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Original Research

24-Methylenecycloartanyl Ferulate Protects against Expression of Pro-Angiogenesis Related Genes through Peroxisome Proliferator-Activated Receptor Gamma 2 in Human Breast Cancer MCF7 Cells

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Abstract

Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) has currently been considered as molecular target for angiogenesis signaling. Here, we investigated the effect of 24-methylenecycloartanyl ferulate (24-MCF) induced PPARγ2 on expression of angiogenesis-related genes in MCF7 cells. cDNA microarray, real-time PCR revealed that 24-MCF mediated the expression of genes related to angiogenesis in MCF-7 cells. We identified PPAR-response elements (PPRE) located in the LIF promoter regions (-1192 to -802), and VEGF (-452 to +1). Luciferase reporter assay demonstrated that activation of the LIF gene, an anti-angiogenesis factor, was increased upon both 24-MCF treatment and PPARγ2 overexpression; whereas activation of VEGF promoters, known pro-angiogenesis factors, was decreased upon 24-MCF treatment and PPARγ2 overexpression. While these mutations individually appeared to have no effect. Treatment with 24-MCF also decreased VEGF production in MCF7 cells and PMA-stimulated tube formation in HUVECs. Our findings suggest that 24-MCF induces PPARγ2-mediated regulation of angiogenesis-related genes *via* PPRE motifs.

ABBREVIATIONS

24-MCF: 24-Methylenecycloartanyl ferulate; PPARγ2: Proliferator Activated-receptor gamma 2; VEGF: Vascular Endothelial Growth Factor; LIF: Leukemia Inhibitory Factor

INTRODUCTION

24-Methylenecycloartanyl ferulate (24-MCF), a gamma oryzanol (γ-oryzanol) compound, is a non-toxic dietary compound that exhibits important pharmacological activities, such as anti-cholesterol [1], anti-platelet aggregation [2], and anti-tumor properties [3]. Our previous studies showed that 24-MCF-induced peroxisome proliferator-activated receptor gamma 2 (PPARγ2) activation increased parvin-β expression, leading to the inhibition of oncogenic integrin-linked kinase (ILK), which is associated with tumor angiogenesis signaling in MCF7 cells [3].

PPAR represents a nuclear hormone receptor superfamily of ligand-inducible transcription factors that regulate genes in various metabolic processes. The PPAR family comprises three isoforms: PPARα, PPARβ/δ, and PPARγ [4]. Activated PPARα regulates genes involved in the β-oxidation pathway [5]; PPARβ/δ enhances glucose metabolism; and activation of PPARγ causes insulin sensitization and enhances glucose metabolism [6]. Three different isoforms of PPARγ have been identified and were generated as a result of alternative splicing: PPARγ1, PPARγ2, and PPARγ3 [7]. PPARγ2 is expressed exclusively in

adipose tissue and accelerates adipocyte differentiation [8]. A potential role for PPARγ2 in carcinogenesis was highlighted by the ability of the ligands to affect cellular proliferation and differentiation [9]. Because of its potent effects on the regulation of cell fate, PPARγ2 has attracted interest as a potential target for cancer therapy [3,7]. Furthermore, numerous studies have shown that PPARγ2 activation leads to the regulation of anti- and pro-angiogenic pathways [10].

Although several key components of the angiogenesis process and the angiogenic switch have been reported [11], the complete underlying molecular mechanisms are not yet fully known. Previous studies showed that the promoter sequence of the pro-angiogenic factor VEGF contained a PPAR response element (PPRE) motif [12], and demonstrated that PPAR-γ2 activation decreased pro-angiogenic VEGF [12]. Although 24-MCF has recently been shown to possess anti-tumor properties [3,4], its molecular mechanism is not fully understood and its potential effects on angiogenesis have remained largely unknown.

In this study, we hypothesized that 24-MCF regulated PPARγ2-mediated angiogenesis. We first examined whether angiogenesis-related genes identified by microarray in MCF-7 cells treated with 24-MCF were PPARγ2 target genes.

MATERIALS AND METHODS

The rice bran compound 24-MCF was provided by the Korean National Institute of Crop Science (Jeollabuk-do, Korea). Each gene on the array Agilent Human Whole Genome 44K v2 chip was provided by E-Biogen Inc. (Seoul, Korea). Total RNA was extracted from human breast cancer MCF7 cells using a TRIzol reagent kit (Invitrogen, San Diego, CA) following the manufacturer's instructions. Antibodies against VEGF and β-actin antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Cell culture

Human breast cancer MCF7 cells were cultured in Dulbecco's Modified Eagle's Medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD) were cultured in EBM-2 medium supplemented with EGM-2 Single Quots. EBM-2 and EGM-2 Single Quots were purchased from Cambrex Company (Lonza, Walkersville, MD).

Plasmid constructs

We collected the putative PPRE motifs using the Dragon PPAR Response Element Spotter v.2.0 (www.cbrc.kaust.edu.sa/ppre/). To construct promoter-reporter plasmids, promoter fragments containing PPRE motifs were amplified and ligated into the *Xho*I and *Hind* III sites of the pGL3-basic vector, which contains firefly luciferase coding sequences but lacks eukaryotic promoter or enhancer elements. Oligonucleotides used in this experiment were as follows: LIF were 5′-TTGATCTCAGGTCAGGGTCAGGCGGAA-3′ (wild type-forward), 5′-TTGATCTCAAATAAGAATTTGGCGGAA-3′ (mutant-forward); and 5′-GTCGCTGGTCCCTTCCAGAC-3′(reverse). VEGF were5′- GGGGAGAAGGCCAGGGGTCACTCCA-3′ (wild type-forward) and 5′-GGGGAGAAGGTTAGGTATTTCTCCA-3′(mutant-forward); and 5′-TCCTCCCCGCTACCAGCCGA-3′ (reverse). Const-ruction of pcDNA-human PPARγ2 (accession no. NM_015869) expression plasmid was described previously [3]. Plasmids were confirmed by DNA sequencing.

Microarray

Microarrays were generated and cRNA probes were hybridized using the Low RNA Input Linear Amplification Kit (Agilent Technology, Santa Clara, CA) according to the manufacturer's instructions. Labeled cRNA was purified on a cRNA Cleanup Module (Agilent Technology, Santa Clara, CA) according to the manufacturer's instructions. Fragmented cRNA was resuspended with 2× hybridization buffer and directly pipetted onto the assembled Human Oligo Microarray (44K; Agilent Technology). Arrays were hybridized at 65°C for 17 h using a hybridization oven (Agilent Technology, Santa Clara, CA). Hybridized microarrays were washed according to the manufacturer's protocol (Agilent Technology).

Data analysis

Hybridized images were scanned using a DNA microarray scanner (Agilent Technology, Santa Clara, CA) and quantified with Feature Extraction software (Agilent Technology). All data normalization and selection of genes whose expression changed were performed using Gene Spring GX 7.3 (Agilent Technology). The averages of normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity. Functional annotation of genes was performed according to the Gene Ontology Consortium (http://www.geneontology.org/index.shtml) using Gene Spring GX 7.3. Genes were classified based on searches performed by BioCarta (http://www.biocarta.com/), GenMAPP (http://www.genmapp.org/), DAVID (http://david.abcc.ncifcrf.gov/), and Medline (http://www.ncbi.nlm.nih.gov/).

Western blot analysis

Western blot analysis was performed according to a standard method as previously described [3]. Protein samples (20 μg) were separated on a 4-12% NuPAGE gel (Invitrogen, San Diego, CA) and then transferred to a nitrocellulose membrane using standard procedures. The membrane was blotted with anti-VEGF antibody or anti-β-actin antibody as a control, and analyzed using the Multi Gauge V3.1 program (Fujifilm Tokyo, Japan).

RNA isolation and real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Total RNA (1 μg) was converted to cDNA using oligo-dT primer and AccuPower RT premix (Bioneer, Korea) in a 20 μl reaction. Primers for real-time PCR are shown in Table 2. The reactions were dispensed into into 96-well optical plates and amplification was carried out using 10 ul amplification mixtures containing Power SYBR Green PCR Master MIX (BioRad, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve: 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec. Three replicates were performed per cDNA sample. Reactions were run on a CFX96 Real-Time System (BioRad, USA). A threshold cycle was observed in the exponential phase of amplification, and quantification of relative expression

levels was determined by the $\Delta\Delta C_t$ method, according to the manufacturer's recommendation.

Luciferase assay

MCF7 cells (30,000 cells / well) were seeded in 24-well plates 12-16 h before transfection. Cells were co-transfected using Lipofectamine LTX (Invitrogen, San Diego, CA) according to the manufacturer's instruction with the following plasmids: 500 ng of WT-reporter plasmid (LIF or VEGF) or its mutant *firefly* luciferase reporter plasmid, 50 ng of *Renilla* luciferase reporter plasmid (pRL-TK) (Promega, Madison, WI), and either 500 ng of pcDNA empty or pcDNA-PPARγ2. Twenty-four hours later, cells were incubated with a medium containing dimethyl sulfoxide (DMSO) or 24-MCF (25, 50, and 100 μM). After 24 h, both *firefly* and *Renilla* luciferase activities were quantified using a Dual-Luciferase Reporter Assay system (Promega, Madison, WI) according to the manufacturer's instructions. Relative luciferase activity was calculated as the ratio of *firefly* /*Renilla* activity. All experiments were performed in triplicate.

Quantification of VEGF protein

VEGF was quantified using the Quantikine human VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, MCF7 cells were seeded in 12-well plates and cultured until 90 % confluent. Cells were switched to fresh serum-free medium containing 24-MCF. After 12 h, the conditioned medium was collected and the number of cells in each well was counted. The secretion of VEGF in the conditioned medium (200 μl) was determined and normalized against the number of cells in the well. A serial dilution of human recombinant VEGF was performed in each assay to obtain a standard curve. All experiments were performed in triplicate.

Tube formation assays

Endothelial tube formation was assessed by an *in vitro* cell-based assay (Cayman Chemical, Ann Arbor, MI) in HUVECs according to the manufacturer's instructions. Phorbol 12-myristate 13-acetate (PMA), known as a stimulator of angiogenesis, pretreatment enhanced the ability of HUVECs to organize into a cell networking structure (termed tube formation). A suspension of HUVECs in medium was then seeded in 96-well plates coated with cell-based extracellular matrix gel at a density of 10^5 cells/ml and treated with 24-MCF in the presence of 1 μM PMA. After 12 h, tube formation was observed by calcein staining under an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan).

Statistical analyses

All data were evaluated by a one-way analysis of variance (ANOVA). The results were considered statistically significant when p values were *$p \leq 0.05$, **$p \leq 0.001$.

RESULTS

Gene expression profiles of 24-MCF treated MCF7 cells

We analyzed changes in global gene expression profiles in MCF7 cells treated with 24-MCF (100 μM) and those treated with DMSO control using a microarray; and identified 30,552 differentially expressed genes out of 44,000 arrayed genes (69.4 %), by microarray hybridization (Supplementary 1). Genes with a fold change of 2.0 or above were considered positively regulated, whereas those with a fold change of 0.5 or below were considered negatively regulated. Figure 1A shows a scatter plot representing the average log2-fold changes; a total of 914 upregulated and 642 downregulated genes were identified in MCF7 cells treated with 24-MCF. Quantitatively, unmodified genes by an average log2-fold change of ≤ 2.0 or ≥ 0.5 were assigned a rate of 94.9 % (Figure 1A). Scatter plot analyses showed 5.1 % (1,556 genes) of the differentially expressed under 24-MCF treatment. To examine the biological functions affected by 24-MCF treatment, we performed functional annotation clustering analysis of the differentially expressed genes using the Gene Ontology Consortium program. The differentially expressed genes were associated with inflammation, angiogenesis, extracellular matrix, immune response, cell proliferation, and cell migration (Figure 1B), indicating that several cellular metabolic pathways are linked directly or indirectly to the response to 24-MCF treatment.

Validation of microarray data using real-time PCR

From the functional annotation clustering analysis, we then selected 10 genes in the angiogenesis categorization that were differentially expressed. Genes with fold change > 2 were upregulated and genes with fold change < 0.5 were downregulated in the MCF7 cells treated with 24-MCF (Figure 1C and 1D). Table 1 provides further characterization of 10 genes that were more strictly responsive to 24-MCF; literature search for these genes classified them as anti- and pro-angiogenesis related genes based on their reported functions. For validation of 10 genes that were differentially expressed among all genes in the angiogenesis category, we next analyzed expression levels of these genes using real-time PCR with the primers listed in Table 2. These results showed that at 12 h after 24-MCF treatment, anti-angiogenesis-related genes (LIF, NPPB, EGR1, and PPARγ2 genes) were significantly upregulated(DMSO control *vs* $p \leq 0.001$) (Figure 1C), whereas pro-angiogenesis-related genes (MMP11, L1Cam, Pbx1, HOXA7, RHOT1, and VEGF genes) were markedly downregulated in MCF7 cells (DMSO control *vs* $p \leq 0.001$) (Figure 1D). Furthermore, these effects occurred in a dose-dependent manner. These findings help validate the microarray results.

PPARγ2 modulates the promoter activity of angiogenesis-related genes via PPRE motifs

To investigate whether the PPARγ2 can directly regulate gene expression through peroxisome proliferator response elements (PPREs), we pursued the computational prediction of PPREs on the promoter region of the angiogenesis-related genes (Table 1). Putative PPRE was identified in the promoter region of candidate genes containing a single half-site (GGGTCA or AGTTCA). We performed luciferase assays using promoter sequences from two of the identified genes (Figure 2A and 2D). We observed that the activation of the LIF reporter vector by 24-MCF or PPARγ2 expression was significantly increased in comparison with the control (control *vs* $p \leq 0.001$, empty vector *vs* $p \leq 0.001$) (Figure 2B and 2C). The activation of VEGF reporter vector by 24-MCF or PPARγ2 were significantly decreased in comparison with the control (control *vs* $p \leq 0.05$, empty vector *vs* $p \leq 0.001$) (Figure 2E and 2F); while these mutation sites had no effect (Figure 2B, 2C, 2E, and 2F), indicating that PPARγ2 mediated transcriptional

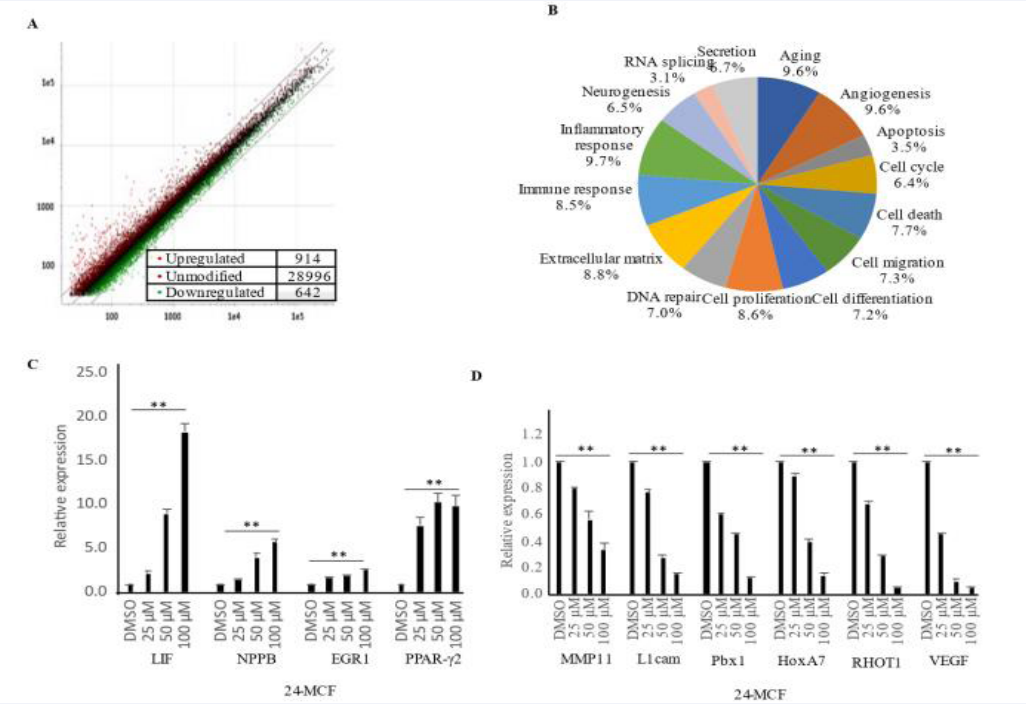

Figure 1 cDNA microarray analysis of gene expression in MCF-7 cells in response to 24-MCF. Scatter plot of normalized microarray data for all 44,000 genes. (A) Plot showing the upregulated (2.0-fold or above, red), unmodified (black), and downregulated genes (0.5 or below, green) in MCF7 cells treated with 24-MCF. (B) Diagram chart showing distribution of ontological analysis in MCF7 cells treated with 24-MCF. Gene ontology terms of differentially expressed genes are shown; the numbers of genes in each category are indicated as a percentage. (C; Anti-angiogenesis-related genes and D; Pro-angiogenesis-related genes) Real-time PCR verification of microarray results. A list of the top 10 genes from the array data with the greatest differences in expression between MCF7 cells treated with 24-MCF and DMSO control. Real-time PCR analysis of 10 selected genes in MCF7 cells treated with various concentrations of 24-MCF as indicated or DMSO control (DMSO, *n*=3; 24-MCF, *n*=3). The results are presented as the mean ± SE (*n* = 3 per group). ** DMSO control *vs* p ≤ 0.001.

Table 1: Angiogenesis-related genes identified by microarray, their proposed functions and position of putative single half-site PPRE in each gene promoter.

Gene symbol	Functions	Position of putative PPRE motifs (GGGTCA or AGGTCA)	Refs
LIF	Angiogenesis inhibitor that may interfere with VEGF and bFGF	-1451 -935 +1	13
NPPB	Angiogenesis inhibitor that may interfere with MAP kinase pathway	-1331 -773 +1	14
EGR1	Inhibitor angiogenic signaling of VEGF and bFGF	-1373 -767 +1	15
PPARγ2	Causes insulin sensitization and enhances glucose metabolism. Inhibits angiogenesis and cancer	-1232 -211 +1	10
MMP11	Stimulates tumor growth, angiogenesis, invasion and metastasis	-1317 -695 -343 -350 +1	16
L1cam	Induces angiogenesis	-1346 -851 +1	17
Pbx1	Required for pro-angiogenic Hox DNA binding and transcriptional activity in endothelial cells	-1360 -407 +1	18
HOXA7	Stimulates cell migration, proliferation and differentiation. Induces angiogenesis in hMSCs	-1428 -439 +1	19
RHOT1	Stimulates tumor growth and angiogenesis	-1413 -673 +1	20
VEGF	Stimulates tumor growth, migration, invasion, angiogenesis and tube formation	-1347 -674 -432 +1	21

Abbreviations: PPRE: Peroxisome proliferator response element; MAP kinase: Mitogen-activated protein kinases; LIF: Leukemia Inhibitory Factor; NPPB: Natriuretic Peptide B; EGR1: Early Growth Response 1; PPARγ2: Peroxisome Proliferator-Activated Receptor Gamma 2; MMP11: Matrix Metallopeptidase 11; L1cam: L1 Cell Adhesion Molecule; Pbx1: PBX Homeobox 1; HOXA7: Homeobox A7; RHOT1: Ras Homolog Family Member T1; VEGF: Vascular Endothelial Growth Factor; bFGF: basic fibroblast growth factor; hMSCs: Mesenchymal stem cells

Table 2: Sequences of the primers used for real-time PCR in this study.

Gene symbol (Accession)	Sequence (5' – 3')	Product (bp)
Anti-angiogenesis-related genes		
LIF (NM_002309)	F: AGATCAGGAGCCAACTGGCACA R: GCCACATAGCTTGTCCAGGTTG	110
NPPB (NM_002521)	F: TCTGGCTGCTTTGGGAGGAAGA R: CCTTGTGGAATCAGAAGCAGGTG	121
EGR1 (NM_001964)	F: AGCAGCACCTTCAACCCTCAGG R: GAGTGGTTTGGCTGGGGTAACT	133
PPARγ2 (NM_005037)	F: AGCCTGCGAAAGCCTTTTGGTG R: GGCTTCACATTCAGCAAACCTGG	153
Pro-angiogenesis-related genes		
MMP11 (NM_005940)	F: GAGAAGACGGACCTCACCTACA R: CTCAGTAAAGGTGAGTGGCGTC	126
L1cam (NM_000425)	F: TCGCCCTATGTCCACTACACCT R: ATCCACAGGGTTCTTCTCTGGG	123
Pbx1 (NM_001204961)	F: GGAGGATACAGTGATGGACTCG R: GGAGGTATCAGAGTGAACACTGC	144
HOXA7 (NM_006896)	F: GCTGAGGCCAATTTCCGCATCT R: GTAGCGGTTGAAGTGGAACTCC	126
RHOT1 (NM_001033566)	F: GACAAAGACAGCAGGCTGCCTT R: TCGCTGAACACTCCACACAGGT	133
VEGF (NM_001025366)	F: TTGCCTTGCTGCTCTACCTCCA R: GATGGCAGTAGCTGCGCTGATA	126
GAPDH (NM_001256799)	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA	131

Abbreviations: LIF: Leukemia Inhibitory Factor; NPPB: Natriuretic Peptide B; EGR1: Early Growth Response 1; PPARγ2: Peroxisome Proliferator-Activated Receptor Gamma 2; MMP11: Matrix Metallopeptidase 11; L1cam: L1 Cell Adhesion Molecule; Pbx1: PBX Homeobox 1; HOXA7: Homeobox A7; RHOT1: Ras Homolog Family Member T1; VEGF: Vascular Endothelial Growth Factor

induction of LIF and VEGF *via* PPRE motifs.

24-MCF inhibited VEGF production in MCF-7 cells and tube formation in HUVECs

To determine whether 24-MCF treatment exhibits anti-angiogenic activity, we investigated activation of VEGF in 24-MCF-treated MCF-7 cells using ELISA and western blot. 24-MCF treatment gradually decreased secretion of VEGF (DMSO control *vs* $p \leq 0.001$) (Figure 3A) and level of VEGF protein (DMSO control *vs* $p \leq 0.001$) (Figure 3B) in MCF7 cells in a dose-dependent manner.

We also performed tube formation assays in HUVEC cells using an *in vitro* cell-based assay. HUVECs were treated with 25 or 100 μM 24-MCF for 12 h under 1 μM PMA as a stimulator of angiogenesis [(Figure 3C, (b) - (e)] and then tube formation was observed by calcein staining under an inverted fluorescent microscope. We found that 24-MCF treatment [(Figure 3C, (d) - (e); 25 μM and 100 μM, respectively] decreased tube formation in comparison with HUVECs treated with 1 μM PMA alone (as a stimulator of angiogenesis) in Matrigel [(Figure 3C, (b)], similar to an inhibitor control with 1 μM JNJ-10198409 (as an inhibitor of angiogenesis) [(Figure 3C, (c)], indicating that 24-MCF treatment exhibits anti-angiogenic activity.

DISCUSSION

Angiogenesis is the formation of new blood vessels and is a hallmark of tumor development and metastasis [22]. In particular, PPARγ2 targets a set of genes that have a critical impact on numerous diseases including angiogenesis and cancer [23]. Our previous results showed that 24-MCF-induced PPARγ2 increased parvin-β expression, which inhibited oncogenic ILK signaling, anchorage-independent growth, and cell migration [3]. A recent study established PPARs as a group of ligand-activated transcriptional regulators that sit at the intersection of genes and the dietary environment [24]. Thus, PPARγ2 has shown predominately anti-angiogenic properties *in vitro* and in animal models [24]. Therefore, some research has examined the PPARγ2 activating potential of a wide range of natural products originating from traditionally used medicinal plants and dietary sources [25]. The γ-oryzanol compound 24-MCF has been reported to have important pharmacological activities, including anti-tumor properties [3]. It has non-toxicity and is used as a healthy food supplement [3]. Nonetheless, its mechanism is unclear. In addition, 24-MCF-induced gene expression changes have not been fully recognized until now.

Here we identified differentially expressed genes in MCF7 cells treated with 24-MCF using a microarray technique. We found that the genes differentially expressed in response to 24-MCF treatment were involved in various cellular responses, including the inflammatory response, angiogenesis, aging, extracellular matrix, cell proliferation, immune response, cell death, and cell migration. Based on the clustering results, we selected the most significantly angiogenesis-related genes, including four anti-angiogenesis-related genes that were upregulated (NPPB, LIF, PPARγ2 and EGR1 genes) and six pro-angiogenesis-related genes that were downregulated (HoxA7, L1Cam, MMP11, RHOT1, VEGF, and Pbx1 genes).

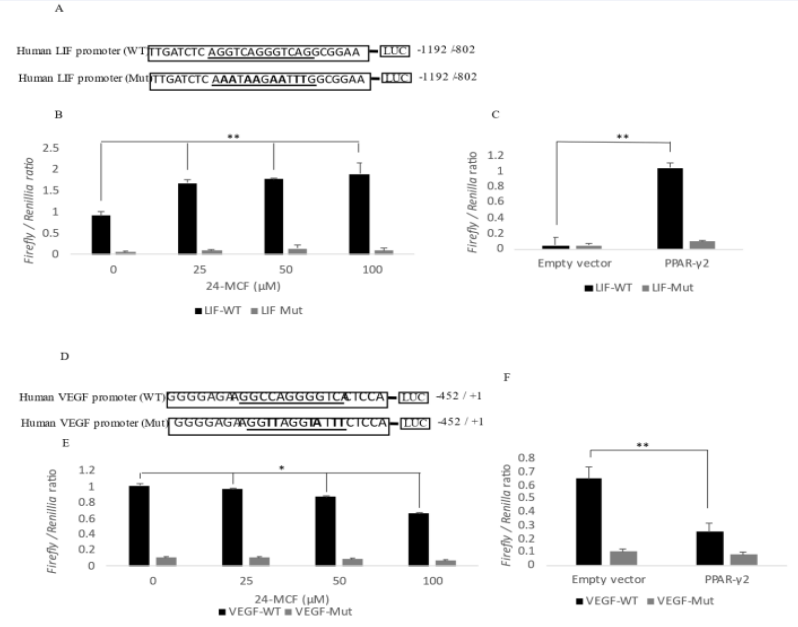

Figure 2 PPARγ2 modulates promoter activity via PPRE motifs. Schematic representations of the LIF (-1192 / -802) promoters (A); VEGF promoter (-452/+1) (D) containing a PPRE motif. Promoter regions were inserted into luciferase plasmids; each wild type (LIF and VEGF) and point mutation (LIF and VEGF). MCF7 cells were co-transfected with the LIF (A), VEGF reporter plasmid (500 ng) (D), pRL-TK (50 ng) and either pcDNA3 empty vector or pcDNA3-PPARγ2 (C and F). For luciferase assays, MCF7 cells (30,000 cells/well) were plated in 24-well plates one day prior to transfection. Transfected cells were treated with 24-MCF (0, 25, 50 and 100 μM) (B and E) for 24 h and the cells were used for reporter gene assays. Luciferase activities from reporter plasmids were normalized by internal *Renilla* luciferase (pRL-TK) activity. Values represent as fold induction of the DMSO control. The results are presented as the mean ± SE (n = 3 per group). DMSO control *vs* *p ≤ 0.05, **p ≤ 0.001.

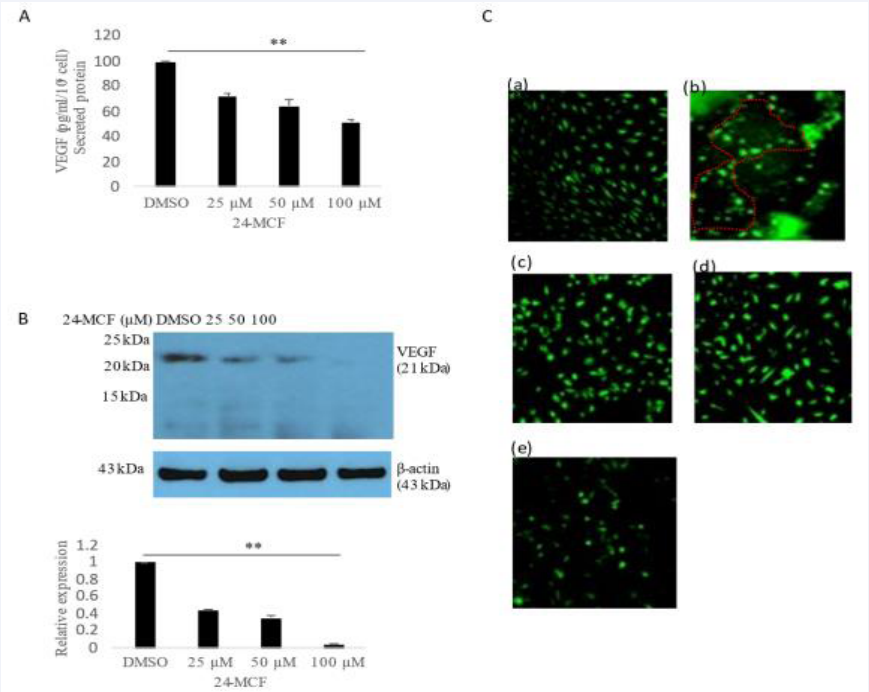

Figure 3 24-MCF inhibited endogenous VEGF production and HUVEC tube formation.
(A) VEGF secretion levels in the supernatant were determined in the presence or absence of 24-MCF using ELISA and normalized against the number of cells in the well. A serial dilution of human recombinant VEGF was performed in each assay to obtain a standard curve. The results are presented as the mean ± SE (n = 3 per group). DMSO control *vs* **p ≤ 0.001. (B) MCF-7 cells were treated with 24-MCF (0, 25, 50, and 100 μM) for 24 h and analyzed by western blotting using the VEGF antibodies. β-actin served as loading control. The results are presented as the mean ± SE (n = 3 per group). DMSO control *vs* **p ≤ 0.001. (C) HUVECs were kept in 1% FBS containing medium for 12 h. (a) Tube formation on Matrigel was performed using HUVECs treated with DMSO control, (b) 1 μM phorbol-12-myristate-13-acetate (PMA) as a positive control (stimulator of angiogenesis), (c) 1 μM PMA with 1 μM JNJ-10198409 as a negative control (inhibitor of angiogenesis), (d) 1 μM PMA with 25 μM of 24-MCF and (e) 1 μM PMA with 100 μM of 24-MCF. Tube formation was observed by calcein staining under an inverted fluorescent microscope (Olympus IX71, Tokyo, Japan).

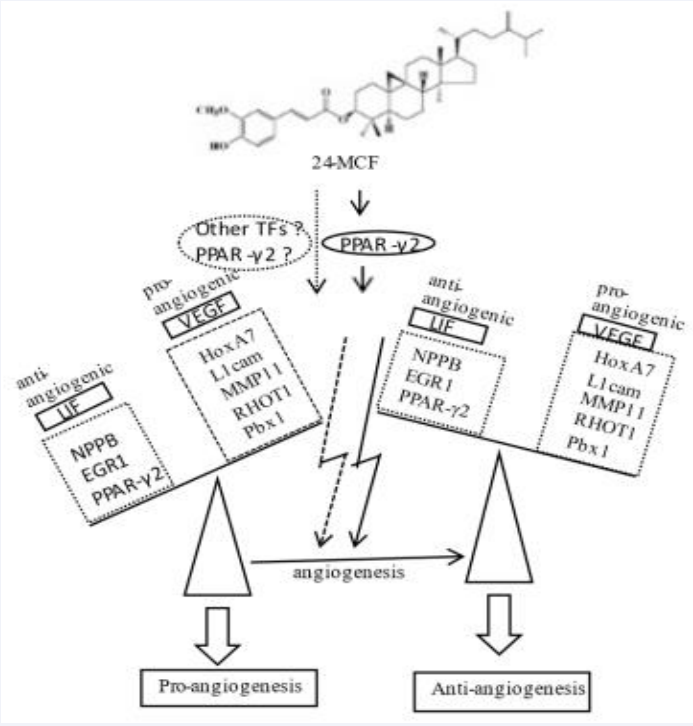

Figure 4 The proposed mechanism by which 24-MCF treatment induces PPARγ2-mediated anti-angiogenesis. 24-MCF induces PPARγ2 to function as an angiogenic switch. Solid rectangle mark: confirmed in this experiment, Dotted rectangle: another transcription factors or PPARγ2 possibility.

Angiogenesis is tightly regulated at the molecular level [26]. The imbalance of pro- and anti-angiogenic signaling within tumors creates an abnormal vascular network that is characterized by dilated, tortuous and hyperpermeable vessels [26]. Based on our results, we propose that 24-MCF induces PPARγ2 to function as an angiogenic switch that mediates the balance between pro- and anti-angiogenic molecular levels (Figure 4).

Our ELISA results showed that VEGF secretion was decreased in MCF7 cells treated with 24-MCF. Based on previous studies that displayed PMA-induced angiogenesis as VEGF-dependent [21], we analyzed the ability of HUVECs to undergo cell tube formation in response to 24-MCF treatment and found that PMA-induced tube formation of HUVEC cells was markedly attenuated by 24-MCF treatment. These results are consistent with previous results showing that VEGF can regulate all of the key steps of the angiogenic process, including proliferation, migration, and tube formation [21].

CONCLUSION

Our microarray analysis of MCF7 cells treated with 24-MCF identified 10 differentially expressed genes correlated with angiogenesis. 24-MCF treatment decreased VEGF production and tube formation. Together, this suggests that 24-MCF may be a new anti-angiogenic agent that could be important for the development of new cancer therapeutics in the future.

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REFERENCES

1. Purushothama S, Raina PL, Hariharan K. Effect of long-term feeding of rice bran oil upon lipids and lipoproteins in rats. Mol Cell Biochem. 1995; 146: 63-69.

2. Seetharamaiah GS, Krishnakantha TP, Chandrasekhara N. Influence of oryzanol on platelet aggregation in rats. J Nutr Sci Vitaminol. 1990; 36: 291-297.

3. Kim HW, Lim EJ, Jang HH, Kang DR, Lee SH, Kim HR, et al. 24-Methylenecycloartanyl ferulate, a major compound of γ-oryzanol, promotes parvin-β expression through an interaction with peroxisome proliferator-activated receptor γ2 in human breast cancer cells. Biochem Biophys Res Commun. 2015; 468: 574-579.

4. Panigrahy D, Huang S, Kieran MW, Kaipainen A. PPARγ as a therapeutic target for tumor angiogenesis and metastasis. Cancer Biol Ther. 2005; 4: 687-693.

5. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc Natl Acad Sci. 1994; 91: 11012-11016.

6. Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, et al. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. Pharmacol Rev. 2006; 58: 726-741.

7. Tachibana K, Kobayashi Y, Tanaka T, Tagami M, Sugiyama A, Katayama T, et al. Gene expression profiling of potential peroxisome proliferator-activated receptor (PPAR) target genes in human hepatoblastoma cell lines inducibly expressing different PPAR isoforms. Nucl Recept. 2005; 3: 1-17.

8. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 1994; 8: 1224-1234.

9. Tonini T, Rossi F, Claudio PP. Molecular basis of angiogenesis and cancer. Oncogene. 2003; 22: 6549-6565.

10. Xin X, Yang S, Kowalski J, Gerritsen ME. Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis *in vitro* and *in vivo.* J Biol Chem. 1999; 274: 9116-9121.

11. 11. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996; 86: 353-364.

12. Venkatachalam G, Kumar AP, Yue LS, Pervaiz S, Clement MV, M.K. Sakharkar MK. Computational identification and experimental validation of PPRE motifs in NHE1 and MnSOD genes of human. BMC Genomics. 2009; 10: 1-13.

13. Pepper MS, Ferrara N, Orci L, Montesano R. Leukemia inhibitory factor (LIF) inhibits angiogenesis *in vitro.* J Cell Sci. 1995; 108: 73-83.

14. Kozulin P, Natoli R, O'Brien KM, Madigan MC, Provis JM. Differential expression of anti-angiogenic factors and guidance genes in the developing macula. Mol Vis. 2009; 15: 45-59.

15. Lucerna M, Pomyje J, Mechtcheriakova D, Kadl A, Gruber F, Bilban M, et al. Sustained expression of early growth response protein-1 blocks angiogenesis and tumor growth. Cancer Res. 2006; 66: 6708-6713.

16. Kumar BVS, Kumar KA, Padmanath K, Sharma B, Kataria M. Heterologous expression and functional characterization of matrix metalloproteinase-11 from canine mammary tumor. Anim Biotechnol. 2013; 24: 31-43.

17. Magrini E, Villa A, Angiolini F, Doni A, Mazzarol G, Rudini N, et al. Endothelial deficiency of L1 reduces tumor angiogenesis and promotes vessel normalization. J Clin Invest. 2015; 124: 4335-4350.

18. Charboneau A, East L, Mulholland N, Rohde M. Pbx1 is required for Hox D3-mediated angiogenesis. Angiogenesis. 2005; 8: 289-296.

19. Tang B, Qi G, Sun X, Tang F, Yuan S, Wang Z, et al. HoxA7 plays a critical role in metastasis of liver cancer associated with activation of Snail. Mol Cancer. 2016; 15: 1-12.

20. Li Q, Yao L, Wei Y, Geng S, He C, Jiang H. Role of RHOT1 on migration and proliferation of pancreatic cancer. Am J Cancer Res. 2015; 5: 1460-1470.

21. Xu H, Czerwinski P, Hortmann M, Sohn HY, Förstermann U, Li H. Protein Kinase C alpha promotes angiogenic activity of human endothelial cells via induction of vascular endothelial growth factor. Cardiovasc Res. 2008; 78: 349-355.

22. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer. 2003; 3: 401- 410.

23. Han SW, Roman J. Anticancer actions of PPARγ ligands: Current state and future perspectives in human lung cancer. World J Biol Chem. 2010; 1: 31-40.

24. Nava-Villalba M, Nuñez-Anita RE, Bontempo A, Aceves C. Activation of peroxisome proliferator-activated receptor gamma is crucial for antitumoral effects of 6-iodolactone. Mol Cancer. 2015; 14: 1-11.

25. Wang L, Waltenberger B, Pferschy-Wenzig EM, Blunder M, Liu X, Malainer C, et al. Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ), Biochem Pharmacol. 2014; 92: 73-89.

26. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev. 2011; 91: 1071-1121.

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