg/well vector SR70-1, SR70-2, SR70-3 or negative control plasmid pcDNA™6.2-GW/-EmGFP-miR-neg (Invitrogen) was transfected into a 6-well plate. One day post-transfection, the mRNA level of PPARγ was assessed by semi-quantitative RT-PCR.

The PPARγ knockdown efficiency of siRNA sequences was also examined by PPRE-SV40-luc reporter gene assay. Briefly, MDA-MB-231 cells were co-transfected with 0.1 μg/well PPRE-SV40-luc reporter plasmid and 0.03 μg/well SR70-1, SR70-2, SR70-3 or pcDNA™6.2-GW/-EmGFP-miR-neg control plasmid in the 96-well plate, respectively. After transfection for 6 h, the PPARγ agonist (rosiglitazone) was added to the cells, and luciferase activity examined 24 h later.

Stable PPARγ-knockdown clonal cell lines were generated in a selection medium containing 4 μg/mL of blasticidin according to the instructions of the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (www.invitrogen.com). A cell line transfected with the pcDNA™6.2-GW/±EmGFP-miR-neg control plasmid was used as the negative control. The knockdown efficiency of PPARγ in the stable cell line was analyzed by semi-quantitative RT-PCR.

2.12. Statistical Analyses

Each experiment was carried out at least thrice. Results are means S.D. Differences between two groups were analyzed by the Student's *t*-test. $P<0.05$, and $P<0.01$ were considered to be statistically significant.

3. Results

3.1. MPA Efficiently Activates the Transcription Function of PPARγ

The GAL4-hPPARγ (LBD) chimeric receptor assay and PPRE×3-luc reporter gene assay systems were developed as shown in Fig. 2A. The potency of MPA to activate PPARγ was assayed using the two different reporter gene assays. The activity of MPA was much lower than that of the reference compound rosiglitazone (ROS), but MPA increased luciferase activities with a maximum induction fold of 4.6 ± 0.6 and an EC_{50} of 5.2 ± 2.1 μM (Fig. 2B) in the PPRE×3-luc reporter gene assay. In the GAL4-hPPARγ (LBD) chimeric receptor assay, MPA produced a maximum fold-induction of 13.1 ± 0.7 and an EC_{50} of 9.3 ± 1.7 μM (Fig. 2C).

3.2. MPA Inhibits the Proliferation of Breast Cancer Cells at the G1/G0 Stage of the Cell Cycle

The anti-proliferation effects of MPA on breast cancer cells were examined on two cell lines: MCF-7 and MDA-MB-231. The proliferation of MDA-MB-231 cells and MCF cells was significantly inhibited by MPA in a dose-dependence manner with an IC_{50} of 1.8 μM and 4.6 μM, respectively (Fig. 3A). Cell-cycle analyses (Fig. 3B) showed that treatment with MPA resulted in an increase in the proportion of the G0/G1 stage in MDA-MB-231 and MCF-7 cells. After treatment with MPA for 5 days, the proportion of the G0/G1 stage of MDA-MB-231 cells increased from 49.5% (vehicle) to 59.4% (1 μM) and 60.6% (10 μM), and the proportion of the G2/M stage decreased from 16.6% (vehicle) to 6.7% (1 μM) and 3.1% (10 μM), whereas the proportion of the S stage increased only slightly. MCF-7 cells showed a similar cell-cycle profile. These results suggested that MPA arrested the cell cycle of MDA-MB-231 and MCF-7 cells at the G0/G1 stage.

3.3. MPA Induces Adipocyte-like Differentiation of Breast Cancer Cells

To investigate the morphological changes induced by MPA, the cells were fixed and stained with Oil-red O/hematoxylin after treatment with MPA. As shown in Fig. 3C, the treated MDA-MB-231 and MCF-7 cells underwent dramatic adipocyte-like morphological conversion: enlarged cell volume with more and larger lipid droplets accumulated in the cytoplasm. Quantification of lipid content in cells showed that the lipid content in cells treated with 10 μM MPA was nearly 3–4-fold more that in control cells (Fig. 3D).

PPARγ, aP2, and adipsin D are three well-known adipocyte markers (MacDougald and Lane, 1995; Demetri et al., 1999). Next, we analyzed the mRNA levels of the genes of these three agents in MPA-treated MDA-MB-231 and MCF-7 cells by semi-quantitative RT-PCR. MPA clearly upregulated the expression of PPARγ, aP2 and adipsin D in treated MDA-MB-231 and MCF-7 cells (Fig. 3E). The expressions of adipocyte markers as well as G1 phase arrest, enlarged cell volume and accumulation of lipid droplets indicated that MPA could efficiently induce the breast cancer cells to undergo adipocyte-like differentiation.

3.4. MPA Reverses the Clonogenic Formation of Breast Cancer Cells

To further determine the ability of MPA to reverse the malignancy of breast cancer cells, the in-vitro colony-forming capacities of MDA-MB-231 and MCF cells in response to MPA were further investigated. Untreated control cells developed numerous, large, and dense colonies, but colonies were not observed in MPA-treated cells (Fig. 4). When MDA-MB-231 and MCF cells were pretreated with 10 μM MPA for 1 week to induce differentiation and then re-plated the cells lost the ability to form colonies in fresh medium lacking MPA. These results suggested that MPA treatment results in the permanent withdrawal of the cell cycle in breast cancer cells.

3.5. PPARγ-knockdown Partially Blocks the Differentiation Induced by MPA

RNA interference (RNAi) is an effective tool for the study of the biological and physiological function of target genes. To further confirm the role of PPARγ in the efficiency of the induction of differentiation of MPA, we knock-downed the PPARγ of MDA-MB-231 cells using RNAi. First, we identified the efficient siPPARγ sequences (Fig. 5A) by detecting the mRNA levels of PPARγ in MDA-MB-231 cells after transfection with PPARγ pre-siRNA expression vectors. SR70-1 and SR70-3 could efficiently reduce the expression of PPARγ in MDA-MB-231 cells (Fig. 5B). The expression of PPARγ is critical to the PPRE-luc reporter gene assay, so we confirmed the efficiency of PPARγ siRNA using this assay. Cells transfected with the negative control vector exhibited the highest luciferase activity, and cells transfected with PPARγ pre-siRNA expression vector SR70-1 or SR70-3 had only ~20–30% luciferase activities compared with those of the control (Fig. 5C). These data were consistent with the results obtained from the mRNA levels of PPARγ in cells. Both experiments suggested that SR70-1 and SR70-3 were efficient siPPARγ sequences.

Next, we used the optimal siRNA vector SR70-1 and pcDNA™ 6.2-GW/±EmGFP-miR-neg control vector to generate a stable PPARγ-downregulated clonal cell line and a negative control cell line, respectively. Using the two stable cell lines, the morphological changes and gene expression between PPARγ-knockdown cells and control cells were compared after treatment with MPA for 5 days. Compared with negative control cells, these patterns of morphologic differentiation (Fig. 5D) and lipid accumulation (Fig. 5E) were notably decreased in PPARγ-knockdown cells after treatment with MPA. However, they were in a visibly differentiated state when compared with untreated negative control cells. At the molecular level (Fig. 5F), gene expression was very consistent with the morphological observations. In addition to PPARγ suppressed in PPARγ-knockdown MDA-MB-231 cells, the mRNA levels of aP2 and adipsin D were markedly lower in MPA treated PPARγ-knockdown MDA-MB-231 cells than that in the treated control cells. However, the mRNA level of aP2 in PPARγ-knockdown cells was still higher than that in untreated control cell.

4. Discussion

Treating cancer through inducing differentiation is an attractive and promising alternative to conventional chemotherapy for certain

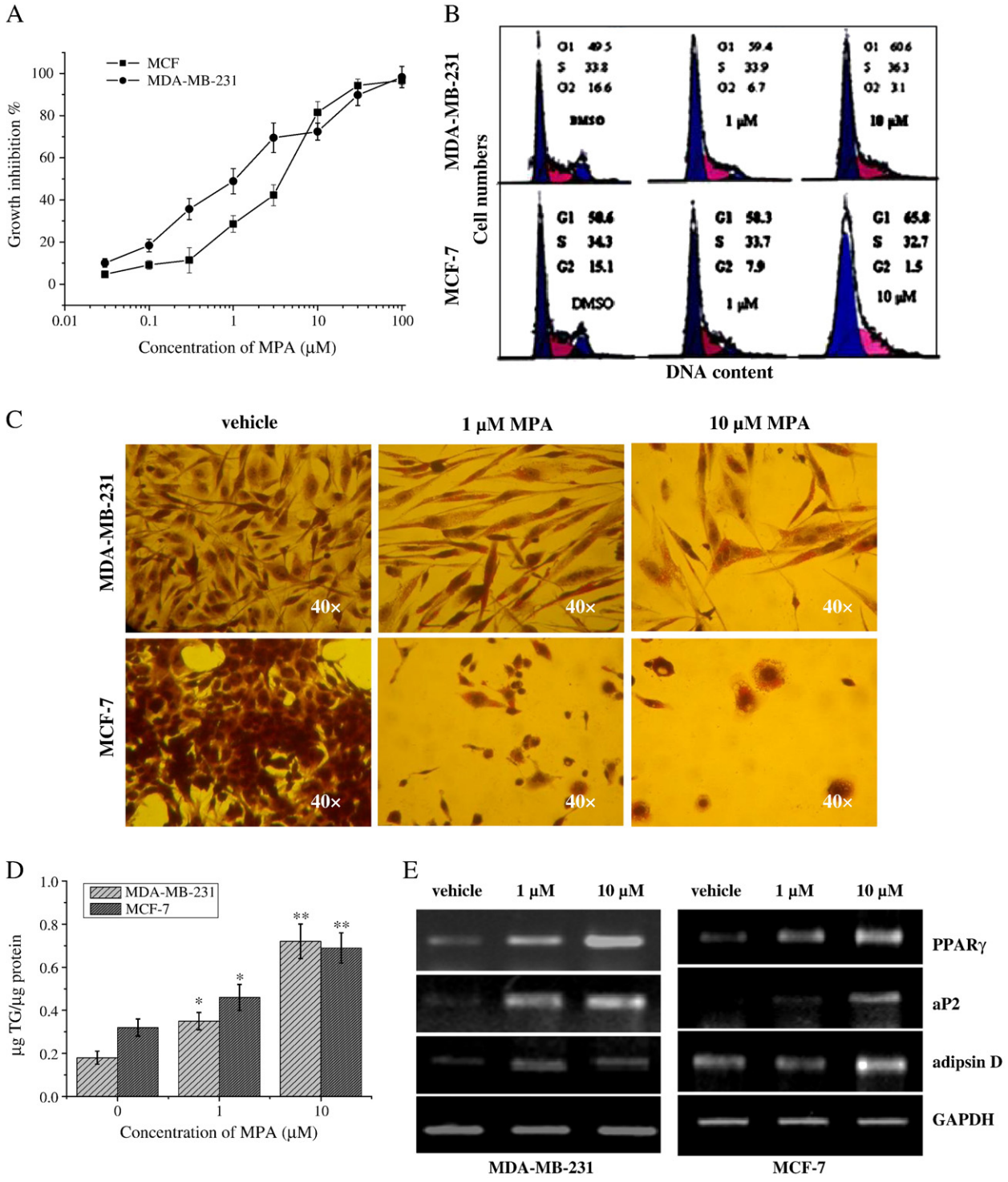


Fig. 3. MPA inhibits the proliferation of breast cancer cells and induces adipocyte-like differentiation. (A) MPA inhibits the proliferation of MDA-MB-231 and MCF-7 cells. Cells were plated on a 96-well plate and treated with MPA (0.01–100 μM) for 5 days. Cell proliferation was measured using the MTT assay. Results are shown as % inhibition. (B) MPA arrests the cell cycles of MDA-MB-231 and MCF-7 cells at the G1 stage. MDA-MB-231 and MCF-7 cells were plated in a T-25 flask and treated with 1 μM MPA, 10 μM MPA, or vehicle when cell confluence was >80%. Cells were harvested after 5 days for analysis of the profile of the cell cycle using flow cytometry. (C) MPA induces breast cancer cells to undergo adipocyte-like phenotypic alterations. After incubation with MPA for 7 days, MDA-MB-231 and MCF-7 cells were fixed and stained with Oil-red O/hematoxylin. Images were captured at 40× magnification. (D) Lipid accumulation increased in MPA-treated breast cancer cells. Lipid content in cells was quantified as described in the "Materials and Methods" section. *P<0.05, **P<0.01, compared with control cells. (E) MPA induces the expression of adipocyte differentiation markers in breast cancer cells. MDA-MB-231 and MCF-7 cells were treated with MPA for 5 days. Total RNA was extracted for the analysis for the mRNA level of human PPARγ, aP2, and adipsin D genes by semi-quantitative RT-PCR. Human GAPDH was as internal reference gene. Experiments were repeated thrice. Results show representative experiments.

malignancies. All-trans retinoic acid, an agonist of the retinoic acid receptor (RAR), has been successfully applied in differentiation therapy to patients with acute promyelocytic leukemia (Frankel et al., 1994). However, the development of differentiation-inducing agents to treat malignant tumors (especially solid tumors) has been limited. Breast cancer is the commonest form of cancer in women. It accounts for an estimated 26% of all cancer diagnosed in women in 2008 according to the American Cancer Society. Studies have shown that PPARγ is not only

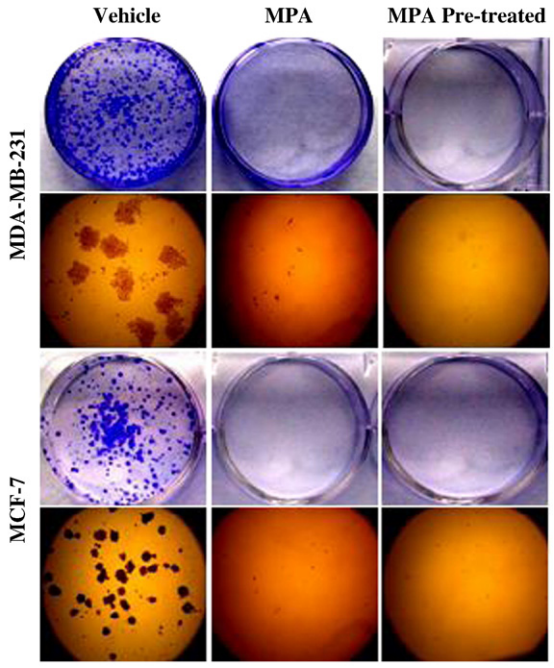


Fig. 4. MPA reverses the clonogenic proliferation of breast cancer cells. MDA-MB-231 and MCF-7 cells or cells pre-treated with MPA for 1 week were plated in 3.5 cm^2 dishes at 10^3 cells per dish, and cultured in fresh medium with or without 10 μM MPA for 2 weeks for analyses of clonogenic proliferation. Cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet. Experiments were repeated thrice, and the results shown here are representative.

highly expressed in adipocyte cells, but expressed at significant levels in primary and metastatic breast adenocarcinomas in humans (Suh et al., 1999). Treatment with PPARγ agonists such as the well-known ligand of PPARγ, troglitazone, could result in the terminal differentiation of breast cancer cells through the induction of differentiation and apoptosis (Clay et al., 1999; Mueller et al., 1998).

In the present study, MPA was shown to efficiently activate the transcription function of PPARγ by the PPRE-luc reporter assay. GAL4-hPPARγ chimeric receptor assay further supported the notion that MPA acts as a PPARγ agonist. In the cell growth assay and morphological observation experiments, MPA treatment significantly inhibited the growth of the breast cancer cells MDA-MB-231 and MCF-7 at the G1/G0 stage, and led to dramatic morphological conversion to the adipocyte phenotype: enlarged cell volume and extensive accumulation of neutral lipids. Such morphological changes are consistent with observations from other PPARγ ligand-treated cancer cells such as breast cancer cells (Mueller et al., 1998) and liposarcoma (Demetri et al., 1999). The effects of MPA on the induction of differentiation has also been observed in leukemia, lymphomas (Cohn et al., 1999; Gortz et al., 1997; Pankiewicz et al., 2002) and solid carcinoma cells in the prostate gland (Floryk and Huberman, 2006). In addition, clonogenic capacity analyses suggested that MPA treatment could cause terminal differentiation in that differentiated cells lost the ability to form colonies even after the removal of MPA from the culture medium.

PPARγ, aP2 and adipsin D are widely used molecular markers of adipocyte differentiation (Demetri et al., 1999; MacDougald and Lane, 1995). After treatment with MPA, the mRNA levels of aP2, adipsin D and PPARγ were notably upregulated in differentiated MDA-MB-231 and MCF cells. PPARγ acts as the promoter of differentiation, and its expression is significantly induced in differentiated (Lefebvre et al., 1999) or PPARγ ligand-treated cells (Demetri et al., 1999; Mueller et al., 1998). aP2, a well-known target of PPARγ (Tontonoz et al., 1994), is highly expressed in adipocyte and adipocyte-like differentiated cancer cells (Demetri et al., 1999; MacDougald and Lane, 1995). The upregulation of aP2 in MPA-treated cells further supported the notion that PPARγ activation can be induced by MPA. These patterns of gene expression are consistent with a less malignant and adipocyte-like differentiated state.

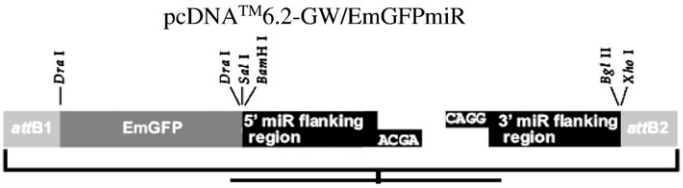
However, when we knock-downed the PPARγ gene in MDA-MB-231 cells by RNAi, although PPARγ-knockdown remarkably lowered the response of the differentiated state of MDA-MB-231 cells to MPA, it could not completely inhibit the MPA-induced differentiation process. This result suggested that PPARγ-knockdown only partially inhibited the MPA-dependent differentiation process. One presumption for such a result may be the limitation of RNAi technology. RNAi is a powerful method to silence gene expression, but the PPARγ-RNAi used in the present study could not completely block PPARγ gene expression in MDA-MB-231 cells, and there remained ~20% of the expression of PPARγ in the PPARγ-knockdown stable cell line. Taken together, these data suggested that MPA could efficiently induce the adipocyte-like terminal differentiation of breast cancer cells, and that the effects were (at least in part) mediated through the PPARγ activation pathway.

In addition, PPARγ activation has been shown to be involved in the apoptosis of many types of cancer cells (Chang and Szabo, 2000; Clay et al., 1999; Martinasso et al., 2007; Takahashi et al., 1999; Ubukata et al., 2000). The effects of MPA on the induction of apoptosis in cancer cells such as human T-lymphocyte, monocyte cell lines (Cohn et al., 1999) and multiple myeloma cells (Takebe et al., 2006) have been reported. Thus, PPARγ activation may contribute to the apoptosis of cancer cells induced by MPA.

Pharmacokinetic and bioavailability studies have shown that single oral administration of 1 g of the pro-drug MMF yielded a dose of 76 mM MPA in the plasma of healthy volunteers (Bullingham et al., 1996). This is much higher than the 1–10 μM MPA used to induce cell-growth arrest and differentiation in MDA-MB-231 and MCF cells in the present study. This suggested that MPA improves PPARγ activation, and that the induction of differentiation could be used as a nontoxic alternative or adjunct to conventional chemotherapy for treating breast cancer via induction of adipocyte-like differentiation. This could be a valuable therapeutic strategy which deserves extensive study.

Fig. 5. PPARγ-knockdown partly inhibits MPA-induced differentiation of MDA-MB-231 cells. (A) Oligonucleotide sequences for siRNA of hPPARγ. (B) Identification of efficient siRNA sequences. MDA-MB-231 cells were transfected with vector SR70-1, SR70-2, SR70-3, or pcDNA™ 6.2-GW/—EmGFP-miR-neg (negative control). One day post-transfection, the mRNA level of PPARγ was assessed by semi-quantitative RT-PCR. Human GAPDH was as internal reference gene. Experiments were repeated thrice, and the results shown here are representative. (C) Confirmation of efficient siRNA sequences by the PPRE × 3-luc reporter gene assay. MDA-MB-231 cells were transfected with the reporter vector PPRE × 3-luc and PPARγ pre-siRNA expression vector or control vector, and treated with 10 μM rosiglitazone. The luciferase activity of cells was assayed after 24 h. Results are expressed as the percentage of firefly luciferase activities of the negative control (means ± S.D.). *$P<0.05$, **$P<0.01$, compared with the control. (D) Effects of PPARγ-knockdown on phenotypic alterations in MDA-MB-231 cells induced by MPA. The stable PPARγ-knockdown MDA-MB-231 clonal cell line and negative control cell line were treated with 10 μM MPA for 5 days. Images were captured after staining with Oil red O/hematoxylin at 40× magnification. (E) Quantification of lipid content in MDA-MB-231 cells. Data are means ± S.D. of three independent experiments. *$P<0.05$, compared with the control. (F) Effects of PPARγ-knockdown on the expression of adipocyte differentiation markers in MDA-MB-231 cells treated MPA. The stable PPARγ-knockdown MDA-MB-231 clonal cell line (kd.) or negative control cell line (con.) was treated with 10 μM MPA for 5 days. Total RNA was extracted for the semi-quantitative PCR analyses of human PPARγ, aP2, and adipsin D genes. Human GAPDH was as internal reference gene. Experiments were repeated thrice, and results are representative.

A



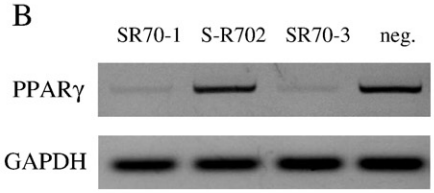
SR70-1: TGCTGTTTAGAAACTCCCTTGTCATGGTTTTGGCCACTGACTGACCATGACAAGAGTTTCTAAA
CAAATCTTTGAGGGAACAGTACCAAAACCGGTGACTGACTGGTACTGTTCTCAAAGATTTGTCC

SR70-2: TGCTGTTCACATTCAGCAAACCTGGGGTTTTGGCCACTGACTGACCCCAGGTTCTGAATGTGAA
CAAGTGTAAGTCGTTTGGACCCCAAAACCGGTGACTGACTGGGGTCCAAGACTTACACTTGTCC

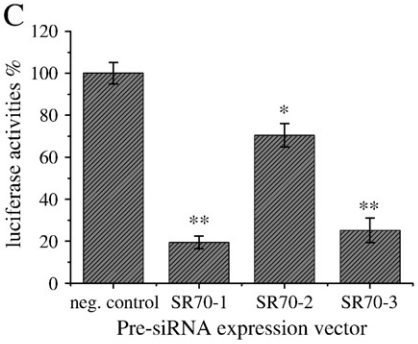
SR70-3: TGCTGCATACTTGTAATCTGCAACCAGTTTTGGCCACTGACTGACTGGTTGCATTACAAGTATG
CGTATGAACATTAGACGTTGGTCAAAACCGGTGACTGACTGACCAACGTAATGTTCATACGTCC

Neg. TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
CTTTACATGACGCACCTCTGCAGTCAGTCACCGGTTTTGCAGAGGTGCGCGTCATGTAAAGTCC

B



C



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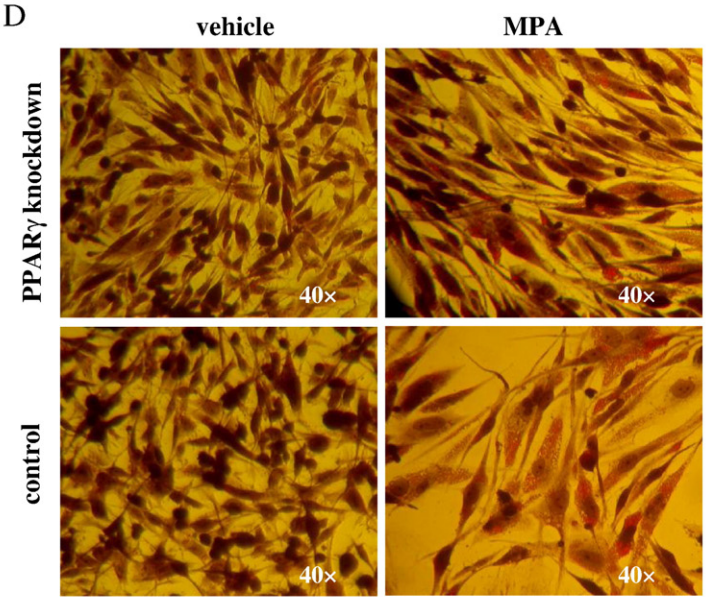
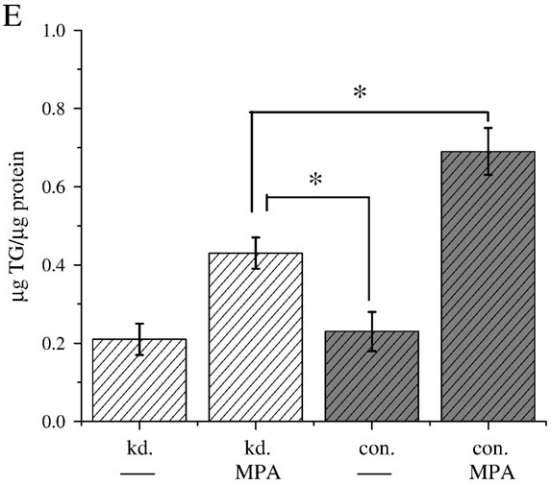
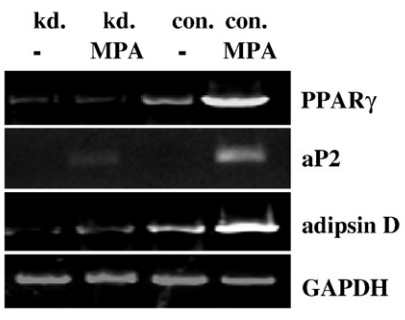


E



F



5. Conclusion

We demonstrated that MPA can efficiently induce adipocyte-like terminal differentiation of breast cancer cells. We also showed that this effect is (at least in part) mediated through the PPARγ pathway, which is a distinct molecular mechanism for the anticancer properties of MPA. We also suggest that MPA improves PPARγ activation, and that the induction of differentiation could be used as a nontoxic alternative or adjunct to conventional chemotherapy for treating breast cancer.

Acknowledgments

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