

Silencing of casein kinase 2 inhibits PKC-induced cell invasion by targeting MMP-9 in MCF-7 cells

JEONG-MI KIM^{1*}, EUN-MI NOH^{1*}, HYUN-KYUNG SONG¹, YONG-OUK YOU², SUNG HOO JUNG³, JONG-SUK KIM⁴, KANG-BEOM KWON⁵, YOUNG-RAE LEE^{1,2,6} and HYUN JO YOUN³

¹Center for Metabolic Function Regulation, Wonkwang University School of Medicine;

²Department of Oral Biochemistry, School of Dentistry, Wonkwang University, Iksan, Jeollabuk 570-749;

³Department of Surgery, Division of Breast and Thyroid Surgery, Chonbuk National University Medical School;

⁴Department of Biochemistry, Institute of Medical Science, Chonbuk National University Medical School, Jeonju, Jeollabuk 560-182; ⁵Department of Korean Physiology, Wonkwang University School of Korean Medicine;

⁶Integrated Omics Institute, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

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Abstract. Casein kinase 2 (CK2) is a serine/threonine protein kinase that has been considered to represent an important factor in mammary tumorigenesis. Increased expression of matrix metalloproteinase-9 (MMP-9) via nuclear factor- κ B (NF- κ B) activation has been demonstrated to promote breast cancer cell invasion. In the present study, the involvement of CK2 in protein kinase C (PKC) induced cell invasion in MCF-7 breast cancer cells was investigated as well as the underlying molecular mechanisms. The mRNA and protein levels of MMP-9 in MCF-7 cells were investigated using reverse transcription-quantitative polymerase chain reaction, western blot analyses and a zymography assay. Cell invasiveness was investigated using a Matrigel invasion assay, and it was revealed that small interfering RNA specific for CK2 suppressed PKC induced cell invasion by regulating MMP-9 expression via activation of the p38 kinase/c-Jun N-terminal kinase/NF- κ B pathway. In addition, it was demonstrated that CK2 inhibitors [apigenin (20 μ M), emodin (20 μ M) or 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole

(2 μ M)] suppressed PKC induced cell invasion and MMP-9 expression. The results of the present study suggested that CK2 is an important factor involved in the induction of MCF-7 breast cancer cell invasion by PKC. Therefore, CK2 may represent novel candidates for therapy intended to inhibit invasion in breast cancer.

Introduction

Casein kinase 2 (CK2) is a ubiquitous serine/threonine protein kinase that is involved in numerous cellular processes, including proliferation and apoptosis, and has been revealed to be overexpressed in a number of human cancer cell lines (1,2). A previous study suggested that CK2 overexpression creates a favorable environment for the development of the tumor phenotype (3). CK2 has been associated with mammary tumorigenesis, as the levels of CK2 protein and activity are increased in breast cancer tissue compared with normal breast tissue (1,4). Furthermore, overexpression of CK2 in the mammary glands of transgenic mice can cause hyperplasia and breast carcinoma, thus demonstrating the oncogenic potential of CK2 (4).

Invasion and metastasis, the fundamental properties of cancer cells, are the primary causes of poor prognosis in patients with breast cancer. The mechanisms of cancer cell invasion and metastasis comprise a multistep biophysical process (5). In the early stages, the proteolytic degradation of extracellular matrix (ECM) components is facilitated by matrix metalloproteinases (MMPs) (6,7). Apigenin is abundant in common fruits and vegetables, and has notable anti-inflammatory, anti-oxidant and anti-carcinogenic properties (8-13). Emodin is an active constituent isolated from *Rheum palmatum*, a Chinese herb (14) and has been revealed to lead to the inhibition of cell proliferation, cell cycle arrest, inhibition of cell division, and decreased cell motility and invasion (15). TBBz is one of the most efficient inhibitors of CK2 (16), and has been demonstrated to induce apoptosis in cancer cells (17,18). MMP-9 has an important role in the degradation of the ECM during breast cancer cell invasion (19,20). Considering

Correspondence to: Professor Young-Rae Lee, Department of Oral Biochemistry, School of Dentistry, Wonkwang University, 460 Iksandae-ro, Iksan, Jeonbuk 570-749, Republic of Korea
E-mail: mindyr@wku.ac.kr

Professor Hyun Jo Youn, Department of Surgery, Division of Breast and Thyroid Surgery, Chonbuk National University Medical School, 567 Baekje-daero, Deokin-gu, Jeonju, Jeollabuk 560-182, Republic of Korea
E-mail: yhj0903@jbnu.ac.kr

*Contributed equally

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its importance in cancer development and progression, MMP-9 can be suggested to represent an early target in the treatment of breast cancer metastasis (21,22), and the inhibition of MMP-9 expression, activity and/or upstream regulatory activity may represent a potential therapeutic strategy. 12-O-tetradecanoylphorbol-13-acetate (TPA), a selective activator of protein kinase C (PKC) (23), induces breast cancer cell invasion by stimulating MMP-9 synthesis and secretion (21,24) via the activation of transcription factors, including nuclear factor- κ B (NF- κ B) and activator protein-1 (25-27).

Considering that CK2 has been previously revealed to induce nuclear factor- κ B (NF- κ B) activation, the present study aimed to investigate whether the inhibition of CK2 affected TPA induced invasion and MMP-9 expression in MCF-7 human breast cancer cells and determine the associated underlying molecular mechanisms.

Materials and methods

Cells and materials. Human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA) and 1% Anti-Anti (antibiotic-antimycotic) 100X (cat. no. 15240-062; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator. CK2 inhibitors [apigenin, 1,3,8-trihydroxy-6-methylanthraquinone (emodin) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (TBBz)], dimethyl sulfoxide used as the solvent of the CK2 inhibitors and TPA (cat. no. P1585), and anti- β -actin (cat. no. A5441) antibodies were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Matrigel was obtained from Corning Incorporated (Corning, NY, USA). Primary antibodies against CK2 α (cat. no. 2656), p38 kinase (p38; cat. no. 9212), c-Jun N-terminal kinase (JNK; cat. no. 9252), extracellular signal-regulated kinase (ERK; cat. no. 9102), as well as the phosphorylated forms of p38 (cat. no. 9211), JNK (cat. no. 9261) and ERK (cat. no. 9101), were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies specific to MMP-9 (cat. no. 12759), transcription factor p50 (cat. no. 7178) and transcription factor p65 (cat. no. 372) and proliferating cell nuclear antigen (PCNA; cat. no. 7907), as well as horseradish peroxidase (HRP)-conjugated secondary immunoglobulin G (IgG; cat. no. SC-2004, SC-2005), were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

RNA interference. CK2 α specific small interfering (si)RNA and negative control siRNA (cat. no. SN-1003) were obtained from Bioneer Corporation (Daejeon, Korea). The siRNA used were as follows: CK2 α sense, CAUUUAGUACUGGG CAUA(dTdT) and antisense, UAUGCCCAGUAAUAAU G(dTdT). Briefly, human breast cancer MCF-7 cells (5x10⁵) were transfected with 100 pmol siRNA using RNAiMAX Transfection Reagent for 24 h at 37°C (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Determination of cell viability. Cell viability assays were performed using the EZ-Cytox Enhanced Cell Viability Assay

kit (DOGEN, Seoul, Korea; www.dogenbio.com/shop/item.php?it_id=1490923054), according to the manufacturer's instructions. Briefly, 3x10⁴ cells/well were seeded into 96-well plates and incubated at 37°C for 24 h to allow the cells to adhere. The cells were then treated with apigenin (20 μ M), emodin (20 μ M) or TBBz (2 μ M) for 24 h at 37°C. Following this, all cells were incubated with EZ-Cytox Reagent (10 μ l) for 30 min at 37°C. Absorbance at 450 nm was determined using an ELISA plate reader (Sunrise™; Tecan Group, Ltd., Mannedorf, Switzerland).

Western blot analysis. MCF-7 cells (7x10⁵) pretreated with CK2 inhibitors [20 μ M apigenin, 20 μ M emodin, or 2 μ M TBBz] for 1 h, and cells (3.5x10⁵) transfected with CK2 were subsequently incubated with TPA for 24 h at 37°C. The cells were lysed with ice-cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.), and the protein concentration of the resulting lysates was determined using a BioSpec-nano (Shimadzu Corporation, Kyoto, Japan). Total protein samples were resolved via 10% SDS-PAGE analysis and then transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were blocked with 5% bovine serum albumin (cat. no. 160069; MP biomedical Inc.) or 5% skimmed milk in TBS with 0.5% Tween-20 at 4°C for 2 h, and then incubated overnight at 4°C with primary antibodies. All antibodies used were diluted 1:2,000. HRP-conjugated IgGs were used as secondary antibodies and incubated at 4°C for 1 h. Protein expression levels were visualized using a Mini HD6 image analyzer using Alliance 1D software (UVitec Cambridge; Cleaver Scientific Ltd., Rugby, UK).

Gelatin zymography assay. MMP-9 activity in the conditioned medium (serum-free DMEM) was determined by gelatin zymography. MCF-7 cells (7x10⁵) were pretreated with CK2 inhibitors [apigenin (20 μ M), emodin (20 μ M) or TBBz (2 μ M)] for 1 h, and then incubated with TPA for 24 h at 37°C. The conditioned medium was mixed with non-reducing sample buffer (0.5M Tris-HCl (pH 6.8), Glycerol 2 ml, 10% SDS, 0.1% Bromophenol blue, D.W 1 ml up to 10 ml), and subjected to 10% SDS-PAGE analysis containing 0.1% [weight (w)/volume (v)] gelatin. The gel was then incubated in a renaturing buffer (2.5% Triton™ X-100; Sigma-Aldrich; Merck KGaA) with gentle agitation to remove the SDS at room temperature for 30 min. Following this, gels were incubated in a developing buffer (5 mM CaCl₂, 0.02% Brij, pH 7.5 and 50 mM Tris-HCl) overnight at 37°C. The gel was stained for 30 min at room temperature with 0.25% (w/v) Coomassie brilliant blue R-250 at room temperature followed by destaining with washing buffer (10% acetic acid and 10% methanol). Proteolysis was visualized as a white zone in a dark blue field using a digital imaging system (FluorChem R by ProteinSimple; Cell Biosciences, Palo Alto, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Complementary DNA was synthesized from total RNA using a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China).

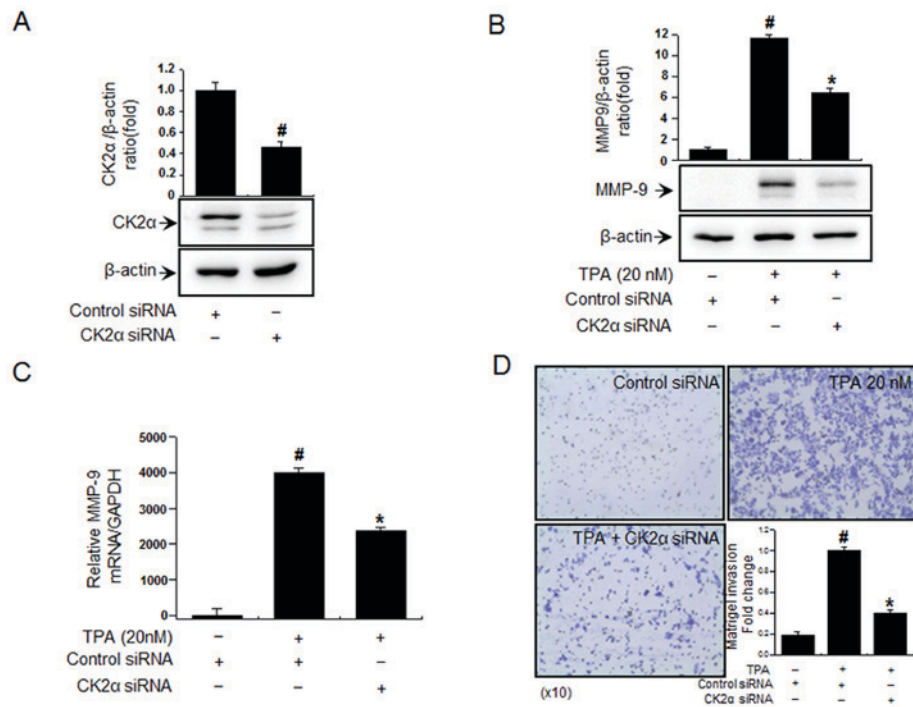


Figure 1. Inhibition of CK2 α suppresses TPA-induced MMP-9 expression in MCF-7 cells. (A) CK2 α siRNA was transfected into MCF-7 breast cancer cells, and cells transfected with the CK2 α inhibitor were revealed to significantly suppress CK2 α expression. (B) Transfected cell lysates were analyzed by western blotting. (C) MMP-9 mRNA levels were analyzed by reverse transcription-quantitative polymerase chain reaction, and GAPDH was used as an internal control. (D) Transfected cell lysates were subjected to Matrigel invasion analysis (magnification, x10). Data represent the mean \pm standard error of the mean of three independent experiments. [#]P<0.005 vs. control; ^{*}P<0.005 vs. control + TPA. CK2 α , casein kinase 2 α ; si, small interfering; TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP-9, matrix metalloproteinase-9.

mRNA expression levels were determined by qPCR analysis using the StepOnePlus™ Real-Time PCR System and SYBR®-Green (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used were as follows: MMP-9 sense, 5'-CCTGGAGACCTGAGAACCAATCT-3' and antisense, 5'-CCACCCGAGTGTAACCATAGC-3'; GAPDH (NM_002046) sense, 5'-ATGGAAATCCCATCACCATCTT-3' and antisense, 5'-CGCCCCACTTGATTTTGG-3'. qPCR was performed with a preliminary incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. MMP-9 mRNA expression levels were quantified relative to GAPDH mRNA expression using the comparative 2^{- $\Delta\Delta C_q$} method (28).

Preparation of nuclear extracts. MCF-7 cells (2x10⁶) were transfected with CK2 siRNA in the presence of 20 nM TPA for 3 h at 37°C and then washed twice with PBS, scraped, resuspended in 1.5 ml ice-cold PBS (pH 7.5), and then centrifuged at 1,500 x g for 4 min at 4°C. Isolation of the nuclear and cytoplasmic extracts was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (cat. no. 78835; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Invasion assay. Invasion of MCF-7 cells was assessed using 24-well chambers with 8 μ m pore membranes coated with 20 μ l Matrigel. The Matrigel coating was rehydrated in 0.5 ml DMEM for 2 h at 37°C immediately prior to experiments. Cell growth medium (0.5 ml) with suspended cells (3x10⁵) and cells (2x10⁵) transfected with CK2 siRNA were added to the upper chambers, and cell growth medium (0.5 ml) with TPA, alone

or with CK2 inhibitors, were added to the bottom well. The upper chambers and bottom well were incubated with DMEM supplemented 10% FBS and 1% antibiotic for 24 h. Following incubation, cells on the upper membrane surface were removed using cotton swabs, and cells that had migrated to the lower membrane surface were fixed by formaldehyde solution (3.6%) at room temperature, stained with crystal violet, and counted in five random fields per chamber using a Leica DM ILLED Inverted Lab microscope (Leica, Wetzlar, Germany) used magnification, x10.

Statistical analysis. Data are expressed as the mean \pm standard error. Statistical significance was determined using one-way analysis of variance followed by Scheffe post hoc test in Excel (Microsoft Excel 2013). P<0.005 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Inhibition of CK2 α inhibits TPA-induced MMP-9 expression in MCF-7 cells. In order to investigate the effects of CK2 α inhibition on TPA-induced cell invasion and MMP-9 expression, intracellular CK2 α expression was suppressed via transfection with siRNA (Fig. 1A). The siRNA-mediated inhibition of CK2 α significantly suppressed the increase in MMP-9 mRNA/protein expression compared with the TPA group (Fig. 1B and C). Furthermore, MCF-7 cells treated with TPA exhibited significantly increased invasion compared with the untreated control cells. However, the inhibition of

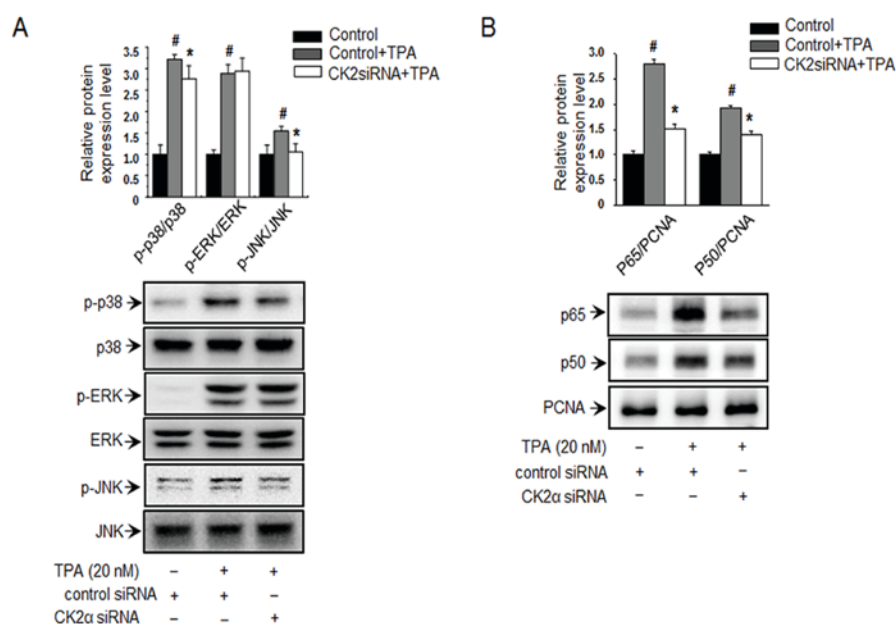


Figure 2. Inhibition of CK2 α suppresses TPA-induced activation of mitogen activated protein kinase signaling pathways and NF- κ B expression in MCF-7 cells. (A) Western blot analysis of p38, JNK, ERK, and their phosphorylated forms in MCF-7 cells following transfection with CK2 α siRNA and treatment with TPA. (B) Nuclear lysates of cells transfected with CK2 α siRNA were subjected to western blot analysis using anti-p65 and -p50 antibodies. # P <0.005 vs. control; * P <0.005 vs. control + TPA. CK2 α , casein kinase 2 α ; si, small interfering; TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP-9, matrix metalloproteinase-9; p-, phosphorylated; p38, p38 kinase; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor- κ B; ERK, extracellular signal-regulated kinase; PCNA, proliferating cell nuclear antigen.

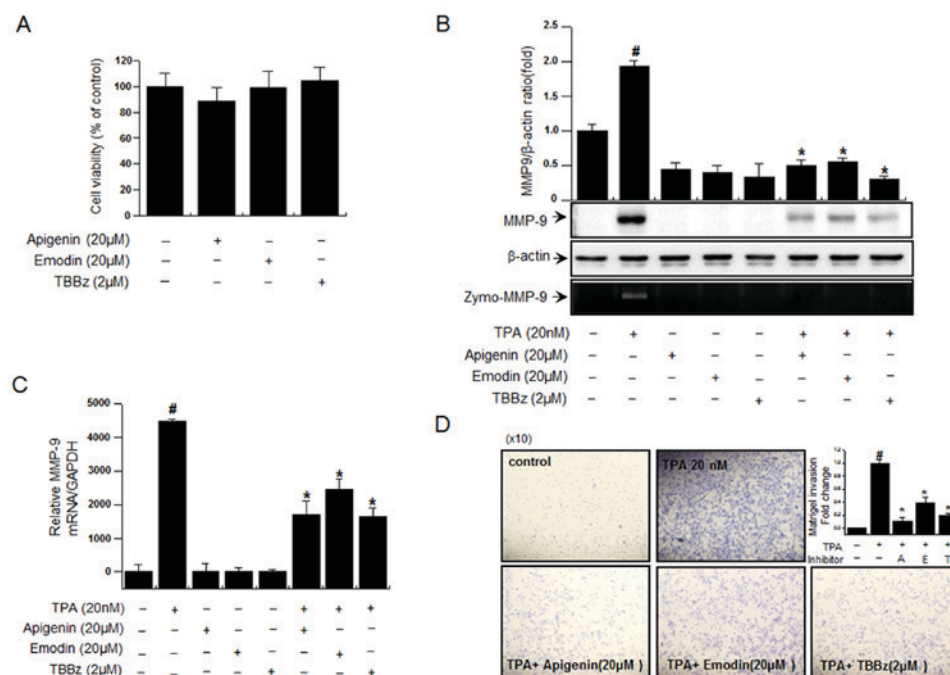


Figure 3. Inhibition of CK2 suppresses TPA-induced invasion and MMP-9 expression in MCF-7 cells. (A) To investigate the cytotoxic effects of the CK2 inhibitors, cell viability following incubation with CK2 inhibitors was determined. The optical density of the control was regarded as 100%. (B) The activity of MMP-9 following treatment with CK2 inhibitors and TPA was investigated using a zymography assay. (C) The mRNA expression of MMP-9 was analyzed via reverse transcription-quantitative polymerase chain reaction. (D) The invasive ability of cells following treatment with CK2 inhibitors and TPA was determined using a Matrigel invasion assay (magnification, x10). Data are presented as the mean \pm standard error of the mean of three independent experiments. # P <0.005 vs. control; * P <0.005 vs. TPA only. CK2 α , casein kinase 2 α ; si, small interfering; TPA, 12-O-tetradecanoylphorbol-13-acetate; MMP-9, matrix metalloproteinase-9; TBBz, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole; zymo, zymography; A, apigenin; E, emodin; T, TBBz.

CK2 α significantly suppressed TPA induced MCF-7 cell invasion (Fig. 1D). These results suggest that CK2 α may

be involved in the underlying mechanism resulting in the TPA-induced increase of cell invasion and MMP-9 expression.

Inhibition of CK2 α suppresses TPA-induced mitogen-activated protein kinase (MAPK) and NF- κ B activation in MCF-7 cells. The role of MAPKs (ERK, p38 and JNK) as upstream modulators of NF- κ B in the activation of MMP-9 expression has been well established (25,26,29). The effects of CK2 α inhibition on the TPA-induced phosphorylation of MAPKs was investigated. Following suppression of CK2 α expression via siRNA knockdown, the phosphorylation levels of p38 and JNK, but not of ERK, were significantly suppressed (Fig. 2A). As revealed in Fig. 2B, treatment with TPA significantly enhanced the level of NF- κ B (p65 and p50 subunits) compared with the control group. However, following suppression of intracellular CK2 α expression, the expression levels of NF- κ B were significantly inhibited (Fig. 2B). These results suggest that CK2 α is an upstream regulator of p38 and JNK in the PKC induced MAPK-NF- κ B signaling pathway responsible for the regulation of MMP-9 expression.

CK2 inhibitors suppress TPA-induced cell invasion and MMP-9 expression in MCF-7 cells. To further elucidate the therapeutic potential of CK2 inhibition for the prevention of PKC induced breast cancer cell invasion, three CK2 inhibitors were investigated. The cytotoxicity of these three CK2 inhibitors was investigated using a EZ-cytox assay. As revealed in Fig. 3A, treatment with 20 μ M apigenin, 20 μ M emodin and 2 μ M TBBz did not cause any significant changes in cell viability. Furthermore, the CK2 inhibitors were revealed to suppress TPA-induced increase in MMP-9 mRNA/protein expression and cell invasion (Fig. 3B-D). These results suggest that CK2 inhibitors have the ability to inhibit cell invasion via regulating MMP-9 expression.

Discussion

The stages of breast cancer are classified as 0-4. Number staging systems usually use the TNM system to divide cancers into stages. The majority of cancer types are classified into 4 stages, numbered from 1 to 4, which are important for diagnosis and treatment (30). From stage 2 onwards, metastases develop and the 5 year survival rate begins to decline (31). Mortality from metastatic breast cancer are on the rise, and thus suppression of metastasis has emerged as a major challenge in breast cancer treatment (32,33). In the early stages of tumor metastasis, individual tumor cells or clusters invade the ECM surrounding the primary tumor (34,35). The ECM consists of type IV collagen and other matrix proteins (19). Type IV collagen is a major component of the basement membrane and type IV collagenase MMP-9 has previously been revealed to be an important molecule in tumor progression and invasion in mammary tumors (36).

Elevated MMP-9 levels are functionally associated with breast cancer metastasis, and MMP-9 serves a role in TPA induced invasion of MCF-7 cells (21,24,37). Therefore, the suppression of MMP-9 expression may represent a novel therapeutic strategy for the prevention of tumor metastasis. TPA upregulates MMP-9 expression in MCF-7 cells; however, the underlying molecular mechanisms have not been fully established. Therefore, the present study aimed to investigate the role of CK2 in the regulation of MMP-9 expression in MCF-7 cells. The results of the present study revealed that the inhibition of CK2 α suppressed TPA-induced MMP-9 expression and invasion in MCF-7 cells. Furthermore, the

results of the present study revealed that CK2 α is a regulator of PKC-induced invasion in MCF-7 cells.

In addition, the inhibition of CK2 was demonstrated to suppress the activation of NF- κ B in MCF-7 cells. The MAPK/NF- κ B signaling cascade serves a role in PKC-mediated MMP-9 expression in MCF-7 cells (23,24,38,39). Furthermore, the MAPK signaling pathway has been previously demonstrated to be important for the activation of NF- κ B (40,41). CK2 has been reported to induce NF- κ B activation (27,42). In the present study, it was revealed that CK2 α inhibition markedly suppresses TPA-induced activation of p38, JNK and NF- κ B. In addition, it was revealed that the treatment of MCF-7 cells with CK2 inhibitors suppressed TPA-induced invasion and MMP-9 expression in MCF-7 cells.

In conclusion, the results of the present study revealed that suppression of CK2 exerts anti-invasive effects in PKC-induction condition via the regulation of MMP-9 expression levels. Therefore, CK2 may represent a novel anti-invasive target for cancer therapy.

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Availability of data and materials

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

Authors' contributions

JMK and EMN performed most of the experiments. HKS, YOY and KBK analyzed the data and provided comments. SJ and JSK helped with the experiments. HJY designed the project. YRL designed the analysis. KBK, YRL and HJY wrote the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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