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Article (Article)

Aurora kinase A induces migration and invasion by inducing epithelial-to-mesenchymal transition in colon cancer cells

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Running title: Aurora kinase induces EMT in colon cancer cells

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Abstract

Aurora kinase is a family of serine/threonine kinases intimately associated with mitotic progression and the development of human cancers. Studies have shown that aurora kinases are important for the protein kinase C (PKC)-induced invasion of colon cancer cells. Recent studies have shown that aurora kinase A promotes distant metastasis by inducing epithelial-to-mesenchymal transition (EMT) in colon cancer cells. However, the role of aurora kinase A in colon cancer metastasis remains unclear. In this study, we investigated the effects of aurora kinase A on PKC-induced cell invasion, migration, and EMT in human SW480 colon cancer cells. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) changed the expression levels of EMT markers, increasing α-SMA, vimentin, and MMP-9 expression and decreasing E-cadherin expression, with changes in cell morphology. TPA treatment induced EMT in a PKC-dependent manner. Moreover, the inhibition of aurora kinase A by siRNAs and inhibitors (reversine and VX-680) suppressed TPA-induced cell invasion, migration, and EMT in SW480 human colon cells. Inhibition of aurora kinase A blocked TPA-induced vimentin and MMP-9 expression, and decreased E-cadherin expression. Furthermore, the knockdown of aurora kinase A decreased the transcriptional activity of NF-κB and AP-1 in PKC-stimulated SW480 cells. These findings indicate that aurora kinase A induces migration and invasion by inducing EMT in SW480 colon cancer cells. To the best of our knowledge, this is the first study that showed aurora kinase A is a key molecule in PKC-induced metastasis in colon cancer cells.

Introduction

Aurora kinase is a family of serine/threonine kinases associated with mitotic progression. The overexpression or amplification of aurora kinases is generally detected in human cancers and is associated with a poor prognosis. Recent data show that aurora kinase A promotes distant metastasis by inducing EMT in breast cancer cells (1-5). Thus, aurora kinases have become promising therapeutic targets for cancer treatment.

Colorectal cancer (CRC) is frequently diagnosed in men and the second in women. Although CRC mortality has been decreasing for a long time, metastasis is the leading cause of cancer-related deaths (6, 7). EMT is when epithelial cells transdifferentiate into mesenchymal cells during embryonic development (8, 9). As a consequence, epithelial cells adopt the morphology of mesenchymal stromal cells and become motile and invasive. In cancer, EMT induces the migration and invasiveness of cancer cells, increasing their metastatic potential. Following this transformation, a decreased expression of E-cadherin and an increased expression of vimentin and α-SMA have been observed (10, 11).

PKC is known to be an important factor in signal transduction. PKC activation promotes carcinogen-induced tumorigenesis and is associated with specialized cellular functions, including adhesion, invasion, and metastasis (12). Treatment with a phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA) induced cell migration and invasion in cancer cells (11). Recently, it has been reported that PKC activation includes the regulation of EMT (14-16). Although studies have implicated PKC in EMT, the underlying molecular mechanisms remain poorly understood. We recently reported that aurora kinase positively regulates invasion and MMP-9 expression induced by PKC in breast cancer cells (15).

Here, we show the involvement of aurora kinase A in PKC-induced EMT in CRC cells. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) altered the expression EMT markers, such as increased α-SMA, vimentin, and MMP-9, and decreased E-cadherin expression, accompanying changes in cell morphology. However, the silencing of aurora kinase A by siRNAs and inhibitors suppressed TPA-induced cell invasion, migration, and EMT in SW480 human colon cells. These findings indicate that aurora kinase A induces migration and invasion by inducing EMT in SW480 colon cancer cells. To the best of our knowledge, this is

the first study to show that aurora kinase A is a key molecule in PKC-induced metastasis in colon cancer cells.

Results

PKC treatmemt induced EMT in SW480 colon cancer cells

SW480 colon cancer cells were derived from a primary tumor and had no metastatic properties. To determine whether TPA induces EMT in colon cancer cells, we treated SW480 cells with TPA. As shown in Figures 1A and 1B, treatment of cells with TPA, a PKC activator, induced changes in EMT markers, such as decreased E-cadherin and increased α-SMA, vimentin, and MMP-9 expression, with changes in cell morphology. We then confirmed the migration and invasion potential of cells treated with TPA, and observed that cells developed strong migration and invasive ability after 24 h of TPA treatment (Fig. 1C). Next, to investigate whether the induction of EMT by TPA is PKC-dependent, cells were treated with GF 109203X, a PKC inhibitor, 1 h before TPA treatment. Pretreatment with GF 109203X blocked TPA-induced EMT (Fig. 1C and 1D). These results confirmed that TPA induced EMT in a PKC-dependent manner.

Aurora kinase A is involved in PKC-induced EMT in SW480 colon cancer cells

We explored whether the expression of aurora kinase A was altered in SW480 colon cancer cells after exposure to TPA. TPA treatment increased aurora kinase A expression in SW480 colon cancer cells (Fig. 2A). Therefore, the role of aurora kinase A in PKC-induced EMT was examined in SW480 cells. The protein expression of aurora kinase A was determined to confirm that it was silenced by siRNA treatment (Fig. 2B). Next, we examined the alteration of EMT markers induced by aurora kinase A expression. The inhibition of aurora kinase A using siRNA and specific inhibitors (reversine and VX-680) blocked the TPA-induced alteration of vimentin, MMP-9, and E-cadherin expression (Fig. 2C and 2D). Therefore, these results imply that aurora kinase A plays an important role in PKC-induced EMT in colon cancer cells.

Aurora kinase A regulates PKC-induced cell invasion and migration in SW480 colon cancer cells

To investigate the effect of aurora kinase A inhibition on PKC-induced cell invasion and migration of colon cancer cells, we performed *in vitro* Matrigel invasion and scratch migration assays. The inhibition of aurora kinase A using siRNA suppressed TPA-induced invasion and migration (Fig. 3A). Additionally, we checked the effect of aurora kinase inhibitors such as reversine and VX680 in cell invasion. We observed that both agents inhibited TPA-stimulated cell invasion (Fig. 3B). These results suggest that the inhibition of aurora kinase A suppressed PKC-induced invasion and migration in SW480 cells.

Aurora kinase A regulates PKC-induced NF-κB/AP-1 activation in SW480 colon cancer cells

NF-κB and AP-1 are important transcription factors that regulate EMT (17, 18). As shown in Figure 4, TPA increased p65 (NF-κB subunit) expression and levels of phosphorylated c-Jun (AP-1 subunit) in the nucleus. To elucidate whether aurora kinase A expression was involved in NF-κB/AP-1 activation, suppression of aurora kinase A in SW480 cells by treatment with siRNA or inhibitors was performed. Treatment was done after exposing the cells to TPA for 3 h, and then the total nuclear protein was extracted to examine transcription factor activity. Inhibition of aurora kinase A suppressed TPA-induced expression of p65 and p-c-Jun in the nucleus (Fig. 4A and 4B). Indeed, TPA-induced NF-κB DNA-binding activity was partially blocked by treatment with aurora kinase inhibitors (Fig. 4C). These data suggest that aurora kinase A regulates the activation of NF-κB and AP-1 in PKC-stimulated SW480 cells.

Discussion

Aurora kinases are serine/threonine kinases that play a role in maintaining chromosome stability as mitotic regulators. Moreover, recent studies have shown that aurora kinase A activates EMT, stemness reprogramming (19), and the stabilization and accumulation of aurora kinases, which drives the transition of breast cells from an epithelial to a highly invasive mesenchymal phenotype (20). Aurora kinase expression in patients with CRC liver metastases is associated with poor prognosis (21, 22). These findings strongly suggest that aurora kinases are key negative regulators of the highly invasive mesenchymal phenotype in cancer cells. Our study suggests that aurora kinase A induces migration and invasion by inducing EMT in SW480 colon cancer cells. To the best of our knowledge, this is the first study to show that aurora kinase A is a key molecule in PKC-induced metastasis in colon cancer cells.

EMT is a stage of phenotypic alteration in cancer cells, in which cells undergo morphological transformation. During EMT, cells lose their epithelial characteristics and acquire a mesenchymal phenotype. Phenotypic changes are thought to be derived from a shift in the expression between epithelial (E-cadherin) and mesenchymal (N-cadherin, Snail, and Twist-1) factors (8, 9). EMT markers have been a major potential target for the prevention and treatment of various human cancers (19, 22). As a result, the transition from an epithelial to a mesenchymal phenotype in cancer cells increases their ability to invade and migrate to surrounding tissues.

MMP-9 is a key enzyme that plays a major role in metastasis. It acts through the degradation of the extracellular matrix (ECM), resulting in cancer and endothelial cell migration (23, 24). In addition, the induction of invasiveness and EMT has been associated with the activation of NF-κB and AP-1, which are major transcriptional factors of MMP-9 expression in cancer cells (25, 26, 27), suggesting that MMP-9 expression leads to EMT in cancer. However, the signaling systems that lead to EMT in colon cancer cells remain unclear. A previous study found that the inhibition of aurora kinases strongly suppressed TPA-induced MMP-9 expression in breast cancer cells (15). This suggests that activating aurora kinases is necessary for PKC-mediated induction of MMP-9 gene expression in colon cancer cells.

In this study, we found that the inhibition of aurora kinases by their respective siRNAs and

inhibitors suppressed TPA-induced cell invasion, migration, MMP-9 expression, and EMT in human colon cells (Fig. 2 and 3). These findings indicate that aurora kinase induces migration and invasion by inducing EMT and MMP-9 expression in SW480 colon cancer cells. Furthermore, the knockdown of aurora kinase A decreased the transcriptional activity of NF-κB and AP-1 in PKC-stimulated SW480 colon cancer cells (Fig. 4). Our results indicate that aurora kinase plays a role in PKC-induced MMP-9 expression through the NF-κB and AP-1 signaling pathways in colon cancer cells. Supporting this hypothesis, another study suggested that aurora kinase is a promising therapeutic target in cancer through the inhibition of the EMT pathway (19). These findings suggest that aurora kinase is a major regulator of EMT expression in colon cancer metastasis.

In conclusion, this study demonstrates that MMP-9 expression and the invasion of colon cancer cells may be modulated through an aurora kinase A-induced EMT signaling mechanism. Our data showed that aurora kinase A might be a novel target in the EMT signaling cascade for the prevention and treatment of colon cancer.

Materials and methods

Cell culture and materials

SW480 cells were purchased from the Korean Cell Line Bank. The cells were cultured in high-glucose DMEM containing 10% bovine serum albumin and 1% antibiotics (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) at 37 °C in an incubator with 5% CO_2. TPA, DMSO, and β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). GF 109203X and reversine were obtained from Calbiochem (St. Louis, MO, USA). VX-680 was purchased from Selleck (Houston, TX, USA). Primary antibodies against α-SMA, vimentin, MMP-9, E-cadherin, p65, and PCNA were purchased from Santa Cruz (CA, USA), and aurora kinase A (AURKA) and p-c-Jun were obtained from Cell Signaling Technology (Beverly, MA, USA).

Western blotting

SW480 cell extracts were isolated using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protease inhibitor (Millipore, Billerica, MA, USA). Twenty micrograms of protein was separated using 7-12% SDS-PAGE and transferred onto PVDF membranes (GE Healthcare Life Sciences, Buckinghamshire, UK). Membranes were incubated with the corresponding primary antibodies at 4 °C for overnight and HRP-conjugated secondary antibodies at 4 °C for 1 h. HRP was detected using an imaging analyzer (LAS 1000; FujiFilm Corporation, Japan).

Immunofluorescence analysis

Cell fixation was carried out with 4% formalin for 30 min at room temperature, and cell permeability was measured using 0.2% Triton-X100 in PBS for 20 min. Next, the cells were blocked with 2% bovine serum albumin in PBS to prevent nonspecific antibody binding. The cells were then incubated with anti-E-cadherin and anti-vimentin antibodies, followed by Alexa Fluor-400 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). Confocal images were obtained using a Carl Zeiss laser confocal microscope (Model no. LSM 510

META).

Matrigel invasion assay

Invasion assays were conducted using a Transwell insert chamber (8 μm pore size) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). SW480 cells were added to the upper chamber, and a chemoattractant was added to the bottom well. The wells were filled with a conditioned medium containing TPA for 24 h. After incubation, the invading cells were fixed, stained with toluidine blue, and photographed (40× magnification). Three independent experiments were conducted.

Scratch migration assay

A scratch migration assay was performed to compare the migratory abilities of normal and aurora kinase A (AURKA)-knockdown SW480 cells. All cells were cultured to confluency, scratched, photographed at 0 h, and then photographed after treatment with TPA for 24 h using a light microscope at 40× magnification. All experiments were performed in triplicate.

Electrophoretic motif shifting assay

Nuclear proteins were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA). Extraction was performed according to the manufacturer's protocol. Oligonucleotides containing the κ-chain (κB, 5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) binding site were synthesized and used as probes for the gel retardation assays. The NF-κB oligonucleotide was labeled with [α-^{32}P]dCTP using a Rediprime II DNA Labeling System (Amersham Life Science, Amersham, UK). In competition assays, a 100-fold excess of cold κB oligonucleotide was added. The DNA-protein complexes were analyzed via electrophoresis on a 4% polyacrylamide gel. After electrophoresis, the gel was dried and examined using autoradiography.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

Figure Legends

Fig. 1. PKC is a potent inducer of EMT in colon cancer cells. (A) SW480 cells were treated with 100 nM TPA for 24 h and then photographed to determine cell morphology. E-cadherin (green), vimentin (green), and nuclear DNA (blue; DAPI) were stained. (B) Cells were treated with 100 nM TPA for the indicated times. To determine their protein expression, EMT markers (E-cadherin, α-SMA, vimentin, and MMP-9) were analyzed via western blot. β-actin was used as a loading control. (C) GF pretreatment for 1 h and after treatment with TPA for 24 h. For the cell invasion assay, invading cells that passed through the Matrigel coating chamber were stained with toluidine blue. SW480 cells were plated in a 6-well plate and a scratch migration assay was performed. Cells were scratched at 0 h and photographed at 0 h and 24 h. (D) Cells were treated with GF for 1 h and then with TPA. After 24 h, cell lysates were analyzed via western blotting to determine the protein expression of E-cadherin, vimentin, MMP-9. β-actin was used as a loading control.

Fig. 2. Expression of AURKA during TPA-induced EMT in SW480 colon cancer cells. (A) Cells were treated with 100 nM TPA for the indicated times. Expression of AURKA was analyzed via western blotting. (B) Expression of AURKA in SW480 cells transfected with AURKA siRNA was confirmed via western blotting. (C) Cells were transfected with AURKA-siRNA for 24 h and then treated with TPA for 24 h. The expression of E-cadherin, vimentin, and MMP-9 were analyzed using western blotting. (D) SW480 cells were pretreated with AURK inhibitors (reversine and VX-680) for 1 h and then stimulated with TPA for 24 h. The expressions of EMT markers were determined via western blotting. All western blots used β – actin as the loading control.

Fig. 3. AURKA regulates TPA-induced invasion and migration in SW480 colon cancer cells. (A) Cells were transfected with AURKA-siRNA and were transferred into a 24-well chamber and treated with 100 nM TPA. After 24 h, the cells were fixed and stained with a toluidine blue solution. For the scratch migration assay, confluent cell monolayers were scratched at 0 h. (B) Cells were seeded in a 24-well chamber and treated with 100 nM TPA. After 24 h, the cells were fixed and stained with a toluidine blue solution.

Fig. 4. AURKA inhibits TPA-induced NF-κB and AP-1 activation in SW480 colon cancer

cells. (A) Cells were transfected with AURKA-siRNA for 24 h and then treated with TPA for 3 h. The expressions of p65 and p-c-Jun were analyzed using western blotting. PCNA was used as the nuclear loading control. (B) SW480 cells were pretreated with AURK inhibitors (reversine and VX-680) for 1 h and then stimulated with TPA for 3 h. Cell nuclear extracts were obtained and subjected to western blotting to determine the nuclear levels of NF-κB (p65) and AP-1 (p-c-Jun) subunits. PCNA was used as the nuclear loading control. (C) Cells were treated with AURK inhibitors (reversine and VX-680) for 1 h and then stimulated with TPA for 3 h. The DNA binding activity of NF-κB was determined through an electrophoretic mobility gel shift assay (EMSA).

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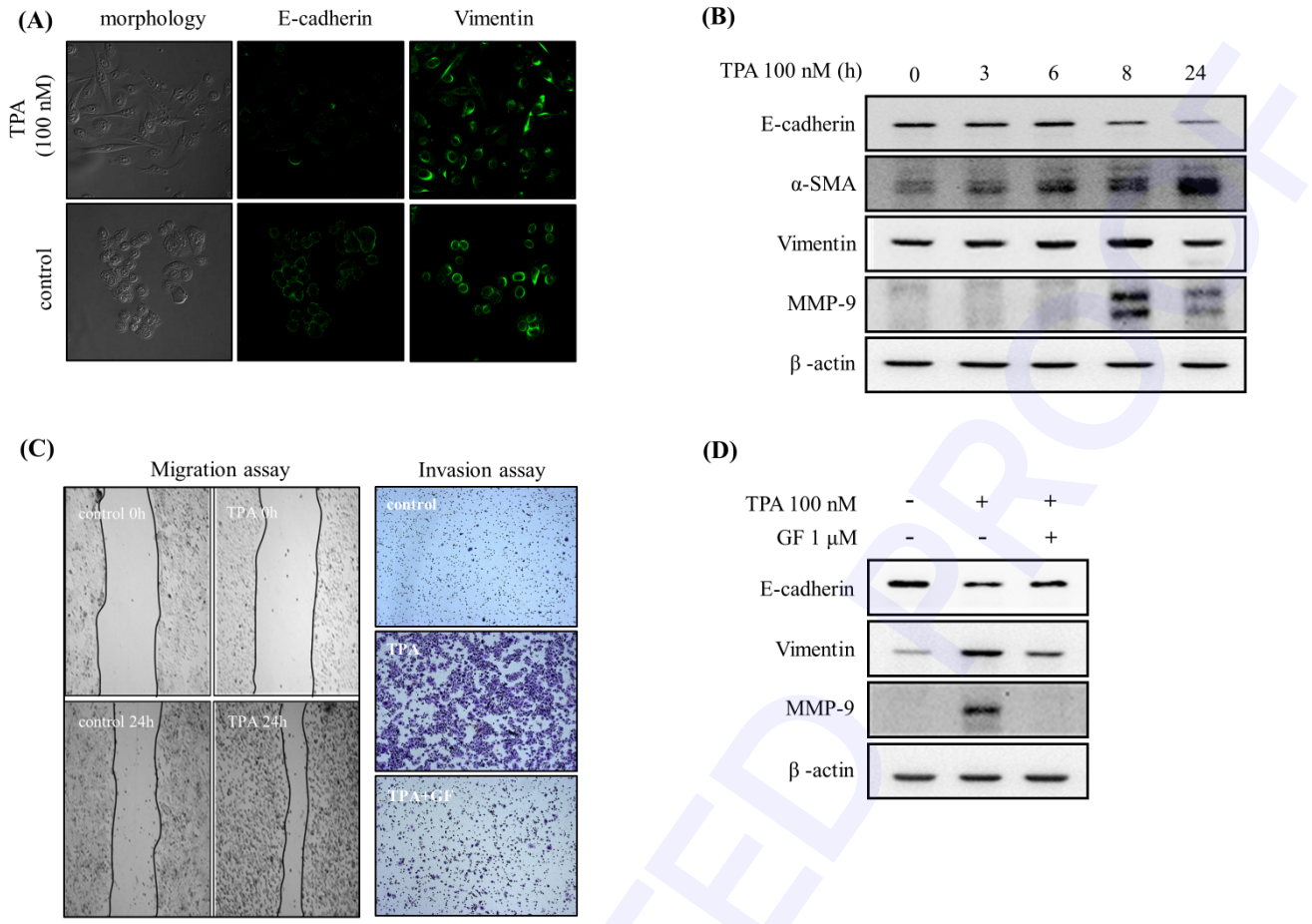
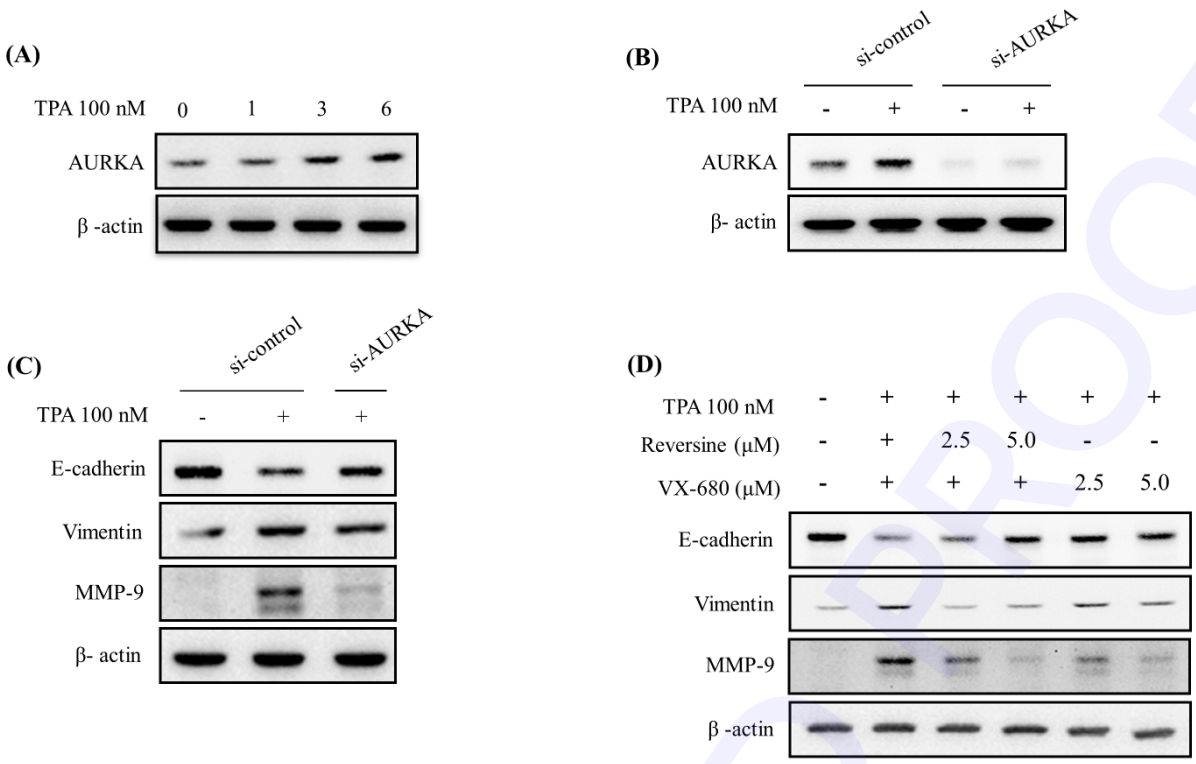


Fig. 1. PKC is a potent inducer of EMT in colon cancer cells. (A) SW480 cells were treated with 100 nM TPA for 24 h and then photographed to determine cell morphology. E-cadherin (green), vimentin (green), and nuclear DNA (blue; DAPI) were stained. (B) Cells were treated with 100 nM TPA for the indicated times. To determine their protein expression, EMT markers (E-cadherin, α-SMA, vimentin, and MMP-9) were analyzed via western blot. β-actin was used as a loading control. (C) GF pretreatment for 1 h and after treatment with TPA for 24 h. For the cell invasion assay, invading cells that passed through the Matrigel coating chamber were stained with toluidine blue. SW480 cells were plated in a 6-well plate and a scratch migration assay was performed. Cells were scratched at 0 h and photographed at 0 h and 24 h. (D) Cells were treated with GF for 1 h and then with TPA. After 24 h, cell lysates were analyzed via western blotting to determine the protein expression of E-cadherin, vimentin, MMP-9. β-actin was used as a loading control.



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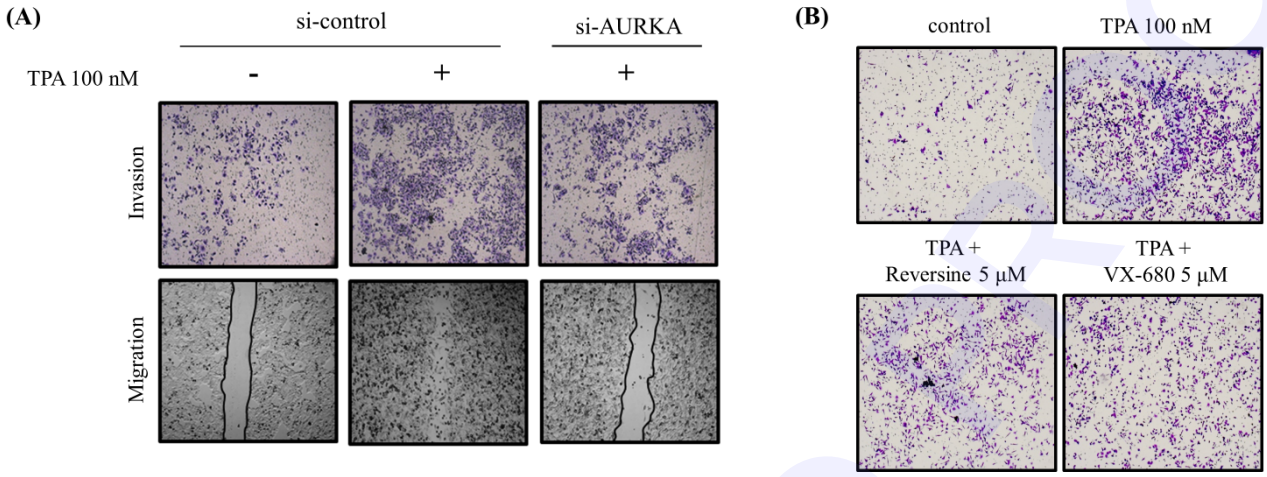

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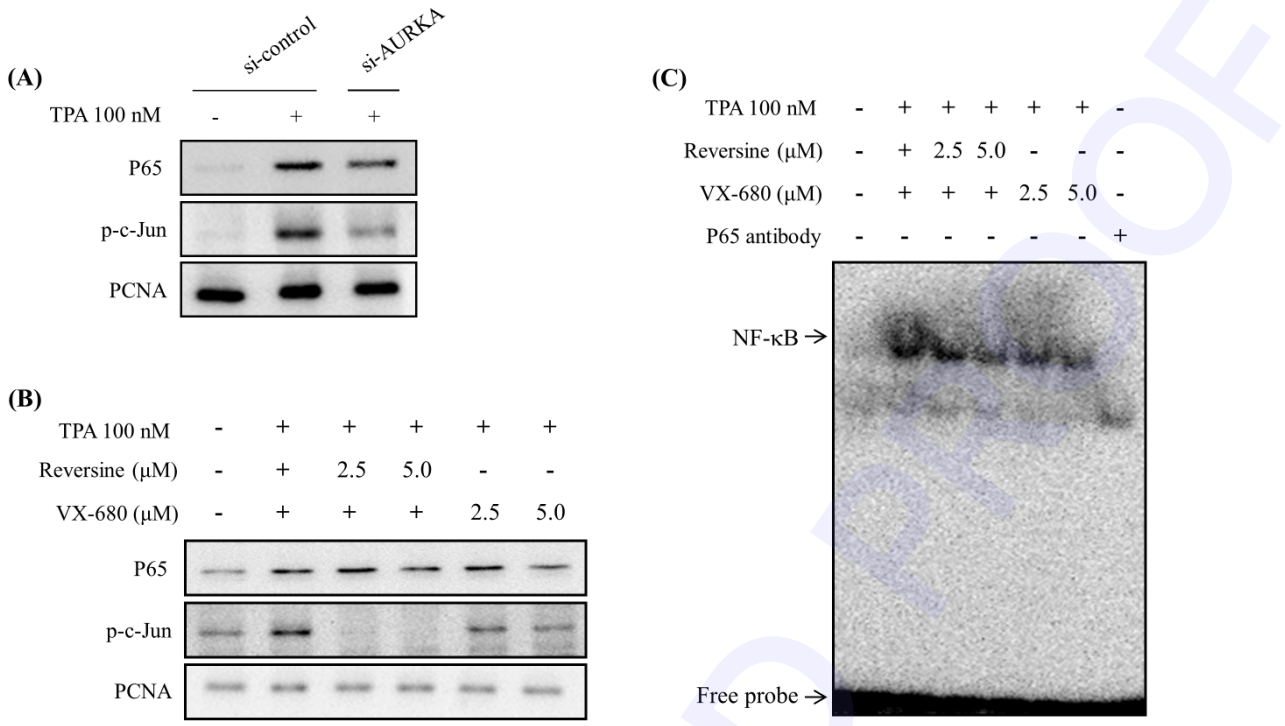

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