

Original Article

p21-activated kinase 4 promotes proliferation, migration, and invasion of human gastric cancer cells

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Abstract: Background/Aim: p21-activated kinase 4 (PAK4) overexpression has been observed in many cancers, including metastatic gastric cancer (GC). However, the precise role of PAK4 in GC remains unclarified. The aim of this study was to elucidate the role of PAK4 in GC development and progression. Methods: PAK4 was knocked down in MKN-28 cells using RNA interference (RNAi). Stable transfection of MKN-45 cells with PAK4 vector was used in overexpression experiments. PAK4 mRNA and protein levels were detected by qPCR and Western blot. PAK4 influence on cell proliferation, clone formation, migration, and invasion were evaluated. Results: PAK4 downregulation inhibited cell growth, clonogenicity, migration, and invasion, while PAK4 overexpression resulted in increased cell proliferation, migration, and invasion. Moreover, PAK4 regulated activation of the c-Src/EGFR/cyclin D1 and MEK-1/ERK1/2/MMP2 pathways. Conclusion: PAK4 overexpression promotes proliferation, migration, and invasion of GC cells and thus its down-regulation might be used as new strategy for GC treatment.

Keywords: p21-activated kinase 4, gastric cancer, human, tumorigenesis

Introduction

The incidence of gastric cancer (GC) has decreased during recent decades, especially in the Western world, but remains the second leading cause of cancer mortality worldwide [1]. Despite recent advances in the molecular understanding of GC, development of new targets and therapeutic approaches remains essential to cope with this illness.

p21-activated kinase 4 (PAK4) (chromosome 19q13.2) is a member of the serine/threonine kinase family of p21-activated kinases (PAKs). PAKs are involved in a variety of cellular processes related to tumor development and progression, including cytoskeletal dynamics, cell motility, gene transcription, death, and survival signaling, as well as cell cycle progression [2]. PAK4 was first identified as an effector molecule for Cdc42Hs and was found to be essential for regulating cytoskeleton reorganization [3]. Overexpression of PAK4 was observed in various cancer cell lines [4] and cancer tissues, including pancreatic [5], gallbladder [6], breast

[7], ovarian [8], hepatocellular [9], gastric [10] and oral squamous cell cancer [11]. PAK4 has also been shown to increase the growth and invasion of tumor cells [8, 11, 12]. Despite recent advances in the understanding of PAK4 biology, the functional role or mechanism of its action in GC remains poorly understood. Ahn et al. showed that PAK4 protein was strongly expressed in five gastric cell lines (AGS, MGK-28, MKN-74, SNU-216, and SNU-601) and was overexpressed in 8.1% (4/49) of metastatic gastric cancer specimens [10]. However, down-regulation of PAK4 expression using small interfering RNA induced apoptosis in PAK4-overexpressing GC AGS cells has also been tested [10]. Knockdown of PAK4 using the inhibitor LCH-7749944 suppressed proliferation in GC MKN-1, BGC823, SGC7901 and MGC803 cells and inhibited cell migration and invasion in GC SGC7901 and BGC823 cells [13]. Thus, PAK4 appears to be a potential therapeutic target for GC.

In the present study, the functional role and molecular mechanisms of PAK4 activity in GC was investigated.

Material and methods

Cell culture and reagents

The human GC cell lines MKN-28 and MKN-45 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). GC cells were cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified chamber containing 5% CO₂.

Stable overexpression of PAK4 in MKN-45 cells

The empty plasmid vector pcDNA3.1 (Invitrogen) or the plasmid vector containing PAK4 cDNA was transfected into MKN-45 cells using Lipofectamine 2000 (Life Technologies Co. PAK4 cDNA was transfected into MKN-45 cells using Lipofectamine 2000 (Life Technologies Corporation, USA). Multiple clones were selected in the presence of 0.8 mg/mL G418. PAK4-transfected clones were screened for PAK4 expression, and stably transfected clones were chosen and maintained in medium containing 0.1 mg/mL G418. To avoid clonal variations, six positive clones were pooled for further studies.

Stable knockdown of PAK4 expression in MKN-28 cells

The DNA sequence of PAK4 RNAi was designed to hybridize and destroy human PAK4 mRNA (accession no. NM_005884) as previously described [14]. The DNA sequence of RNAi for PAK4 was as follows: (sense, 5'-GATCCCCA-TGTCGGTGACACGCTCCTCAAGAGAGGAGCGT-GTCACCGAGATGTTTTTA-3'; antisense, 5'-AGCT-TAAAAACATGTCGGTGACACGCTCCTCTTGAA-GGAGCGTGTACCGAGATGGGG-3') and was synthesized and cloned into the pSUPER.neo vector (OligoEngine, USA) according to the manufacturer's instructions. A negative control siRNA-vector unrelated to the PAK4 coding region was also prepared. Next, negative control siRNA vector or siRNA-PAK4 vector were transfected into MKN-28 cells using Lipofectamine 2000 (Life Technologies Corporation, USA). Multiple clones were selected in the presence of 0.3 mg/mL G418, and PAK4-RNAi clones were screened for PAK4 expression. Stable RNAi

clones were chosen and maintained in the medium containing 0.1 mg/mL G418. To avoid clonal variations, six positive clones were pooled for further studies.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

For MTT assay, cells were plated in 96-well plates at a density of 1×10⁵ cells per well for five days. One hundred microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (1 mg/mL, Sigma) were added to each well daily, and the cells were further incubated at 37°C for four hours. Next, the supernatant was discarded, and 200 µL dimethyl sulfoxide (DMSO) was added for dye extraction. The absorbance at wavelength 570 nm was measured with a micro-ELISA reader. Every sample was evaluated in triplicate.

Clonogenic assay

For the clonogenic assay, cells (5×10⁴ cells per well) were plated in six-well plates. After 48 hours, the cells were trypsinized, and 10,000 cells were plated in a 100-mm petri dish. Next, the cells were incubated for 10 to 12 days at 37°C in a humidified chamber containing 5% CO₂, and colonies were then stained with 2% crystal violet and counted.

Wound healing assay

For the wound healing assay, cells (5×10⁵ cells per well) were plated in six-well plates. After 24 hours, the confluent monolayer cells were scratched manually with a plastic pipette tip, and after being washed with PBS, wounded monolayers of cells were allowed to heal for 12-24 hours. All experiments were conducted in triplicate.

Cell invasion assay

Cell invasion assay was performed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, USA) with an 8.0-µm pore size according to the manufacturer's instructions. Cells (2.5×10⁵) were added to the upper side of chambers and incubated for 24 hours at 37°C. After incubation, non-invaded cells remaining on the upper side of chambers were cleared with a cotton swab. The invaded cells on the lower side of the chambers were stained with

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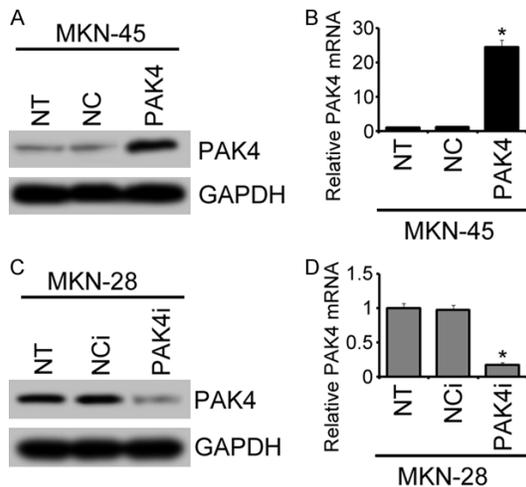


Figure 1. Stable overexpression of PAK4 in MKN-45 cells and knockdown of PAK4 in MKN-28 cells. (A & B) Increases in PAK4 protein (A) and mRNA (B) levels in MKN-45 cells revealed by Western blot (A) or real-time RT-PCR (B). NT, untransfected MKN-45 cells; NC, MKN-45-NC cells; PAK4, MKN-45-PAK4 cells. (C & D) Decreases in PAK4 protein (C) and mRNA (D) levels in MKN-28 cells revealed by Western blot (C) or real-time RT-PCR (D). NT, untransfected MKN-28 cells; NCi, MKN-28-NCi cells; PAK4i, MKN-28-PAK4i cells. Each bar represents the mean \pm SD. All experiments were repeated three times with similar results. * $P < 0.05$.

0.2% crystal violet in 10% ethanol. Three independent fields of invasive cells per well were photographed under the phase contrast microscope. The number of cells per field was counted. All experiments were conducted in triplicate.

RNA isolation and reverse transcription polymerase chain reaction

Total RNA was purified from cells using the Trizol reagent (Life Technologies, USA). First-strand cDNA was synthesized using 2.5 μ g RNA and AMV retroviridase (Promega, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed using the Bio-Rad iCycler iQ real-time PCR system (Bio-Rad, USA) and following primers: PAK4-L: 5'-ATGTGGTGGAGATGTACAACAGCTA-3', PAK4-R: 5'-GTTCATCCTGGTGTGGGTGAC-3'; GAPDH-L: 5'-CCACCATGGCAAATCCATGGCA-3', GAPDH-R: 5'-TCTAGACGGCAGG-TCA GGTCCACC-3'.

Protein isolation and Western blot

For Western blot analysis, cells at 70-80% culture confluence were harvested, lysed and their

protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, USA). Next, cell lysates (50 μ g protein/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (HyClone, Logan, USA). Membranes were blocked with 5% (v/v) skim milk and probed with primary antibody at 4°C overnight. The next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. Primary antibodies used were specific for phospho-c-Src, c-Src, phospho-EGFR, EGFR, phospho-ERK1/2, ERK1/2, phospho-MEK, MEK, cyclin D1, MMP2, and GAPDH (Cell Signaling Technology, Inc., USA). The bound antibodies were visualized using an electrochemiluminescence system (Amersham Pharmacia Biotech, UK).

Statistical analysis

Data are presented as the mean \pm SD. Differences in between-group variables were analyzed by Student's *t* test. Differences were considered significant when $P < 0.05$.

Results

Stable knockdown and overexpression of PAK4 in GC cells

The baseline expression of PAK4 was determined in two gastric cancer cell lines (Figure 1). The MKN-28 cell line showed higher expression of PAK4 protein while the MKN-45 cells showed little or no detectable expression of PAK4 protein in Western blot analysis (Figure 1A). The relative PAK4 mRNA levels in two cell lines were examined by qPCR, and results were consistent with those of Western blot analysis (Figure 1B).

To explore the role of PAK4 expression in GC cells, vectors carrying PAK4 cDNA were used to overexpress PAK4 in MKN-45 cells (MKN-45-PAK4 cells), and siRNA vectors were used to knockdown PAK4 expression in MKN-28 cells (MKN-28-PAK4i cells). Western blot and qPCR analysis confirmed that PAK4 expression was significantly increased in MKN-45-PAK4 cells and reduced in MKN-28-PAK4i cells compared with control cells (Figure 1).

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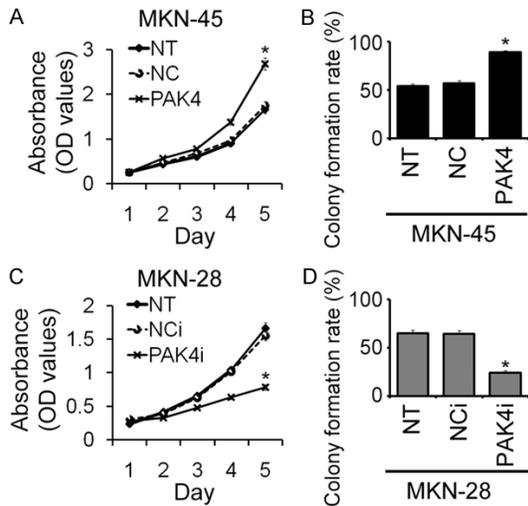


Figure 2. Effect of PAK4 expression on GC cell growth and colony formation. (A & B) PAK4 overexpression induced significant growth promotion of MKN-45 cells as revealed by MTT assay (A) and clonogenic assay (B). NT, untransfected MKN-45 cells; NC, MKN-45-NC cells; PAK4, MKN-45-PAK4 cells. (C & D) PAK4 knockdown caused a significant growth inhibition of MKN-28 cells as revealed by MTT assay (C) and clonogenic assay (D). NT, untransfected MKN-28 cells; NCi, MKN-28-NCi cells; PAK4i, MKN-28-PAK4i cells. Each bar represents the mean \pm SD. All experiments were repeated three times with similar results. * $P < 0.05$.

Overexpression of PAK4 promotes proliferation of GC cells

To determine the effect of PAK4 on cell proliferation of GC cells, MTT assay and clonogenic assay were performed. PAK4 stable overexpression increased cell growth and colony formation rate of MKN-45-PAK4 cells as measured by MTT assay taken over a 5-day period, as well as by clonogenic assay ($P < 0.05$, **Figure 2A, 2B**). No apparent difference was found between MKN-45 cells and the MKN-45-NC cells. However, down-regulation of PAK4 expression caused cell growth inhibition in MKN-28 cells (**Figure 2C**). The effect of PAK4 down regulation on MKN-25 cell growth inhibition was also assessed by clonogenic assay, and the results were consistent with the MTT assay (**Figure 2D**).

Overexpression of PAK4 improves migration and invasion potential of GC cells

Next, the effects of PAK4 expression on GC cell migration and invasion were examined. Wound

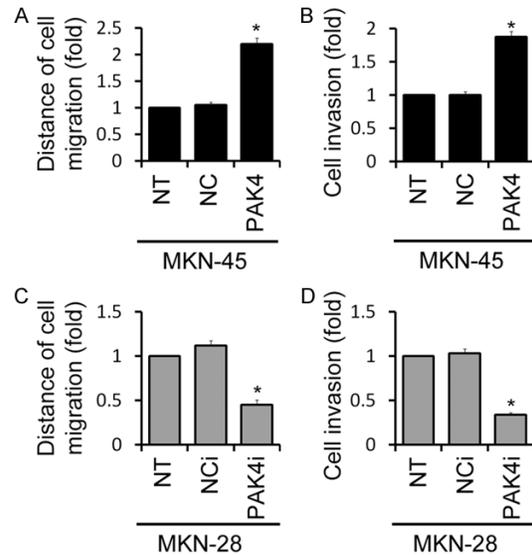


Figure 3. Effect of PAK4 expression on GC cell migration and invasion. (A & B) PAK4 overexpression caused a significantly increased cell migration (A) and invasion (B) in MKN-45 cells. NT, untransfected MKN-45 cells; NC, MKN-45-NC cells; PAK4, MKN-45-PAK4 cells. Data are represented as the fold change of control MKN-45 cells. (C and D) PAK4 knockdown significantly inhibited cell migration (C) and invasion (D) in MKN-28 cells. NT, untransfected MKN-28 cells; NCi, MKN-28-NCi cells; PAK4i, MKN-28-PAK4i cells. Data are represented as the fold change of control MKN-28 cells. Each bar represents the mean \pm SD. All experiments were repeated three times with similar results. * $P < 0.05$.

healing assay was carried out to test cell migration, and in our study, MKN-45-PAK4 cells had a substantially faster wound-healing rate compared with control cells ($2.23 \pm 0.11:1$, $P < 0.05$) (**Figure 3A**). Furthermore, Matrigel invasion chambers assay was used to determine the effects of PAK4 on cell invasion. MKN-45-PAK4 cells showed an approximate 1.89-fold higher invasion rate through the Matrigel-coated membrane compared with control cells (**Figure 3B**). Conversely, MKN-28-PAK4i cells showed a marked decrease in cell migration (0.43%) and invasion (0.31%) compared with control cells (**Figure 3C, 3D**).

Overexpression of PAK4 activates signaling networks downstream of the c-Src/EGFR/cyclin D1 and MEK-1/ERK1/2/MMP2 pathways

To further study the mechanism of how PAK4 influences cell proliferation, several signaling proteins involved in cell cycle control were

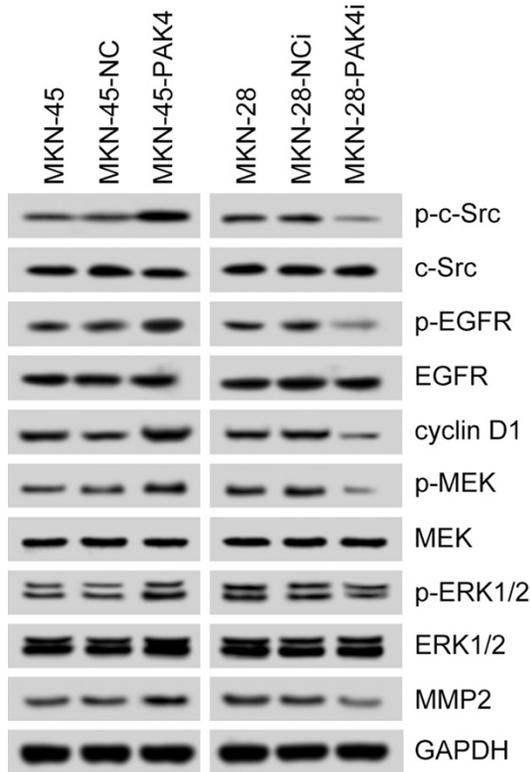


Figure 4. Effect of PAK4 expression on activation of the c-Src/EGFR/cyclin D1 pathway and MEK-1/ERK1/2/MMP2 pathway in GC cells. The protein expression of phosphor-c-Src (p-c-Src), c-Src, phosphor-EGFR (p-EGFR), EGFR, cyclin D1, phosphor-MEK (p-MEK), MEK, phosphor-ERK1/2 (p-ERK), ERK1/2, and MMP2 was determined by Western blot. All experiments were repeated three times with similar results.

examined. Previous studies showed that PAK4 regulated cell proliferation and was involved in activation of the c-Src/EGFR/cyclin D1 pathway. In agreement with these previous findings, Western blot analysis showed that phospho-c-Src, phospho-EGFR and cyclin D1 protein levels were increased in the MKN-45-PAK4 cells (**Figure 4**). In contrast, downregulation of PAK4 led to a decrease in the phospho-c-Src, phospho-EGFR and cyclin D1 protein expression in MKN-28-PAK4i cells (**Figure 4**).

To further explore the mechanism of the role of PAK4 in the regulation of GC cell migration and invasion, several signaling pathways were examined. Western blot analysis showed that increased PAK4 expression activated the MEK-1 and ERK1/2 signaling pathways, leading to the up-regulation of MMP2 expression in

MKN-45-PAK4 cells (**Figure 4**). In contrast, PAK4 silencing in MKN-28-PAK4i cells resulted in significant inhibition of the MEK-1/ERK1/2/MMP2 pathway (**Figure 4**). Representative Western blots of different groups are shown for p-c-Src, p-EGFR, cyclinD1, p-MEK, p-ERK1/2, and MMP2 protein expression in MKN-45 cells and MKN-28 cells (**Figure 5**).

Discussion

PAK4 is an important regulator for cell proliferation, migration, and invasion [8, 11, 12], and an abnormal overexpression of PAK4 protein is a hallmark of many human cancers [4-11]. Therefore, PAK4 appears to be an attractive target for anti-tumor therapy, and inhibiting this factor it should enable the targeting of multiple facets of tumorigenesis [4, 8, 13]. In GC, one of the earliest pieces of evidence for the involvement of PAK4 was provided by Ahn et al. [10], who performed an immunohistochemical analysis of PAK4 protein expression in metastatic GC. In their study, PAK4 overexpression was found in 4 (8.1%) of 49 metastatic GC specimens, suggesting that PAK4 may have some involvement in the progression of GC. Using the PAK4 inhibitor LCH-7749944, Zhang et al. [13] reported that exposure of GC cell lines to the inhibitor results in a dose-dependent inhibition of cell proliferation, migration, and invasion. Despite these previous results indicating an important role for PAK4 in GC, there is little information regarding a simultaneous comparison of the consequence of PAK4 exogenous overexpression or down-regulation in GC cell lines. Therefore, in this study, the role of PAK4 in cell proliferation, migration, and invasion was examined in the low and high PAK4 expressing cell lines MKN-45 and MKN-28, respectively. According to our results, up-regulation of PAK4 resulted in a significant increase in the ability of GC cells to proliferate as well as to migrate and invade, which is in contrast to the inhibitory effect on cell proliferation, migration and invasion induced by PAK4 silencing. Collectively, these findings provide evidence in support of the role of PAK4 as an oncogene in GC cells.

EGFR is a cell-surface receptor that is activated through the binding of its specific ligands, which are members of the epidermal growth factor (EGF) family of extracellular protein ligands [15, 16]. EGFR has been established as an impor-

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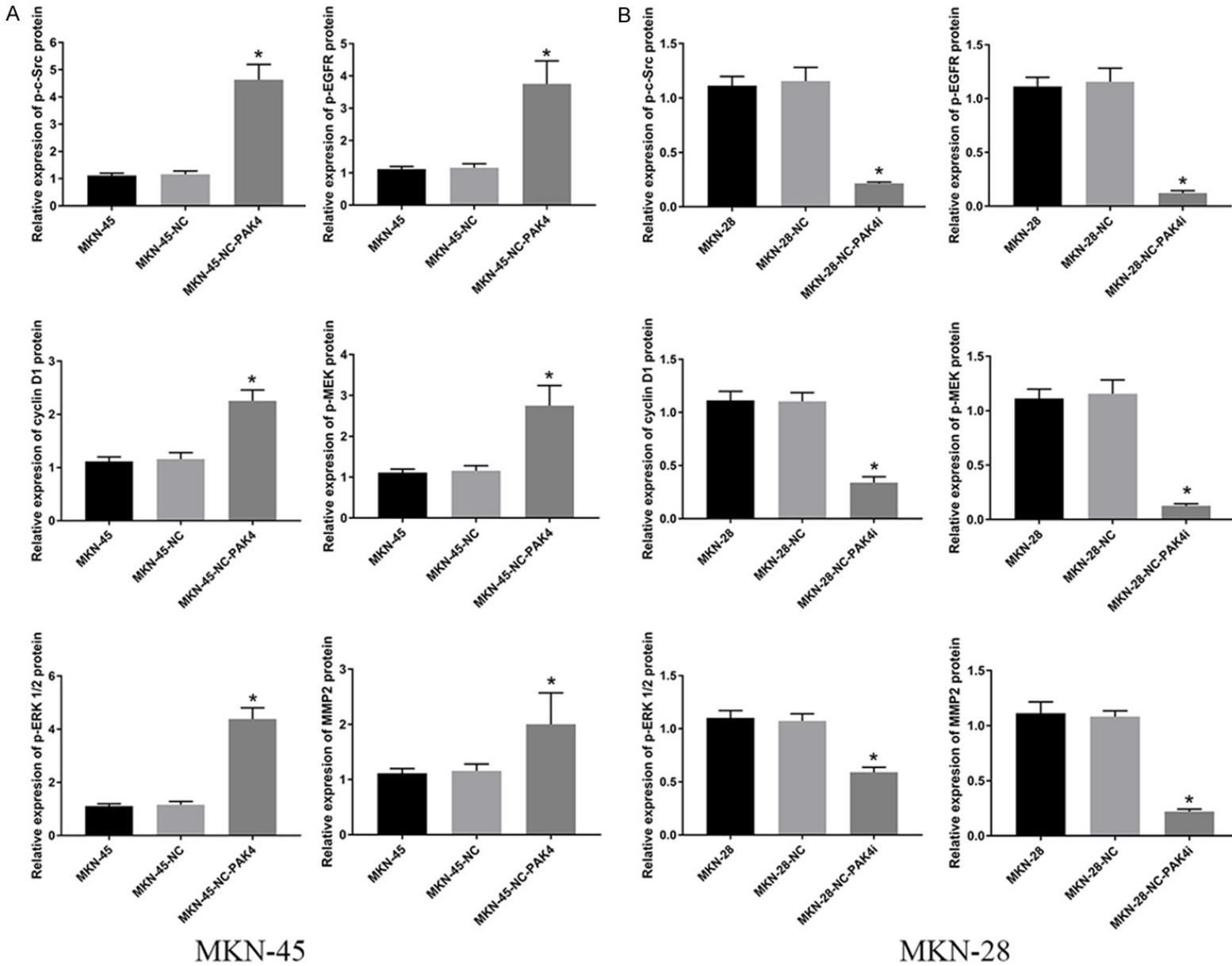


Figure 5. Representative Western blot showing expression of p-c-Src, p-EGFR, cyclin D1, p-MEK, p-ERK1/2, and MMP2 proteins in MKN-45 (A) and MKN-28 (B). *P<0.05.

tant mediator of tumorigenesis in the etiology of several common human cancers [17-19]. Overexpression and activation of EGFR have both been reported in GC and have been associated with poor patient outcomes [20-22]. c-Src is a nonreceptor tyrosine kinase that is highly activated in a wide spectrum of human tumors, and its expression was correlated with malignant progression of various tumors [23]. In addition, c-Src contributes to transformation, proliferation, survival, and motility of malignant cells as well as to tumor angiogenesis [24]. In this study, an association between Pak4 overexpression, c-Src and EGFR activation, and cyclin D1 up-regulation and gastric cancer cell proliferation is shown.

The importance of the ERK signaling pathway and MMP family in cancer invasion has been thoroughly recognized [25-27]. In this study, a downstream pathway of PAK4, which included the MEK-1 to ERK1/2 pathway that controls GC cells migration and invasion was identified. MMPs and especially MMP2 play a crucial role in tumor invasion and metastasis [28]. Consistent with previous studies, the observations suggest that the activation of MEK-1 and ERK1/2 induced by PAK4 also results in the up-regulation of MMP2. Furthermore, using RNAi technology, down-regulation of PAK4 inhibited the invasive potential of GC cells involving the inhibition of the MEK-1/ERK1/2/MMP2 pathway.

In summary, these results provide mechanistic insight in support of the role of PAK4 in proliferation, migration, and invasion of GC cells. The study also provides evidence that the up-regulation of PAK4 can be mechanistically associated with the promotion of GC tumorigenesis mediated by the activation of the c-Src/EGFR/cyclin D1 and MEK-1/ERK1/2/MMP2 pathways. Thus, PAK4 may represent an effective target for antitumor therapy and PAK4 down-regulation can likely result in cell growth inhibition, reduced migration and less invasion by GC cells.

Disclosure of conflict of interest

None.

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