WISP-2/CCN5 Is Involved As a Novel Signaling Intermediate in Phorbol Ester-Protein Kinase Cα-Mediated Breast Tumor Cell Proliferation†

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ABSTRACT: PMA and active phorbol esters stimulate the proliferation of various tumor cells, including ER-positive human breast tumor cell lines. However, the specific signaling pathways involved in the PMA-induced mitogenic effect on breast tumor cells have not been fully elucidated. In the present study, we explored the mechanisms associated with the mitogenic influence of PMA on breast tumor cells. Following an acute exposure (i.e., within 2 to 6 h) to PMA (50 nM), a mitogenic effect was observed on WISP-2/CCN5-positive breast tumor cell lines, including MCF-7, ZR-75-1 and SKBR-3 cells, and induction of WISP-2/CCN5 mRNA expression paralleled the observed mitogenic proliferation. This effect was undetected in WISP-2/CCN5 negative MDA-MB-231 breast tumor cells or human mammary epithelial cells with or without ER-α transfection. The mitogenic effect of PMA was perturbed by short hairpin RNA (shRNA)-mediated inhibition of WISP-2/CCN5 signaling in MCF-7 cells. Moreover, the upregulation of WISP-2/CCN5 by PMA is not ER dependent but is instead mediated through a complex PKCα-MAPK/ERK and SAPK/JNK signaling pathway, which leads to growth stimulation of MCF-7 breast tumor cells. These series of experiments provide the first evidence that WISP-2/CCN5 is a novel signaling molecule that critically participates in the mitogenic action of PMA on noninvasive, WISP-2/CCN5-positive breast tumor cells through PKCα-dependent, multiple molecular signal transduction pathways.

Phorbol esters are plant products that are exclusively extracted from a few members of the family *Euphorbiaceae* (*1*), and they are present as both active (*β*-phorbols) and inactive forms (α-phorbols) (*1*). The most widely used active phorbol esters are PMA[1] (phorbol 12-myristate 13-acetate)/TPA (4*β*-12-*O*-tetradecanoyl-phorbol-13-acetate), and PDBu (4*β*-phorbol-12, 13-dibutyrate). PMA and other active phorbol esters are potent tumor promoters in vivo, and they are compelling regulators of growth of many different cell types (*2*). Regulation of cellular growth by phorbol esters is a complex event and is usually mediated through the modulation of its receptor protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinase (*3*). PKC plays a critical role in controlling many signal transduction pathways associated with physiological and patho-physiological functions (*1, 3*−*8*). Moreover, PMA and active phorbol esters can exhibit dual effects (stimulatory or inhibitory) on cellular proliferation in various tumor cells including human breast tumor cell lines (*1, 9*−*14*). In breast tumor cells, the dual effects are dependent on the length of exposure (incubation periods). Prolonged exposure (20 h or more) to PMA and other phorbol esters cause the growth arrest of estrogen-receptor (ER)-positive and -negative breast cancer cells (*11*, *12*, *14*, *15*), whereas short exposure (less than 20 h) to PMA stimulates cell proliferation in various ER-positive breast tumor cells (*10*, *12*). The problem of how phorbol esters promote divergent impacts on ER-positive breast tumor cells has attracted a number of studies in recent years. However, the molecular mechanisms of the dual impact of phorbol esters remain unclear.

PMA mimics the action of estrogen in vivo (*16*) and in vitro (*17*), and it upregulates the cytosolic ER levels and their binding activity (*18, 19*), Therefore, it is reasonable to anticipate that the proliferative impact of PMA on ER-positive breast tumor cells may be mediated through estrogen-dependent signaling pathways. To evaluate this possibility, the current studies utilized the WISP-2/CCN5 (Wnt1-induced secreted protein/cysteine-rich 61, connective

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[1] Abbreviations: CCN, connective tissue growth factor/cysteine-rich 61/neuroblastoma overexpressed family of growth factors; DIG, digoxigenin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetracetate; ER-α, estrogen receptor-α; ERK, extra cellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMEC, human mammary epithelial cells; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonylfluoride; shRNA, short hairpin RNA; WISP-2, wnt-1 induced secreted protein-2.

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tissue growth factor/nephroblastoma overexpressed 5) signaling system.

WISP-2/CCN5 encodes a member of the CCN family of growth factors (*20*) and exhibits divergent roles in a tissue-specific manner (*20*−*24*). Structurally, WISP-2/CCN5 is very similar to the other members of the CCN family, except for the cysteine knot (CT) domain, which is absent in this protein (*25*−*27*). Expression of the WISP-2/CCN5 gene has been identified in a variety of human breast tumor derived cell lines, including MCF-7, ZR-75-1, T-47D, and SKBR-3 as well as human breast tumor samples (*28*). In contrast, the expression of this gene is virtually undetected or minimally detected in normal human mammary epithelial cells and normal breast samples (*21*, *28*). Subsequent studies have demonstrated that WISP-2/CCN5 is a serum-inducible gene and its expression is upregulated by sex steroid hormones such as 17β-estradiol (a natural estrogen) and progesterone in steroid receptor-mediated pathways (*29*−*31*, *21*). Moreover, previous studies also demonstrated that the alteration of WISP-2/CCN5 signaling is associated with significant changes in the proliferative capacity of ER-positive breast cancer cells (*21*, *22*).

Establishing whether WISP-2/CCN5 gene expression correlates with PMA-induced modulation of cell growth is important, given the significant influence of WISP-2/CCN5 gene expression on the proliferation of breast tumor cells. In the present study, we demonstrate that PMA upregulates WISP-2/CCN5 expression in a time-dependent manner, and it is critical to controlling the proliferative effects of PMA on breast tumor cell proliferation. However, the action of PMA on WISP-2/CCN5 expression is independent of ER-α. The results indicate that PKCα is a key mediator of PMA-induced upregulation of WISP-2/CCN5 expression through the activation of MAPK/ERK1/2 and SAPK/JNK pathways.

MATERIALS AND METHODS

Reagents. Wortmannin, Aprotinin, PMSF, leupeptin, and β-actin specific monoclonal antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Estrogen receptor antagonist ICI 182,780 and U0126 were purchased from Tocris (Ellsville, MO) and Promega Corp. (Madison, WI), respectively. PD98059 and SP600125 were obtained from Calbiochem (San Diego, CA). PMA and the antibodies specific to the phosphorylated and nonphosphorylated forms of the MAP kinase family, that is, p44/42 MAPK (ERK1 and ERK2) and SAPK/JNK and p38 MAPK, were purchased from LKT Laboratories, Inc. (St. Paul, MN) and Cell Signaling Technology, respectively (Beverly, MA). Anti-calnexin monoclonal antibody was purchased from BD Biosciences (San Jose, CA). PKCα siRNA/siAb assay kit was purchased from Upstate (Lake Placid, NY). Anti-WISP-2/CCN5 rabbit polyclonal antibody was custom-made (Alpha Diagnostic, San Antonio, TX) as described earlier (*29*). Lipofectin reagent and ECL western blotting detection reagents were obtained from Invitrogen (Carlsbad, CA) and Amersham Pharmacia Biotech, Inc. (Piscataway, NJ), respectively.

Cell Culture. Human breast tumor-derived ER-positive MCF-7, ZR-75-1, ER-α-variant-positive SKBR-3 and ER-negative MDA-MB-231 cells were purchased from American Type Culture Collections (ATCC, Manassas, VA) and grown in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) containing 10% FBS (HyClone, Road Logan, UT) with 100 U/mL of penicillin and 100 U/mL of streptomycin. Human mammary epithelial (HME) cells were obtained from Clonetics Corp. (San Diego, CA), grown in mammary epithelial growth medium (MEGM) (Clonetics Corp.), in serum free conditions. HME cells with stably transfected ER-α were kindly given as a gift by Dr. Deborah Zajchowski (Berlex Biosciences, Richmond, CA) and grown in DFCI-1 medium as described previously (*21*).

Treatments. To determine the effect of PMA on cell proliferation and WISP-2/CCN5 expression, cells were treated with 50 nM PMA for different times. Then, 60−70% confluent cultures were serum-starved for 3 days in order to deplete the growth factors from the cultures, and then, the cells were exposed to PMA for different times. In this study, we considered only one dose of PMA (50 nM) because previous multiple studies have shown that acute exposure (i.e., 2−6 h) of 50nM or higher concentrations of PMA exhibit proliferative effect on some breast tumor cells, whereas prolonged exposures have antiproliferative action. Cultures treated with 0.1% DMSO were considered as untreated controls. For inhibitor assays, the serum-starved cultures were pretreated with appropriate concentrations of inhibitors for 30 or 60 min before exposure to 50 nM PMA for 2 h.

To determine the effect of PMA on ER-α transfected or nontransfected HME cells, the cells were washed with serum-free basal medium (MEBM, Clonetics Corp.) and treated with 50 nM PMA for different periods as indicated.

Cell Proliferation Assay. Cell proliferation assays were carried out according to the previous method described earlier (*21*). Briefly, breast tumor cells and HME cells were plated in 12-well-plates at densities of ∼20 000 cells per well, respectively, and incubated at 37 °C with 5% CO_2 overnight for cell attachment and spreading. In each set of experiments, cells were plated in quadruplicate. Cells were treated with 50 nM PMA under serum-starved conditions for different times. Control and treated cultures were pulsed with 1 μCi [^{3}H]-thymidine/mL (NEN Research, Wilmington, DE) for respective periods of PMA treatments, and 0.1% DMSO was added to the control cultures. One μCi radioactive thymidine may not be sufficient for long-term incubation. It is possible for cells to run out of radioactive thymidine within a 24 h assay period. Therefore, additional radioactive thymidine (0.5 μCi) was added after 18 h of initial incubation into the culture media to get interpretable data. The washed cells were precipitated with 10% trichloroacetic acid, filtered through glass fiber filters, and dried. The amount of incorporated [^{3}H]-thymidine was measured in a 2200CA Tri-Carb liquid scintillation counter (Packard Instrument, Meriden, CT).

shRNA Insert Preparation and Transfection in MCF-7 Cells. Preparation of WISP-2/CCN5-specific shRNA inserts and ligation of the inserts with the pSilencer 1.0-U6 expression vector were carried out following the instruction provided by the manufacturer (Ambion Inc., Austin, TX). Briefly, the double stranded shRNA insert was prepared by incubating the WISP-2/CCN5-specific oligonucleotides at 90 °C for 3 min, followed by 1 h at 37 °C. Thereafter, the annealed shRNA insert was ligated with linearized pSilencer 1.0-U6 expression vector at 16 °C overnight. In this study, we used four pairs of nucleotide sequences to prepare WISP-

2/CCN5-specific four shRNA inserts. The sequences are **shRNA 1**: 5′-GTT TTC TGG CCT TGT CTC TTT CAA GAG AAG AGA CAA GGC CAG AAA ACT GTT TTT T-3′ (**sense**) and 5′- AAT TAA AAA ACA GTT TTC TGG CCT TGT CTC TTC TCT TGA AAG AGA CAA GGC CAG AAA ACG GCC-3′ (**antisense**); **shRNA 2**: 5′-GAC CCA CCT CCT GGC CTT CTT CAA GAG AGA AGG CCA GGA GGT GGG TCT TTT TT-3′ (**sense**) and 5′AAT TAA AAA AGA CCC ACC TCC TGG CCT TCT CTC TTG AAG AAG GCC AGG AGG TGG GTC GGC C-3′ (**antisense**); **shRNA 3**: 5′AGG T GC GTA CCC AGC TGT GTT CAA GAG ACA CAG CTG GGT ACG CAC CTT TTT TT-3′(**sense**) and 5′-AAT TAA AAA AAG GTG CGT ACC CAG CTG TGT CTC TTG AAC ACA GCT GGG TAC GCA CCT GGC C-3′ (**antisense**); **shRNA 4**: 5′-GGG GGG CCC TGT GCC TCT TTT CAA GAG AAA GAG GCA CAG GGC CCC CCT TTT TT-3′ (**sense**) and 5′-AAT TAA AAA AGG GGG GCC CTG TGC CTC TTT CTC TTG AAA AGA GGC ACA GGG CCC CCC GGC C-3′ (**antisense**). The shRNA sequences were either obtained from Ambion, Inc. or selected from our previous studies when the potency of multiple sequences was tested to inhibit the WISP-2/CCN5 expression (*22*). The negative control ligation reaction was performed with the linearized expression vector with no insert. Expression vectors, with or without an shRNA insert, were transformed into the competent cells, DH5α. Plasmids containing an expression vector, with or without an shRNA insert, were purified by QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA).

The transfection procedure was the same as that described previously (*21*, *22*). Briefly, ∼40% confluent cultures were serum starved for 3 days in OPTI-MEM (Invitrogen, Carlsbad, CA). Thereafter, cells were transfected with 10 μg/mL of plasmid with or without an shRNA insert in the presence of 20 μg/mL of lipofectin for 24 h. Following transfection, the cells were further maintained in serum-free conditions for 24 h, and the cells were treated with 50 nM PMA in phenol-red-free DMEM for indicated period.

PKCα siRNA transfection in MCF-7 cells was carried out by using a PKCα siRNA/siAb Assay kit (Upstate, Lake Placid, NY). Approximately, 30−40% confluent MCF-7 cells were serum starved in OPTI-MEM for 72 h and transfected with 200 nM SMARTpool PKCα siRNA in the presence of 20 μg/mL of lipofectin for 24 h. Simultaneously, the MCF-7 cells were transfected with 200 nM pooled nonspecific siRNA duplexes, which are treated as the negative control. Following transfection, the cells were incubated with OPTI-MEM for another 72 h and treated with or without 50 nM PMA for 2 h.

RNA Extraction and Northern Blot Analysis. Total RNA extraction, cDNA synthesis, and DIG-labeled nonradioactive probe preparations were essentially the same as that previously described (*21*). RNA was separated on 1% agarose gel containing 2.2 M formaldehyde in MOPS buffer and blotted on super charged nylon membranes (Schleicher & Schuell Inc., Keene, NH). Blots were probed with a nonradioactive DIG-labeled human WISP-2/CCN5-specific cDNA probe. The rest of the procedure was carried out according to the protocols provided by the DIG high prime DNA labeling and detection kit (Roche Diagnostics GmbH, Indianapolis, IN). To check normalization and even loading of RNA in each lane, the blotted membranes were stripped in 0.05X SSC and 0.1% SDS buffer and re-probed with DIG-labeled GAPDH-specific cDNA probe.

The sequences of primers are as follows: WISP-2: 5′-CCT ACA CAC ACA GCC TAT ATC-3′ (forward) and 5′-CCT TCT CTT CAT CCT ACC C-3′ (reverse); GAPDH: 5′-ATG AGA AGT ATG ACA ACA GCC-3′ (forward) and 5′-TGA GTC CTT CCA CGA TAC C-3′ (reverse). Relative expressions of WISP-2/CCN5 mRNAs were calculated by densitometric analyses using 1D Image Analysis Software version 3.6 (Eastman Kodak Company, Rochester, NY).

RT-PCR Analysis. The RT-PCR technique is as described previously (*22*). Briefly, 5 μL of cDNA from each sample was used for PCR amplification. The efficiency of cDNA synthesis from each sample was estimated by coamplification of the GAPDH gene with specific primers. The primers used for this experiment were WISP-2 forward, 5′-CCT ACA CAC ACA GCC TAT ATC-3′ and reverse, 5′- CCT TCT CTT CAT CCT ACC C-3′ and GAPDH forward, 5′-ATG AGA AGT ATG ACA ACA GCC-3′ and reverse, 5′-TGA GTC CTT CCA CGA TAC C-3′. A low DNA mass ladder DNA molecular weight marker was used to determine the size of the reaction.

Western Blot Analysis. The immuno-Western blot analysis was same as that described earlier by Sengupta et al. (*32*). Briefly, cells were washed with chilled phosphate buffered saline and lysed in 10 mM Tris-HCl at pH 7.5, 0.4 mM EDTA, 1% Triton X-100, 0.2% SDS, 10 mM Sodium Fluoride, 0.4 mM Sodium Ortho-vanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/mL of leupeptin, and 1μg/mL of Aprotinin and incubated on ice for 20 min with occasional vortexing. The lysates were spun at 14 000*g* for 1 h at 4 °C, and the supernatants were collected for immunodetection. Protein concentrations were measured by using the coomassie blue reagent assay (Pierce, Rockford, IL). Equal amount of proteins were resolved in 10% SDS−PAGE, transferred onto nitrocellulose membranes, and reacted with specific primary antibodies at 4 °C, overnight. The antigen−antibody reactions were probed with HRP-conjugated anti-rabbit or anti-mouse IgG. Immunoreactions were detected by ECL chemiluminescence's reagent kit. Relative expressions of WISP-2/CCN5 protein were calculated by densitometric analyses using 1D Image Analysis Software version 3.6 (Eastman Kodak Company, Rochester, NY).

Subcellular Fractionation and Immunodetection. The isolation of membrane and cytoplasmic fractions from breast tumor cells were carried out accourding to our previous method (*33*). Briefly, cells were harvested by trypsinization from 70 to 80% confluent cultures; the membrane and cytosolic fractions were isolated using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL). PKCα was detected in subcellular fractions by Western blot analysis using specific antibodies.

To confirm equal protein loading and to evaluate the quality of the cytoplasmic and membrane fractions, the expression of β-actin protein and an integral membrane protein, Calnexin, were evaluated. They were both detected in the subcellular fractions using specific antibodies. The ratios of these two proteins with PKCα were used to determine the level of expression of PKCα in the cytoplasm and membrane of these cells. The existence of β-actin in membrane fractions or Calnexin in cytoplasmic fractions was

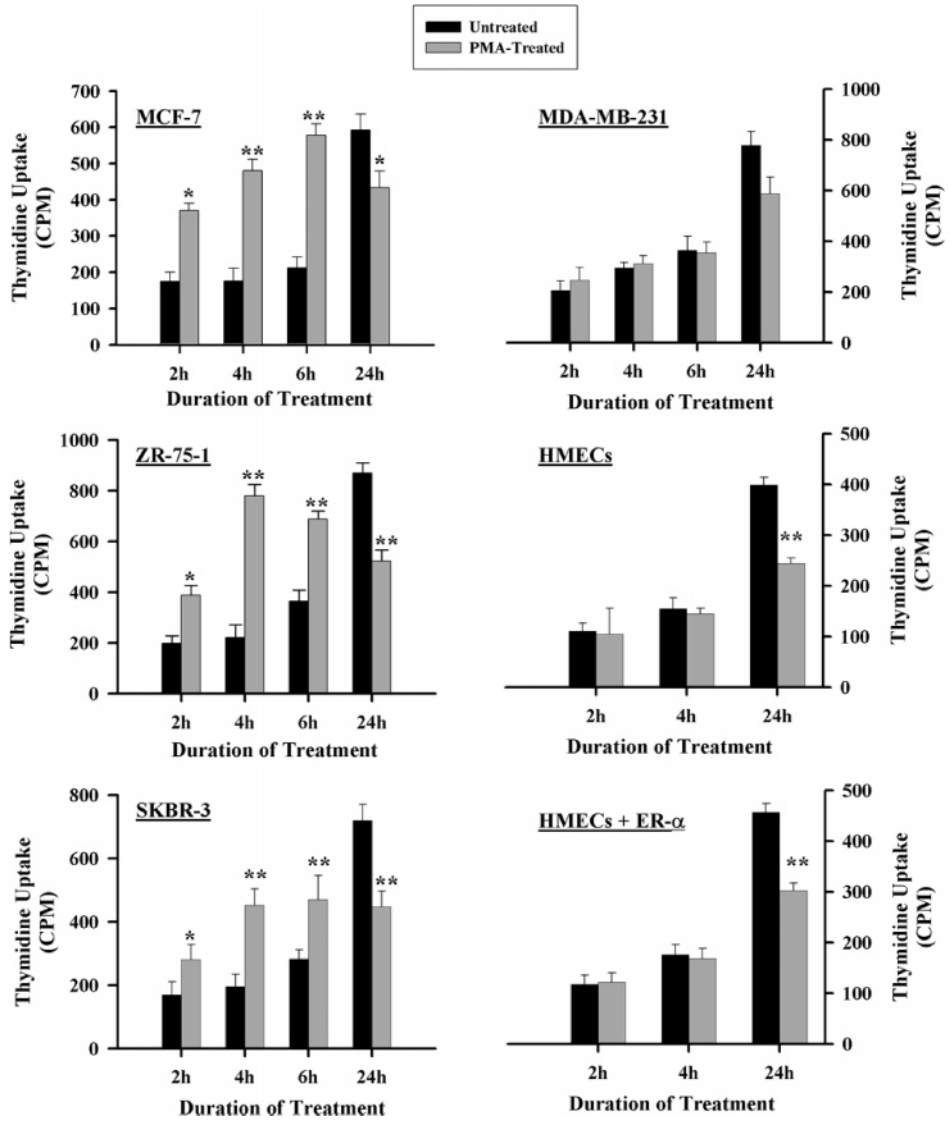

FIGURE 1: Time-dependent effect of PMA on cell proliferation in breast tumor cells. ER-α-positive human breast tumor cell lines (i.e., MCF-7 and ZR-75-1), classical ER-α−negative human breast tumor cell lines (i.e., MDA-MB-231 and SKBR-3), and human mammary epithelial cells (HMEC) with or without ER-α-transfection were used for this study. An equal number of tumor cells or HMECs was plated in 12-well plates, and the cells were either treated with 50 nM PMA or DMSO (vehicle) in appropriate culture conditions as described in the Materials and Methods section. PMA-exposed cells were co-incubated with 1 μci/mL of 3[H]-thymidine for the indicated time periods. Bar diagrams show radioactive thymidine uptake in 50 nM PMA treated and untreated cells. In each experiment, 0.1% DMSO-treated cultures were considered the control. All treated and untreated control cultures were run in quadruplicate in each set of experiments. The results are expressed as the mean ± SD from three sets of experiments. The *P*-values were determined by student's *t*-test. *$p < 0.005$ vs control, **$p < 0.001$ vs control.

an indication of contamination of the two fractions, which occasionally occurs during extractions. Therefore, the determination of these two protein markers was also used to evaluate for possible contamination of the two subcellular fractions.

Statistical Analysis. All data are expressed as the mean ± SD. Statistically significant differences between groups were determined by using the nonpaired Student's two-tailed *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Differential Regulation of Cellular Proliferations by PMA in Human Breast Tumor Cells Is Time-Dependent. The effects of PMA treatment on the cellular proliferation in different human breast tumor-derived cell lines (i.e., ER-positive cell lines: MCF-7 and ZR-75-1, ER-α (variant)-positive cell line: SKBR-3 (*34*), and ER-negative cell line: MDA-MB-231) and ER-negative nontransformed human mammary epithelial (HME) cells with or without ER-α were evaluated using [^{3}H]-thymidine incorporation assay. As depicted in Figure 1, when serum-starved MCF-7 cells were treated with 50 nM PMA for different times, significant induction of radioactive thymidine incorporation was observed at early times (4−6 h). After 24 h of treatment, the incorporation of [^{3}H]-thymidine decreased significantly lower than the basal level (vehicle-treated control). A similar effect of PMA on proliferation was also found on ZR-75-1 cells and SKBR-3 cells. In contrast, the radioactive thymidine uptake was not elevated in MDA-MB-231 breast tumor cells and HME cells after short exposure of PMA (Figure 1). However, prolonged exposure of PMA significantly reduced the radioactive thymidine uptake in these cells. The later part

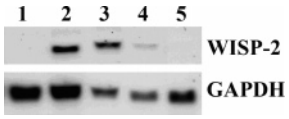

FIGURE 2: Detection of WISP-2/CCN5 mRNA expression by Northern blot analysis in normal and different breast tumor-derived breast tumor cell lines. Lane 1, HME cells; lane 2, MCF-7; lane 3, ZR-75-1; lane 4, SKBR-3, and lane 5, MDA-MB-231.

of this work is in agreement with previous work (*12*). The radioactive thymidine uptake can be rarely altered by changes in transport and metabolism of the nucleoside, and therefore, the magnitude of changes does not correlate proportionally with the changes in cell number. Moreover, PMA is known to alter metabolite transport in cells. Therefore, we verified our radioactive thymidine experiments with cell counting (data not included). Taken together, these studies suggest that estrogen receptor-α or its variants may be required to enhance the proliferation of breast cells by PMA.

To test the above hypothesis, ERα-transfected HME cells were exposed to PMA for different times. Interestingly, the studies overruled the hypothesis and indicated that like ER-α-negative MDA-MB-231 and HME cells, PMA did not exhibit any mitogenic effect on ERα-transfected HME cells (Figure 1).

Effect of PMA on WISP-2/CCN5 mRNA Expression Is Different in Breast Tumor Cells and Normal Breast Epithelial Cells. The main objective of this study is to evaluate whether PMA is able to modulate the expression of WISP-2/CCN5 in various breast tumor cells and nontransformed human mammary epithelial (HME) cells with or without ER-α transfection. To explore this, first, we determined the mRNA expression profile of the WISP-2/CCN5 gene in various breast tumor cell lines (i.e., MCF-7, ZR-75-1, SKBR-3, and MDA-MB-231) and in HME cells using Northern blot analysis. As shown in Figure 2, the WISP-2/CCN5 gene was constitutively expressed in MCF-7, ZR-75-1, and SKBR-3 breast tumor cells. However, MCF-7 and ZR-75-1 cells exhibited more WISP-2/CCN5 mRNA expression than SKBR-3 cells, which show minimum mRNA expression by Northern blot analysis. WISP-2/CCN5 expression was undetected in MDA-MB-231 and HME cells. Next, we determined the impact of PMA on WISP-2/CCN5 expression in MCF-7, SKBR-3, MDA-MB 231, and ER-negative or ER-positive HME cells. To explore this, starved cells were treated with 50 nM PMA for different times as indicated, and WISP-2/CCN5 mRNA expressions were evaluated using nonradioactive Northern blot analysis or semiquantitative RT-PCR analysis. Given that the expression level of WISP-2/CCN5 is minimum in SKBR-3 and undetected in MDA-MB-231 cells and HME cells, we used both Northern blot and RT-PCR analysis for the quantitation of RNA expression. As shown in Figure 3A and B, WISP-2/CCN5 mRNA expression was significantly increased by 50 nM PMA with a peak at 2 h in both MCF-7 and SKBR-3 cells. Densitometric analysis of Northern blot and RT-PCR showed 2.4- and 2.8-fold increases in WISP-2/CCN5 mRNA expression in PMA treated MCF-7 and SKBR-3 cells, respectively, compared to that of the vehicle (0.1% DMSO) treated controls (Figure 3). PMA is unable to alter the WISP-2/CCN5 mRNA levels in ER-negative or ER-α-transfected HME cells (Figure 3) as well as MDA-MB-231 (data not included). Collectively, these data indicate that PMA is only

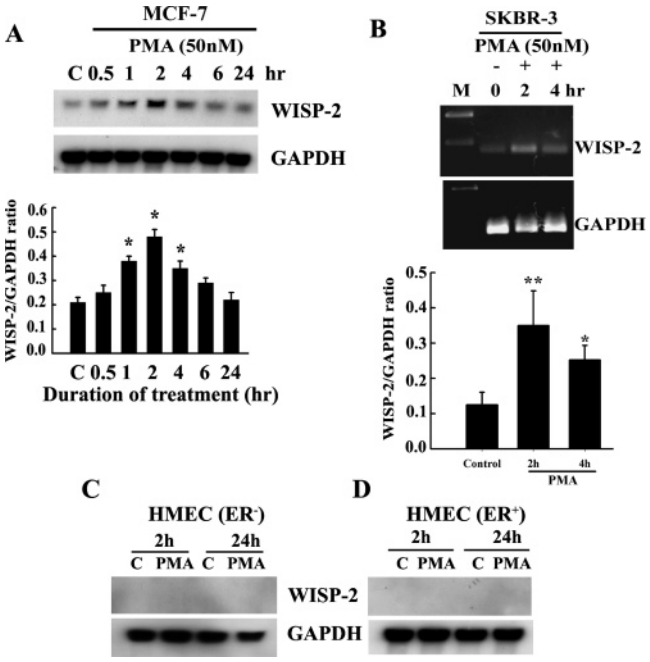

FIGURE 3: WISP-2/CCN5 mRNA expression in PMA-induced breast tumor cells. Cells were treated with 50 nM PMA for the indicated time periods in serum-starved culture conditions. The 0.1% DMSO-treated cultures were considered as the vehicle-treated control. Total cellular RNA was extracted from the control and treated cultures by the Trizol extraction method, and WISP-2/CCN5 mRNA expressions were evaluated by nonradioactive Northern blot and RT-PCR analyses. (A and B) Single representative Northern blot and RT-PCR shows the time course effect of 50 nM PMA on WISP-2/CCN5 mRNA expressions in serum-starved MCF-7 cells and SKBR3 cells, respectively. The arbitrary values indicate the ratio of WISP-2/CCN5 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. (C and D) Representative Northern blots show virtually undetected WISP-2/CCN5 mRNA expression in the vehicle-treated control and 50 nM PMA-treated ER-negative and ER-α-transfected HME cells, respectively; C, vehicle-treated control. The *P*-value was determined by student's *t*-test. $*p < 0.005$ vs control.

able to augment WISP-2/CCN5 expression in WISP-2/CCN5-positive MCF-7 and SKBR-3 cells in a time-dependent manner parallel with the induction of proliferation of these cells. Therefore, it is assumed that WISP-2/CCN5 signaling might play an important role in PMA-induced modulation of WISP-2/CCN5-positive breast tumor cell proliferation.

PMA-Induced WISP-2/CCN5 mRNA Expression and Cellular Proliferation Are Suppressed by WISP-2/CCN5 shRNA. To test the above hypothesis that WISP-2/CCN5 signaling may be involved in PMA regulation of cell proliferation, WISP-2/CCN5-specific shRNA inserts (i.e., shRNA 1, shRNA 2, shRNA 3, and shRNA 4) and mismatched shRNA were designed and cloned in a pSilencer 1.0-U6 expression vector. The transfection efficiency of each shRNA was evaluated by determining the WISP-2/CCN5 mRNA and protein expressions in transfected cells. Differential inhibitory effects on mRNA and protein levels were observed in shRNA's transfected cells. Also, 90−95% inhibition of WISP-2/CCN5 protein expression was achieved upon shRNA transfection in MCF-7 cells (Figure 4). After confirmation of the efficacy of WISP-2/CCN5 shRNAs, the transfected cells were treated with PMA for 2 h, and WISP-2/CCN5

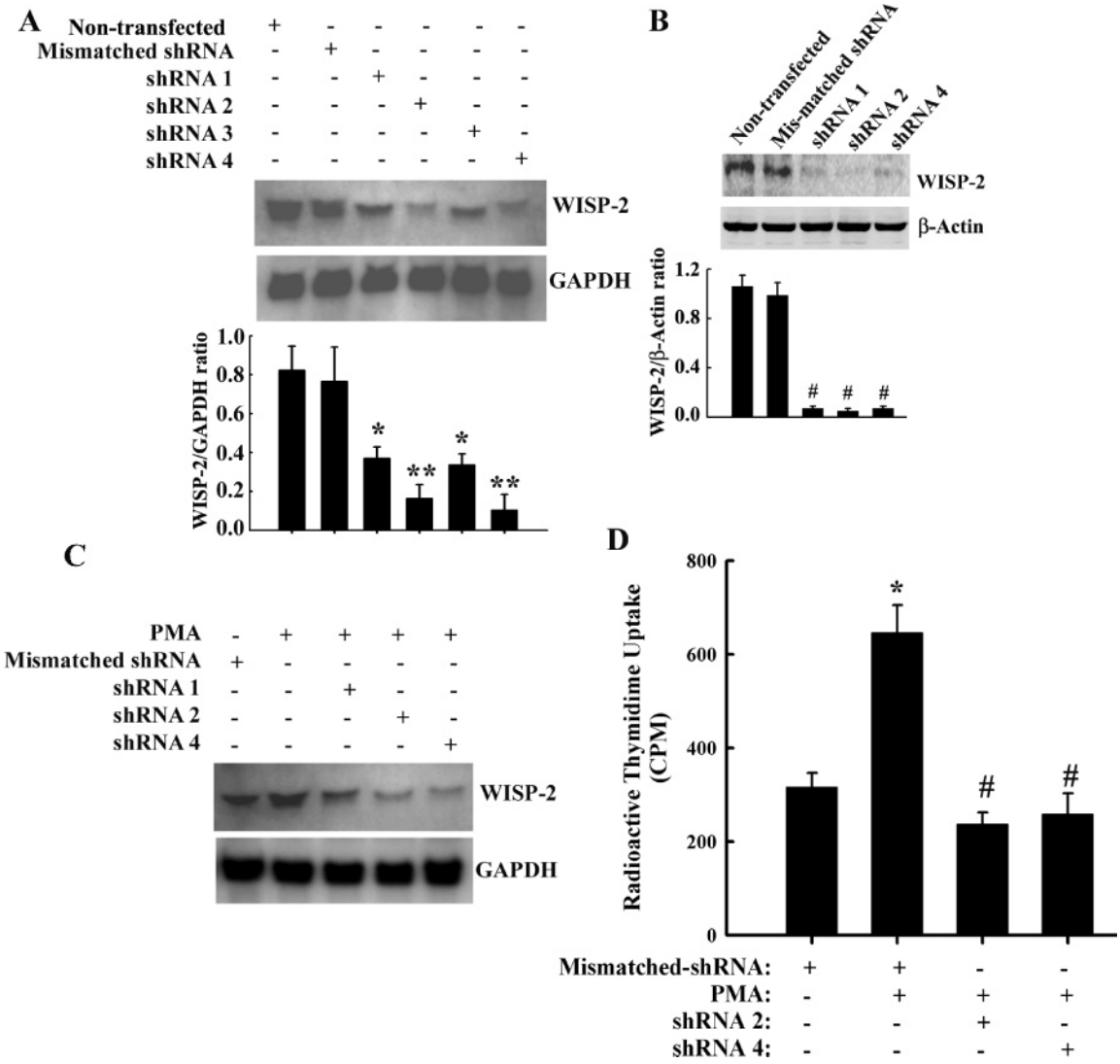

FIGURE 4: PMA-induced WISP-2/CCN5 mRNA expression and cellular proliferation suppressed by WISP-2 shRNA. MCF-7 cells were grown in OPTI-MEM for 3 days and then transfected with WISP-2/CCN5 and different shRNA for 24 h, and the transfected cells were incubated with 50 nM PMA for 2 h. Mismatched vector-transfected cultures were considered as the control. (A and B) Single representative Northern blot and immuno Western blot assays show the WISP-2/CCN5 mRNA and protein expressions in nontransfected (control), mismatched control vector-transfected, and WISP-2/CCN5-shRNA 1-, 2-, 3-, or 4-transfected MCF-7 cells. (C) A single representative Northern blot assay shows the WISP-2/CCN5 mRNA expressions in PMA-treated MCF-7 cells transfected with vector alone or WISP-2/CCN5 shRNA1, shRNA2, and shRNA4. The arbitrary values indicate the ratio of WISP-2/CCN5 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. (**D**) An equal number of MCF-7 cells was transfected with either WISP-2/CCN5 mRNA-specific shRNA 2 or 4 containing the vector or mismatched vector for 24 h. The PMA-induced cellular proliferations were assessed in either vector alone or shRNA-transfected MCF-7 cells using the 3[H]-thymidine incorporation assay. The bar diagram depicts the cellular proliferation in transfected cells with vector alone in the absence or presence of 50 nM PMA for 4 h (1 and 2) and shRNA 2 and 4 transfected cells followed by 50 nM PMA treatment for 4 h (3 and 4). In each set of experiments, the control and treated cultures were run in quadruplicate. The results are expressed as the mean ± SD from three separate sets of experiments. The *P*-value was determined by student's *t*-test; $*p < 0.005$ vs control.

mRNA levels were evaluated. As shown in Figure 4C, PMA significantly induces WISP-2/CCN5 mRNA expression in mismatched shRNA-transfected MCF-7 cells than in untreated mismatched shRNA-transfected cells. The PMA-induced overexpression of WISP-2/CCN5 mRNA was virtually abolished and back to basal levels or less in WISP-2/CCN5-shRNAs transfected MCF-7 cells.

Subsequently, to assess the functional role of the WISP-2/CCN5 gene in PMA-induced breast tumor cell proliferation, [^{3}H]-thymidine incorporation studies have been conducted in shRNA 2- and shRNA 4-transfected or mismatched shRNA-transfected MCF-7 cells. The results are depicted in Figure 4D, which shows that [^{3}H]-thymidine incorporation into cellular DNA was significantly higher in PMA-treated cells than in vehicle treated controls. The effect of PMA on the induction of cell proliferation was found completely abrogated in both shRNA-transfected cells. Together, these findings indicate that WISP-2/CCN5 signaling is critical for the induction of WISP-2/CCN5-positive MCF-7 cell proliferation by PMA.

PMA-Induced Upregulation of WISP-2/CCN5 mRNA Expression Is Mediated through Estrogen Receptor Independent Pathway. Previous data has shown that PMA is able to enhance WISP-2/CCN5 expression in ER-α-positive MCF-7 breast tumor cells and classical ER-α-negative but ER-α-variant-positive SKBR-3 cells. However, expression

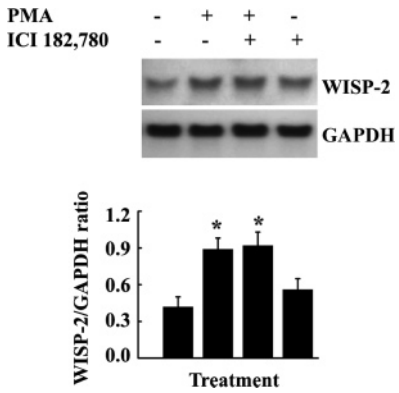

FIGURE 5: Effect of anti-estrogen ICI 182,780 on WISP-2/CCN5 mRNA expression in PMA-induced MCF-7 cells. Serum-starved MCF-7 cells were concomitantly exposed to 50 nM PMA in the presence or absence of 1 μM ICI 182,780 for 2 h. A single representative Northern blot shows WISP-2/CCN5 mRNA expression in PMA-treated MCF-7 cells simultaneously incubated with or without ICI 182,780 for 2 h. The bar diagram shows the ratios of WISP-2/CCN5 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. The *P*-value was determined by student's *t*-test; **p* < 0.01 versus control.

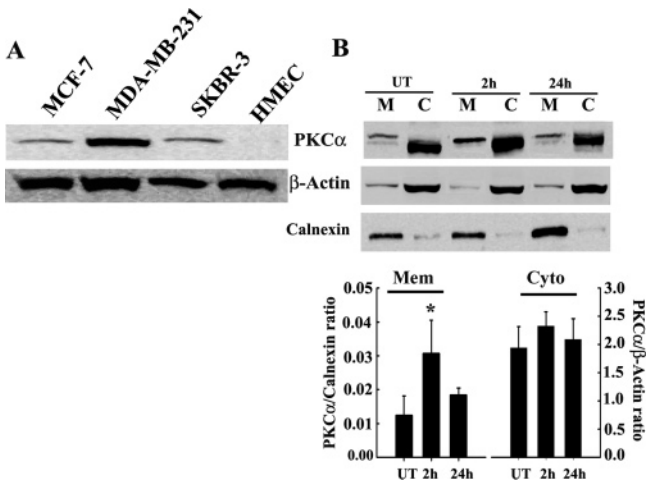

FIGURE 6: PMA-induced WISP-2/CCN5 mRNA overexpression is mediated via PKCα in MCF-7 cells. (A) Single representative immunoblot shows the PKCα expression in different cell extracts as indicated. Protein (50 μg) from each sample was subjected to electrophoresis. Blots were reacted with the antibody specific to PKCα, β-actin, or Calnexin and were detected by an ECL chemiluminescence's detection kit. (B) Representative immunoblots show the level of PKCα protein expression in the membrane (M) and cytosolic (C) fractions in 50 nM PMA-treated MCF-7 cells, respectively, at different time periods as indicated. The untreated (UT) controls received 0.1% DMSO. The blotted membranes were stripped and reacted with anti-β-actin and anti-Calnexin antibodies to check the equal loading for cytoplasmic and membrane fractions, respectively. The bar diagrams show the normalized values indicating the ratios between PKCα and Calnexin on the left side and the ratio between PKCα and β-actin on the right side of each panel. Data are displayed as the mean ± SD from three sets of experiments. The *P*-values were determined by student's *t*-test; **p* < 0.005 vs control.

was undetected in ER-α expressing normal human mammary epithelial cells (Figure 3). Therefore, this study suggests that PMA-induced upregulation of WISP-2/CCN5 is probably mediated through an ER-α-independent signaling pathway. To confirm the premise, an additional experiment was carried out. In this study, MCF-7 cells were exposed to PMA in the presence or absence of ICI 182,780, a pure estrogen receptor antagonist, for 2 h, and WISP-2/CCN5 mRNA levels were determined. We found that PMA treatment significantly enhanced the WISP-2/CCN5 mRNA level in MCF-7 cells than in vehicle-treated cells (Figure 5), and this upregulation by PMA cannot be blocked by ICI 182,780 (Figure 5). Therefore, these findings manifest that PMA induced WISP-2/CCN5 mRNA expression is not ER-α dependent.

PMA-Induced WISP-2/CCN5 mRNA and Protein Overexpressions Are Mediated via PKCα in MCF-7 Cells. Protein kinase C, including PKCα, is the major intracellular receptor for PMA (*35*, *36*). PKCα is differentially expressed in MCF-7, SKBR-3, and MDA-MB-231 breast tumor cells (*37*) and plays a key role in the signal transduction cascade that is involved in diverse cellular functions, such as growth, differentiation, and transformation (*3*, *5*, *6*). Therefore, the present studies were undertaken to determine whether PKCα controls PMA-induced WISP-2/CCN5 signaling in breast tumor cells. To evaluate this, the level of expression of PKCα in cellular extracts of MCF-7, SKBR-3, MDA-MB-231, and HME cells were first established by immuno-Western blot assay using a PKCα−specific antibody. Consistent with a previous study (*37*), PKCα protein expression was detected in all three breast tumor cell lines with different intensities (Figure 6A). However, PKCα was undetected in HME cells (Figure 6A). Next, we determined whether PMA modulates PKCα expression in breast tumor cells. To test this, MCF-7 cells were exposed to 50 nM PMA for 2 or 24 h, and the status of PKCα was determined in subcellular fractions. As depicted in Figure 6B, the level of PKCα expression was increased significantly in the membrane fractions of cells exposed to PMA for 2 h, but such an effect of PMA was not detected at 24 h of exposure. Therefore, this study suggests that PKCα translocates to the membrane upon short exposure of PMA, and this event eventually disappeared after the prolonged exposure of PMA. Because, translocation of PKCα to the membrane fraction is an indication of the activation of this receptor (*38*, *39*), we propose that PMA activates PKCα in MCF-7 cells after acute exposure. Therefore, from this study, we speculated that the upregulation of WISP-2/CCN5 mRNA by PMA might be mediated through the PKCα signaling pathway.

To test this hypothesis, two experiments were undertaken. First, MCF-7 cells were transfected with PKCα siRNA, and transfection efficiency was confirmed by evaluating PKCα protein expression in the transfected cells. Drastic inhibition of PKCα protein expression was perceived in PKCα-siRNA-transfected MCF-7 cells compared to that in nontransfected or control-pooled nonspecific siRNA duplex (negative control)-transfected MCF-7 cells (Figure 7A). After confirmation of efficiency, we determined whether PMA-induced expression of WISP-2/CCN5 could be blocked by PKCα siRNA in MCF-7 cells. To test this, MCF-7 cells were transfected either with PKCα-siRNA or pooled nonspecific siRNA duplexes followed by PMA treatment (as described in Materials and Methods). As expected, PMA induced WISP-2/CCN5 mRNA and protein expression by 2.3- and 2-fold, respectively, in MCF-7 cells compared to that in the untreated negative control. The inducible impact of PMA on WISP-2/CCN5 mRNA or protein was undetected in PKCα siRNA-transfected MCF-7 cells (Figure 7B and C). Together, these findings strongly support the concept that

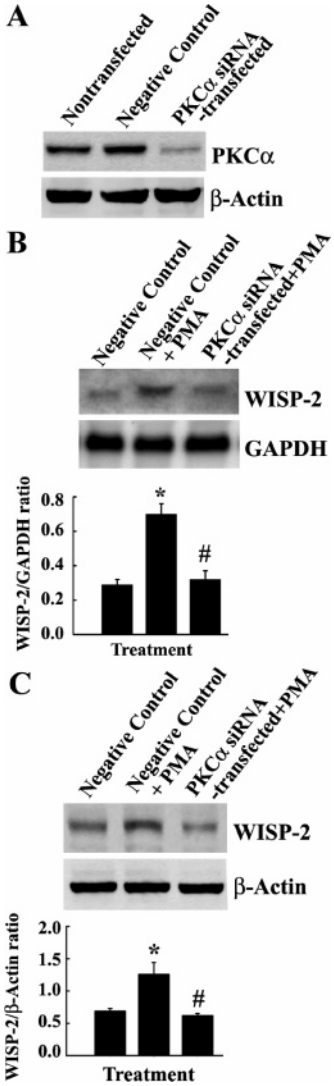

FIGURE 7: Silencing of PKCα-inhibited PMA-induced overexpressions of WISP-2/CCN5 mRNA and protein in MCF-7 cells. MCF-7 cells were grown in OPTI-MEM for 3 days and transfected with PKCα siRNA for 24 h, and the PKCα protein expression was evaluated by immunoblot analysis. (A) A single representative immunoblot shows almost complete abrogation of PKCα expression in MCF-7 cells upon transfection with PKCα siRNA. (B) Serum-starved MCF-7 cells were transfected either with pooled nonspecific siRNA duplexes (negative control) or with PKCα siRNA for 24 h. Total RNA was isolated from the cells treated with or without 50nM PMA for 2 h following transfection, and WISP-2/CCN5 mRNA expression was evaluated by nonradioactive Northern blot analysis. A single representative Northern blot analysis shows PMA-induced WISP-2/CCN5 mRNA expression in pooled nonspecific siRNA duplex (negative control) vector-transfected and PKCα siRNA-transfected MCF-7 cells. The bar diagram shows the values that indicate the ratios of WISP-2/CCN5 and GAPDH mRNA expression derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. (C) Serum-starved MCF-7 cells were transfected with PKCα siRNA and treated with 50 nM PMA as described in panel B. WISP-2/CCN5 protein expression was determined by immunoblot assay as described in Materials and Methods. A single representative immunoblot analysis shows PMA-induced WISP-2/CCN5 protein expression in pooled nonspecific siRNA duplex (negative control)-transfected and PKCα siRNA transfected MCF-7 cells. The bar diagram shows the values that indicate the ratios of WISP-2/CCN5 and β-Actin protein expression derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. The *P*-values were determined by student's *t*-test; *$p < 0.005$ vs control, #$p < 0.002$ vs PMA-treated cells.

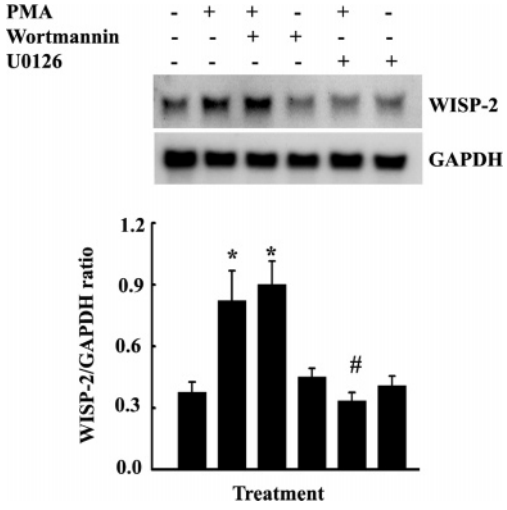

FIGURE 8: PMA-induced WISP-2/CCN5 mRNA upregulation mediated through MAP kinase signaling pathways. Serum-starved MCF-7 cells were pretreated with 100 nM Wortmannin, PI3K inhibitor, and 10 μM U0126, an MEK inhibitor, for 30 min followed by treatment with 50 nM PMA for 2 h. The 0.1% DMSO-treated cultures were considered as the untreated control. A single representative Northern blot shows PMA-induced WISP-2/CCN5 mRNA expression in MCF-7 cells in the presence or absence of Wortmannin and U0126. The arbitrary values indicate the ratio of WISP-2/CCN5 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. The *P*-values were determined by student's *t*-test; *$p < 0.005$ vs control, #$p < 0.001$ vs PMA-treated cells.

PMA-induced WISP-2/CCN5 mRNA and protein expression in MCF-7 cells are mediated through PKCα.

PMA-Induced WISP-2/CCN5 mRNA Expressions Regulated by MAPKK-ERK1/2 and JNK Signaling Pathways. To investigate the signaling pathways involved in PMA-induced WISP-2/CCN5 overexpression, MCF-7 cells were treated with 100 nM wortmannin, a PI3K inhibitor, or 10 μM U0126, an MEK inhibitor for 30 min prior to exposure to 50 nM PMA for 2 h. WISP-2/CCN5 mRNA levels were evaluated using nonradioactive Northern blot analysis. As shown in Figure 8, significant inhibition of WISP-2/CCN5 mRNA expression was achieved by U0126 in PMA-induced MCF-7 cells, whereas Wortmannin was unable to block the PMA-induced overexpression of WISP-2/CCN5 mRNA. Therefore, this finding suggests that the involvement of the MAPK pathway is required for PMA-induced upregulation of WISP-2/CCN5 mRNA expression in MCF-7 cells. Subsequently, the specific enzymes determined in MAPK signaling cascade are activated by PMA in MCF-7 cells during the induction of WISP-2/CCN5 mRNA. To identify these, cells were treated with PMA for different times as indicated in Figure 9. Cell lysates were analyzed for the activities and expressions of ERK1/2 (extracellular signal regulated protein kinase 1 and 2), JNK (Jun-*N*-terminal kinase; p54/p46), and p38 by immuno-Western blot analyses using specific antibodies (Figure 9). Upregulation of activated forms of ERK1/2 and JNK by PMA was identified because it reflected an increase in phosphorylation of ERK1/2 and JNK, whereas the phospho-p38 protein was virtually absent in PMA-treated MCF-7 cells (Figure 9). Total protein levels of ERK1/2, JNK, and p-38 were unaltered by PMA treatment. These studies indicate that in MCF-7 cells, PMA exerts its action through MAPK pathways, specifically via activating the ERK1/2 and

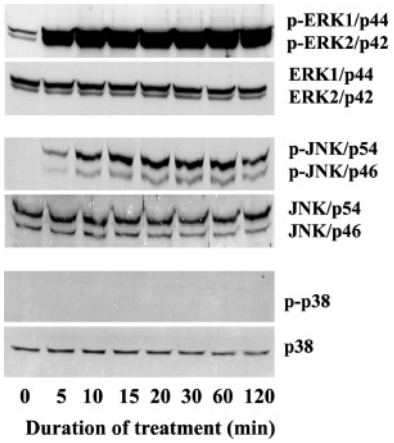

FIGURE 9: PMA activates ERK1/2 and JNK in human breast tumor cells. Serum-starved MCF-7 cells were treated with 50 nM PMA for indicated time periods. Then, 15 μg/lane of the total cell lysate protein was subjected to electrophoresis. Blots were reacted with the antibodies specific to the phosphorylated and nonphosphorylated forms of ERK1/2, JNK (p54/p46), and p38 as indicated and detected with ECL chemiluminescence's detection kit.

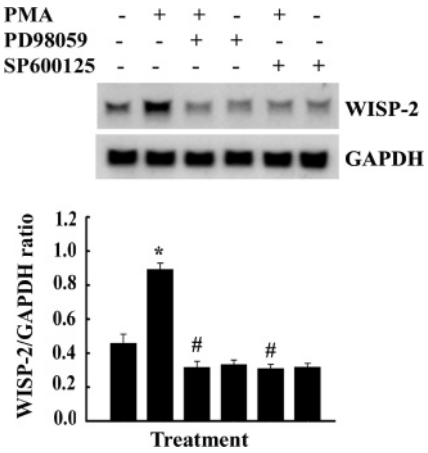

FIGURE 10: PMA-induced WISP-2/CCN5 mRNA upregulation is suppressed by ERK1/2 and JNK inhibitors. Serum-starved MCF-7 cells were pretreated with either 50 μM PD98059, a specific ERK1/2 inhibitor, or 20 μM SP600125, a specific inhibitor of JNK, for 1 h and followed by treatment with 50 nM PMA for 2 h. The cultures treated with 0.1% DMSO were the untreated control. A single representative Northern blot shows PMA-induced WISP-2/CCN5 mRNA expression in MCF-7 cells in the presence or absence of PD98059 and SP600125. The normalized values indicate the ratio of WISP-2/CCN5 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. Data are displayed as the mean ± SD from three sets of experiments. The *P*-values were determined by student's *t*-test; *$p < 0.01$ vs control, #$p < 0.001$ vs PMA-treated cells.

JNK. To define whether ERK1/2 and JNK activation is required for the overexpression of WISP-2/CCN5 mRNA in MCF-7 cells after PMA exposure, cells were pretreated with either 50 μM PD98059, a specific ERK1/2 inhibitor, or 20 μM SP600125, a specific inhibitor of JNK, for 1 h followed by treatment with PMA for 2 h. As indicated in Figure 10, the overexpression of WISP-2/CCN5 mRNA by PMA was inhibited by both PD98059 and SP600125. Constitutive expression of WISP-2/CCN5 in MCF-7 cells was also inhibited by PD98059 and SP600125.

Silencing of PKCα Inhibits PMA-Induced ERK1/2 and SAPK/JNK Activities. Previous studies showed that PMA activates PKCα, which leads to the phosphorylation of

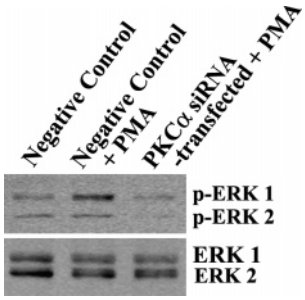

FIGURE 11: Silencing of PKCα inhibits PMA-induced MAPK activation. MCF-7 cells were grown in OPTI-MEM for 3 days and transfected either with PKCα siRNA or pooled nonspecific siRNA duplexes (negative control) for 24 h followed by PMA treatment for 2 h as described in Figure 7B. The Figure depicts a representative immunoblot, showing expressions of phospho-ERK1/2 and total ERK1/2 in PMA-exposed negative control and siRNA-transfected MCF-7 cell lysates.

ERK1/2 via the activation of Raf in endothelial cells (*40*, *41*). Another study showed that phorbol ester induces ZR-75-1 breast tumor cell proliferation in association with the activation of JNK (*10*). Present studies have shown that PMA activates PKCα (Figure 6) in MCF-7 cells to enhance WISP-2/CCN5 expression (Figure 7). However, it is uncertain whether MAPK signaling enzymes, which are upregulated by PMA, are downstream signaling molecules of PKCα. To resolve this issue, we evaluated whether PKCα-specific siRNA is able to block the PMA-induced induction of ERK1/2 and SAPK/JNK phosphorylation in MCF-7 cells. As shown in Figure 11, the level of ERK1/2 phosphorylation was increased upon PMA treatment in pooled nonspecific siRNA duplex-transfected MCF-7 cells (negative control cells). However, this induction of phosphorylation by PMA can be inhibited by PKCα-siRNA transfection. A similar effect is found in SAPK/JNK phosphorylation when PKCα-blocked MCF-7 cells are exposed to PMA (data not shown). Therefore, collectively, this study suggests that the upregulation of WISP-2/CCN5 expression by PMA is mediated through PKCα by activating both ERK1/2 and JNK signaling pathways.

DISCUSSION

The present work describes a novel mechanism of mitogenic action of PMA on human breast tumor cells that is based on the expression profile and function of WISP-2/CCN5 as a molecular switch to dictate the growth response to PMA in breast tumor cells.

Using WISP-2/CCN5-positive and -negative breast tumor cells and normal breast epithelial cells to evaluate PMA activity, we established a positive role of WISP-2/CCN5 in the mitogenic signal of PMA in breast tumor cells (Figure 12). The studies confirm the mitogenic influence of PMA on WISP-2/CCN5-positive breast tumor cells, including MCF-7, ZR-75-1, and SKBR-3 breast tumor cells, that is exposure time-dependent and parallels the induction of WISP-2/CCN5 expression. A comparable PMA effect was not detected in WISP-2/CCN5-negative MDA-MB-231 breast tumor cells or nontransformed ER-negative or ER-transfected HME cells. The mitogenic impact of PMA could be perturbed by blocking WISP-2/CCN5 signaling. Moreover, this study presents several lines of evidence demonstrating that upregulation of WISP-2/CCN5 by PMA is not

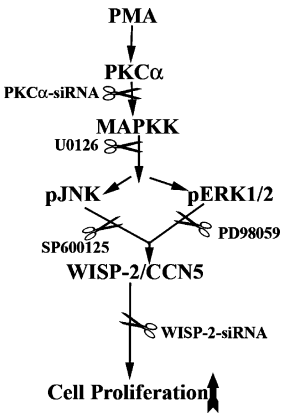

FIGURE 12: Diagrammatic representation depicts the role of WISP-2/CCN5 signaling networks in PMA-induced modulation of breast tumor cell proliferation. The diagram indicates that PMA-induced upregulation of WISP-2/CCN5 signaling is required for the mitogenic switch by PMA. Moreover, induction of WISP-2/CCN5 expression by PMA is mediated through PKCα-MAPK/ERK1/2-JNK signaling pathways because this induction can be blocked by PKCα-specific siRNA, U0126 (MAPKK inhibitor), SP600125 (pJNK inhibitor), or PD98059 (pERK inhibitor). Thick up-arrow, stimulation.

ER dependent but is instead mediated through a complex PKCα-MAPK/ERK and SAPK/JNK signaling pathway, which leads to the growth stimulation of MCF-7 breast tumor cells (Figure 12). Previous studies have demonstrated that PMA is a potent growth regulator of different types of tumor cells including breast cancer cells (*2*). It exerts divergent effects on breast cancer cell proliferation depending on the duration of incubation. However, the molecular signaling networks involved in the PMA mitogenic effect on breast tumor cells were poorly understood because most of the previous experimental data were based on in vitro prolonged exposure studies (*7*, *15*, *42*), which basically dealt with the inhibitory role of phorbol esters and the possible mechanisms associated with them. Therefore, these novel studies represent a paradigm shift regarding the PMA mitogenic mechanism of action in breast tumor cells.

Regardless of the divergent functions of WISP-2/CCN5 in various cell types (*21*−*24*, *43*), the WISP-2/CCN5 growth factor is becoming an increasingly important focus in breast cancer research for multiple reasons. WISP-2/CCN5 is overexpressed in several breast tumor cell lines including those that are estrogen-receptor positive and less aggressive in nature, and it is a critical intermediate growth factor for growth stimulation of MCF-7 breast tumor cells by serum, estrogen, and growth factors such as EGF (*21*, *22*). This novel study provides the initial description of the stimulatory effect of PMA on WISP-2/CCN5 mRNA expression in MCF-7 and SKBR-3 cells that is dependent on the duration of exposure (Figure 3). PMA induces WISP-2/CCN5 expression in MCF-7 and SKBR-3 cells after short exposure, whereas prolonged PMA exposure exhibits no effect on WISP-2/CCN5 expression (Figure 3). PMA effects were not detected on WISP-2/CCN5 expression in ER-α-nontransfected or ER-α-transfected HME cells and ER-α-negative MDA-MB-231. Moreover, the positive effect of PMA on WISP-2/CCN5 expression paralleled the growth regulation of breast tumor cells exposed to PMA. Collectively, these data support the concept that WISP-2/CCN5 has a functional role in regulating PMA stimulated breast tumor cell proliferation.

To resolve the functional role of the WISP-2/CCN5 gene in PMA-induced breast tumor cell proliferation, the RNA silencing technique known as RNA interference (RNAi) or short hairpin RNA (shRNA) was utilized to selectively silence WISP-2/CCN5 gene expression in PMA-treated MCF-7 cells in order to dissect WISP-2/CCN5 function in these cells. These experiments demonstrated that shRNA abolished radioactive thymidine incorporation into PMA-induced MCF-7 cells and suppressed PMA-induced WISP-2/CCN5 expression (Figure 4C). The analogous consequence was also found in other noninvasive ER-positive breast tumor cells (i.e., ZR-75-1 and T-47D) expressing the WISP-2/CCN5 gene (data not included). Therefore, together, these findings lead to a new concept that WISP-2/CCN5 signaling contributes to PMA's mitogenic activity on WISP-2/CCN5-positive breast tumor cells.

PMA behaves like estrogen in estrogen-targeted tissues (*16*, *17*), and it upregulates the expression of WISP-2/CCN5, an estrogen response gene (*21*, *30*) in ER-positive breast tumor cells. Therefore, in this study, we evaluated whether ER-α plays any role in the PMA-induced induction of WISP-2/CCN5 expression. The results obtained from these studies (Figure 3C and D and 5) excluded the possibility of ER-α having a significant role in PMA-induced WISP-2/CCN5 expression.

PKC, a key enzyme in cellular transduction pathways, has been identified as the active receptor for PMA (*7*, *8*). PKC comprises a family of at least 12 isoforms that typically reside in the cytosol in an inactive state. After cell stimulation, they translocate to the plasma membrane where they are activated (*44*−*46*). A recent study has shown that a Ca^{2+}-dependent PKCα isoform undergoes translocation from the cytosol to the membrane in PMA-treated MDA-MB-231 breast tumor cells (*47*). Active involvement of PKCα and PKC*β*I/II has been documented in angiotensin II-induced cell proliferation in breast tumor cells in primary culture (*48*). Leptin and glucose stimulate MCF-7 breast tumor cell progression via the upregulation of PKCα, which could account for the increased risk for developing breast cancer in individuals who are obese and/or diabetic (*49*). Additionally, PKCα has been shown to increase the growth of MCF-7 and other cell lines. These results supported the importance of evaluating the role of PKCα in PMA-induced modulation of WISP-2/CCN5 expression. As a step toward understanding the involvement of PKCα in PMA-induced modulation of WISP-2/CCN5 expression, we provide evidences indicating that PKCα is constitutively expressed in the cell extracts of WISP-2/CCN5-positive breast tumor cells (Figure 6A). PMA is capable of activating PKCα after a short exposure in WISP-2/CCN5-positive MCF-7 cells, and this impact gradually returns to the basal level when exposure time was increased to 24 h (Figure 6B). PKCα activation corresponding to WISP-2/CCN5 induction by short exposure of PMA could argue in support of PKCα being involved in PMA-modulated WISP-2/CCN5 expression in MCF-7 cells. Finally, the present studies convincingly demonstrate that the induction of WISP-2/CCN5 expression by PMA is mediated through PKCα signaling pathways because the silencing of PKCα in MCF-7 cells attenuated PMA-induced WISP-2/CCN5 expression.

PMA is known as a potent activator of MAPK cascades through c-Raf-MEK pathways leading to the direct phosphorylation of c-jun and c-fos and activating mitogenic processes in the nucleus (*7*, *50*). It is evident that in ZR-75-1 breast tumor cells, PMA-enhanced proliferation through the induction of JNK and AP-1 activity (*10*) and, in addition, the induction of $p21^{WAF1}$ by TPA in SKBR-3 breast tumor cells can be attributed to the stimulation of the Raf-1/MEK pathway (*51*). In this study, we found that PMA strongly activated ERK and JNK activities in MCF-7 cells; these activations were blocked by specific inhibitors of MAPKK (U0126), ERK (PD98059), or SAPK/JNK (SP600125), which finally abolished WISP-2/CCN5 induction in PMA-treated MCF-7 cells. Furthermore, in parallel studies, we found that siRNA-mediated inhibition of PKCα significantly reduced PMA-stimulated ERK (Figure 11) and JNK (data not shown) activities in these cells. Together, these studies, therefore, suggest that MAPK/ERK1/2 and SAPK/JNK are downstream target signaling molecules of PKCα, which are associated with PMA-PKCα-mediated regulation of WISP-2/CCN5 expression and ultimately cell proliferation (Figures 7 and 8−11).

In conclusion, the data presented here reveal that WISP-2/CCN5 is a novel signaling molecule that critically participates in the mitogenic action of PMA on noninvasive ER-positive breast tumor cells. The mitogenic action of PMA activates PKCα-MAPK/ERK1/2 and SAPK/JNK signaling cascades leading to the induction of the WISP-2/CCN5 signaling molecule, thus enhancing breast tumor cell proliferation.

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