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Mixed Lineage Kinase 3 is Required for Matrix Metalloproteinase Expression and Invasion in Ovarian Cancer Cells

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Abstract

Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates MAPK signaling pathways and regulates cellular responses such as proliferation, migration and apoptosis. Here we report high levels of total and phospho-MLK3 in ovarian cancer cell lines in comparison to immortalized nontumorigenic ovarian epithelial cell lines. Using small interfering RNA (siRNA)-mediated gene silencing, we determined that MLK3 is required for the invasion of SKOV3 and HEY1B ovarian cancer cells. Furthermore, *mlk3* silencing substantially reduced matrix metalloproteinase (MMP) -1, -2, -9 and -12 gene expression and MMP-2 and -9 activities in SKOV3 and HEY1B ovarian cancer cells. MMP-1, -2, -9 and -12 expression, and MLK3-induced activation of MMP-2 and MMP-9 requires both extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase activities. In addition, inhibition of activator protein-1 (AP-1) reduced MMP-1, MMP-9 and MMP-12 gene expression. Collectively, these findings establish MLK3 as an important regulator of MMP expression and invasion in ovarian cancer cells.

Keywords

MLK3; MMP; invasion; ovarian cancer; MAPK; AP-1

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies and one of the most common causes of death from cancer among women in the western world. Often the diagnosis of ovarian cancer occurs late and the treatment options are limited [1]. Epithelial ovarian cancer arises primarily from neoplastic transformation of the ovarian surface epithelium (OSE), a layer of flat or cuboidal cells that cover the surface of the ovary [2, 3]. Some high grade serous ovarian cancers may also arise from fallopian tubal epithelia [2]. Hormones, growth factors and cytokine-dependent activation of cellular signaling pathways

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are critical for the regulation of OSE proliferation, and a thorough understanding of these signaling pathways is necessary to achieve earlier detection and develop novel therapies [3, 4].

Mitogen-activated protein kinase (MAPK) signaling has an important role in the development of ovarian cancer. Aberrant activation of extracellular-signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) MAPKs has been observed in ovarian cancer cells, and suppression of ERK and JNK MAPK signaling impairs ovarian cell invasion, proliferation and survival [5–8]. Mammalian MAPK signaling pathways are organized in a three-step phosphorelay signaling module in which MAPKs are activated by phosphorylation catalyzed by MAPK kinases (MAP2Ks); and MAP2Ks are activated by phosphorylation catalyzed by MAPKK kinases (MAP3Ks) [9]. Mixed lineage kinase (MLK)-3 is a MAP3K that activates multiple MAPK pathways [10]. Tumor necrosis factor (TNF) stimulation of MLK3 activates JNK and p38 MAPK signaling; and MLK3 is required for epidermal growth factor (EGF) activation of ERK signaling in lung and colon fibroblasts [10–13]. In addition, MLK3 is required for proliferation of normal colon and lung fibroblasts and a number of neoplastic cell lines, including SKOV3 ovarian cancer cells [13].

The major biological outcome of MAPK signaling is the transcriptional regulation of target genes and the control of cellular processes such as proliferation, survival, motility, and differentiation. ERK and JNK MAPK activation and nuclear translocation results in phosphorylation and activation of transcription factors such as activator protein-1 (AP-1) [9].

The AP-1 transcriptional complex is composed of heterodimers of different c-Fos (c-Fos, Fra-1, Fra-2 and FosB) and c-Jun (c-Jun, JunB and JunD) subunits. ERK and JNK phosphorylate AP-1 subunits to enhance their stability and transcriptional activities. Activated ERK phosphorylates the c-Fos C-terminal transactivating domain, and activated JNK phosphorylates c-Jun on residues within the transactivating domain, resulting in c-Jun stabilization and activation [14]. The AP-1 heterodimer binds to TPA responsive elements (TREs) in the promoters of target genes to regulate gene transcription [14]. AP-1 target genes that are critical regulators of ovarian tumor cell invasion include Ezrin, Cathepsin L and the matrix metalloproteinases (MMP)-1, -2, and -9 [15–20].

In the current study, our goal was to gain a better understanding of the role of MLK3 in ovarian cancer cell development. Our results demonstrate that MLK3 total protein and kinase activity are elevated in SKOV3 ovarian cancer cells in comparison to immortalized ovarian epithelial T29 cells. At high cell density, T29 but not SKOV3 cells, exhibit a marked reduction in the level of active MLK3. Small interfering RNA (siRNA)-mediated gene silencing of *mlk3* in SKOV3 and HEY1B ovarian cancer cells substantially reduces MMP-1, -2, -9 and -12 expression and inhibits cell invasion. Furthermore, MLK3 overexpression in SKOV3 and HEY1B results in an elevation of MMP-2 and MMP-9 activities, that is at least partially dependent on ERK and JNK signaling. These results suggest a critical role for MLK3 in the regulation of MMP expression and invasion in ovarian cancer cells.

Materials and methods

Cell lines and cell culture

SKOV3, HEY and HEY1B are human ovarian carcinoma cells. SKOV3 was obtained from the American Type Culture Collection (Manassas, VA, USA). HEY1B cells were a gift from Dr. Douglas Leaman, (University of Toledo). SKOV3, HEY and HEY1B cells were cultured

in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). T29 and T80 cells are immortalized human ovarian epithelial cells as described previously [21]. T29 and T80 cells were cultured in medium 199 (Mediatech, Inc.), with 10% MCDB 105 (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS. All tissue culture media were supplemented with 25 µg/ml streptomycin and 25 I.U. penicillin (Mediatech, Inc.). Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Preparation of RNA from ovarian cell lines

Preparation of RNA from SKOV3, HEY1B and T29 cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, one 6 cm dish of cells was homogenized in 1 ml of TRIzol reagent, and 0.2 ml of chloroform was added followed by centrifugation at 12,000 x g for 15 min at 4°C to separate the phases. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The RNA pellets were washed once with 75% ethanol, dried and dissolved in RNase-free water.

SiRNA and cDNA plasmid transfections

Cells were transfected with double stranded siRNA oligonucleotides using Lipofectamine 2000, or transfected with cDNA plasmids using Lipofectamine as previously described [13]. SiRNA oligonucleotides targeting the human MLK3 coding sequence were: nts 903-923 (siRNA oligo 1) 5'-GGGCAGTGACGTCTGGAGTTT-3', and nts 1198-1218 (siRNA oligo 2) 5'-AAGCGCGAGATCCAGGGTCTC-3'. Non-specific siRNA with non-targeting sequence was from Dharmacon, Lafayette, CO. AP-1 activity was inhibited using a cDNA expression construct encoding a dominant negative N-terminal mutant of c-Jun (Δ1-245) (a gift from Dr. Lirim Shemshedini, University of Toledo). This construct lacks the transactivation domain and the Ser63 and Ser73 JNK phosphorylation sites. The pCMV-FLAG-MLK3 mammalian expression construct contains the coding sequence for human *mlk3*.

Immunoblotting

Immunoblotting was performed as described previously [13]. The antibodies used for immunoblotting were MLK3 (C-20), ERK (C-14), JNK (C-17), β-Actin (C-4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and c-Jun (Thermo Scientific, Rockford, IL, USA). Activation-state phospho-ERK (p-ERK; Thr202/Tyr204), phospho-MLK3 (p-MLK3; Thr277/Ser281), phospho-JNK (p-JNK; Thr183/Tyr185) antibodies were from Cell Signaling Technology, Beverly, MA, USA, and the FLAG antibody was from Agilent Technologies, Santa Clara, CA. Densitometric analysis of immunoblots was performed by using Image J software (National Institutes of Health).

Real-time PCR (RT-PCR)

RT-PCR was performed using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primer sequences (human) were as follows: β-Actin forward 5'-GGACTTCGAGCAAGAGATGG-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3'; GAPDH forward 5'-CGA CCA CTT TGT CAA GCT CA-3' and reverse 5'-AGG GGA GAT TCA GTG TGG TG-3'; MLK3 forward 5'-GTCATGGAATGGCAGTGG-3' and reverse 5'-CACGGTCACCCCTTCCTCA-3'; MMP-1 forward 5'-ATGCTGAAACCCTGAAGGTG-3' and reverse 5'-CTGCTTGACCCTCAGAGACC-3'; MMP-2 forward 5'-TTTCCATTCCGCTTCCAGGGCAC-3' and reverse 5'-TCGCACACCACATCTTCCGTCAC-3' [24]; MMP-9 forward 5'-GCCATTACGTCGTCCTTAT-3' and reverse 5'-TTGACAGCGACAAGAAGTGG-3';

MMP-12 forward 5'-CCTTCAGCCAGAAGAACCTG-3' and reverse 5'-ACACATTTTCGCCTCTCTGCT-3'. Data are presented as the mean, with n representing 3 independent experiments. Comparison of two groups was performed using the unpaired Student's t test. A P value of < 0.05 was considered statistically significant.

Gelatin zymography

MMP-2 and MMP-9 enzymatic activities in the cell culture medium were determined by SDS-PAGE gelatin zymography. Briefly, cells were seeded at 5×10^5 cells per 6 cm dish. Conditioned cell culture medium from each dish was normalized to an equal amount of protein (100 μ g), denatured in the sample buffer in the absence of reducing agents, and subjected to 10% SDS-PAGE containing 0.1% (w/v) gelatin (Acros Organics, Morris Plains, New Jersey, USA). The gel was incubated in the presence of 2.5% Triton X-100 at room temperature for 2 h, and then transferred to digestion buffer containing 1% Triton X-100, 10 mM CaCl_2 , 0.15 M NaCl, and 50 mM Tris (pH 7.5), and incubated for 40 h at 37°C. Gels were stained with Coomassie Blue R-250 and destained. Proteolysis was detected as a white band against a blue background.

Invasion assays

Cell invasion assays were performed with 100 μ l of 1 mg/ml BD Matrigel matrix (BD Biosciences, San Jose, CA, USA) in 24-well Transwells with 8.0 μ m pore size and 24 mm diameter polycarbonate membrane (Corning, Acton, MA, USA). Cells were washed in DMEM containing 0.5% FBS. Ten thousand cells were seeded onto the upper chamber and incubated for 16 h. Matrigel and cells remaining in the upper chamber were removed after the incubation. The cells on the underside surface of the membrane were fixed using the Diff Quick Stain Kit (IMEB Inc., San Marcos, CA, USA) and the number of cells per field of view was counted. The average of 4 fields per experiment was obtained and all experiments were performed in triplicate.

ERK and JNK Inhibitors

U0126 and SP600125 were used to inhibit ERK and JNK activities, respectively. Briefly, cells were seeded at 5×10^5 cells per 6 cm dish and incubated overnight. Inhibitors were diluted in serum-free medium and added to the cells at a final concentration of 10 μ M for U0126 and 50 μ M for SP600125, and cells were incubated with the inhibitors for 4 h. The same volume of the vehicle, DMSO, was added to one dish as a negative control.

Results

Total and phosphorylated MLK3 protein levels in ovarian tumor cell lines

We postulated that inappropriate levels of MLK3 expression and activity in ovarian cancer cells could promote persistent activation of ERK and JNK MAPKs, which are critical contributors to the development of the malignant phenotype. Thus, MLK3 protein levels of human ovarian cancer cell lines, HEY, HEY1B, and SKOV3 were compared to those of immortalized non-tumorigenic ovarian epithelial cell lines, T29 and T80. In addition, to analyze the level of active MLK3, cell extracts were immunoblotted with an antibody that detects MLK3 phosphorylated on residues Thr277 and Ser281 (p-MLK3). Autophosphorylation of these residues within the activation loop is required for MLK3 activity [10]. MLK3 protein levels (total and phosphorylated) in each cell line were expressed as a fold-increase over T29 (Fig. 1A). Both T29 and T80 immortalized cell lines had lower levels of total and active MLK3 than ovarian cancer cell lines. Of the two immortalized cell lines, T80 cells had an approximately 1.5-fold and 2.5-fold higher level of total MLK3 and p-MLK3 respectively, than T29 cells. However, SKOV3, HEY1B and HEY

ovarian cancer cells had a 1.7-2.9-fold higher level of total MLK3, and a 2.7- 3.7-fold higher level of p-MLK3 than T29 cells (Fig. 1A).

Typically, ERK activity is low in non-neoplastic, contact inhibited cells, whereas neoplastic cells do not exhibit a density-dependent inhibition of ERK activity [22]. To determine if the level of active MLK3 is also affected by cell density, p-MLK3 levels were analyzed in T29 and SKOV3 cells at different stages of cell density. P-MLK3 levels decreased with increasing cell density in T29 cells, but remained constant in SKOV3 cells at both low and high density. These results indicate a major difference between the immortalized and neoplastic ovarian cell lines in density-dependent regulation of MLK3 phosphorylation (Fig. 1B).

Cell invasion is impaired in MLK3 knockdown SKOV3 and HEY1B cells

SKOV3 and HEY1B cells have high levels of active MLK3, which we postulate may facilitate an invasive phenotype through persistent activation of MAPK signaling. To determine if ovarian cancer cells require MLK3 for invasion, *mlk3* expression was silenced in SKOV3 (Fig. 2A) and HEY1B (Fig. 2B) cells, and the capacity to invade through Matrigel *in vitro* was analyzed. Invasion was reduced by approximately 2.7-fold (siRNA oligo 1) or 5.6-fold (siRNA oligo 2) in MLK3-knockdown SKOV3 cells (Fig. 2A), and 3.4-fold (siRNA oligo 1) or 4.9-fold (siRNA oligo 2) in MLK3-knockdown HEY1B cells (Fig. 2B) in comparison to cells transfected with non-specific siRNA. These results indicate a specific requirement for MLK3 in the invasion of SKOV3 and HEY1B ovarian cancer cells.

MLK3 knockdown in SKOV3 and HEY1B cells results in reduced MMP expression and activity

MMPs are proteolytic enzymes that degrade components of the extracellular matrix, and increased expression of MMP-2 and MMP-9 is associated with the progression from benign to advanced ovarian cancer [15, 23, 24]. Analysis of MMP gene expression by RT-PCR indicated that MMPs -1, -2, -9 and -12 are expressed at higher levels in SKOV3 cells in comparison to T29 cells (Fig. 3A). To gain mechanistic insight into how MLK3 could promote ovarian cancer cell invasion, we investigated the possibility that MLK3 may regulate MMP gene expression. *MLK3* was silenced with siRNA in SKOV3 cells, and MMP mRNA levels were analyzed by RT-PCR. A 75-90 percent decrease in MMP-1, -2, -9 and -12 mRNA levels was observed in MLK3-knockdown cells in comparison to cells treated with nonspecific siRNA (Fig. 3B, upper panel). Similarly, a 54-76 percent decrease in MMP-1, -2, -9 and -12 mRNA levels was observed in MLK3-knockdown HEY1B cells in comparison to cells transfected with nonspecific siRNA (Fig. 3B, lower panel). Next, we employed gelatin zymography to analyze the effect of silencing *mlk3* on MMP-2 and -9 enzyme activities. We observed that MMP-2 and -9 gelatinase activities in the culture media of MLK3-knockdown (siRNA oligo 1 or 2) SKOV3 and HEY1B cells were substantially lower than in cells transfected with nonspecific siRNA (Fig. 3C). These results indicate that MLK3 is required for MMP-1, -2, -9, and -12 expression, and MMP-2 and -9 activities in SKOV3 and HEY1B cells.

MLK3-dependent increase in MMP-2 and -9 activities requires ERK and JNK in SKOV3 and HEY1B cells

To investigate the mechanism by which MLK3 regulates ovarian cancer cell invasion, we first assessed the impact of silencing *mlk3* on the activities of the MAPKs, ERK and JNK, in SKOV3 cells. Consistent with our previous findings in colorectal and lung fibroblasts, *mlk3* silencing significantly reduced the basal levels of active ERK and JNK in SKOV3 cells (Fig. 4A) [13]. To determine the relative contribution of ERK and JNK signaling to MMP expression, SKOV3 cells were treated with pharmacological inhibitors of ERK (U0126) or

JNK (SP600125), and MMP expression was assessed. As shown in Fig. 4B (right panel), treatment of SKOV3 cells with U0126 or SP600125 reduced ERK and JNK activities, respectively. RT-PCR analysis of SKOV3 MMP mRNA showed an 80–90 percent decrease and a 10–50 percent decrease in MMP-1, -2, -9 and -12 mRNA levels in cells treated with U0126 and SP600125, respectively, as compared to cells treated with DMSO alone (Fig. 4B, left panel).

Next, the effect of MLK3 overexpression on ERK, JNK, MMP-2 and MMP-9 activities was analyzed. Overexpression of FLAG-MLK3 in SKOV3 and HEY1B cells resulted in an increase in activated ERK and JNK (Fig. 5A). In addition, FLAG-MLK3 overexpression led to an increase in MMP-2 and MMP-9 gelatinase activities in SKOV3 and HEY1B cells, which was not observed in cells treated with ERK or JNK inhibitors (Fig 5B). These results indicate that both ERK and JNK signaling pathways contribute to MLK3-dependent regulation of MMP-2 and -9 activities in SKOV3 and HEY1B cells.

AP-1 activity is required for SKOV3 cell invasion and MMP gene expression

MMP-1, -2, -9, and -12 gene expression is regulated by the heterodimeric transcription factor complex, AP-1. Activation of ERK and JNK signaling pathways stimulates AP-1-mediated gene expression [14]. To test if AP-1 activity is required for SKOV3 invasion, a dominant negative mutant of c-Jun lacking the N-terminal residues 1–245 (c-Jun (Δ 1–245)), which includes the Ser63 and Ser73 JNK phosphorylation sites in the transactivation domain, was used to block AP-1 activity, and invasion through Matrigel *in vitro* was analyzed. SKOV3 cells expressing c-Jun (Δ 1–245) had a 3-fold reduction in cell invasion in comparison to cells transfected with an empty vector (Fig. 6A). Furthermore, RT-PCR analysis of MMP gene expression indicated that SKOV3 cells expressing c-Jun (Δ 1–245) had a 10–50 percent reduction in MMP-1, -9 and -12 expression, while the expression of MMP-2 was slightly elevated (Fig. 6B). These results establish a requirement for AP-1 in SKOV3 MMP-1, -9 and -12 expression and invasion.

Discussion

Diagnosis of epithelial ovarian cancer usually occurs when the cancer has already progressed to the advanced stages and treatment options are limited. Therefore, understanding the events involved in ovarian cancer development is of critical importance to the development of novel therapies to treat ovarian cancer. Here we report elevated levels of total and active MLK3 in SKOV3, HEY and HEY1B ovarian cancer cell lines in comparison to T29 and T80 immortalized ovarian epithelial cells, which indicate aberrant regulation of MLK3 protein levels and activity in ovarian cancer cells. The level of active MLK3 decreased in confluent T29 cells, but remained high in confluent SKOV3 cells, indicating that SKOV3 cells may have lost a density-dependent mechanism to down-regulate MLK3 activity.

Mounting evidence points to a role for MLK3 in neoplastic transformation, and recent reports indicate a requirement for MLK3 in the invasion of mammary epithelial tumor cells, and in the migration of gastric and A459 lung tumor cells [25–27]. However, the function of MLK3 in establishing a transformed phenotype in ovarian epithelial cells has not been elucidated. Using RNAi-mediated *mlk3* gene silencing, we determined that SKOV3 and HEY1B cell invasion is impaired in cells lacking MLK3. These results suggest a novel critical function for MLK3 in ovarian tumor cell invasion.

Aberrant Ras/MAPK signaling is frequently observed in ovarian cancer and treatment of ovarian cancer cells with B-Raf or KRAS mutations using the MEK inhibitor CI-1040, induces profound growth inhibition and apoptosis, which emphasizes the importance of

ERK1/2 signaling in tumor growth and survival of ovarian cancers [28]. Our findings indicate that MLK3 is required for ERK and JNK activation in SKOV3 cells, which is consistent with our previous observations in colon and lung fibroblasts [13]. The kinase activity of MLK3 is dispensable for its activation of ERK signaling. However, MLK3 is present in a protein complex with B-Raf and Raf-1, and may function as a scaffold protein in this complex to facilitate optimal mitogen-induced ERK activation [29]. Thus, elevated MLK3 protein levels in ovarian cancer cells may promote ERK signaling through fostering the formation MLK3-B-Raf protein complexes. MLK3 activates JNK by directly phosphorylating and activating the MAP2K, MKK4/SEK1; and high levels of active MLK3 in ovarian cancer cells could lead to constitutive JNK signaling, which is a key contributor to establishment of the malignant phenotype [30–32].

The MMPs are expressed as pro-enzymes that are activated in the extracellular space, and both the level of MMP gene expression and the level of MMP enzyme activation contribute to MMP enzyme activity. We postulated that MLK3 may regulate ovarian cancer cell invasion through modulation of MMP expression and/or activities. Consistent with this, we identified a requirement for MLK3 in MMP -1, -2, -9 and -12 gene expression and MMP-2 and -9 gelatinase activities. ERK inhibition, and to a lesser extent JNK inhibition, reduced MMP -1, -2, -9 and -12 gene expression in SKOV3 cells. However, ERK or JNK inhibition potentially reduced MLK3-dependent MMP-2 and MMP-9 gelatinase activities. The more substantial effect of JNK inhibition on MMP-2 and MMP-9 activities versus gene expression may indicate that, in addition to regulating MMP-2 and MMP-9 gene expression, JNK may also regulate the process of MMP-2 and MMP-9 enzyme activation in SKOV3 and HEY1B cells. Indeed, ERK and JNK pathways have been previously implicated as regulators of MMP-2 and MMP-9 activation [33]. For instance, ERK and JNK modulate the activity of TIMP-2, an enzyme that facilitates MMP-2 activation, and MMP-2 promotes the activation of MMP-9 [33–35].

We observed that AP-1 activity is required for the expression of MMPs -1, -9, and -12, but not MMP-2, which indicates a distinct difference in the transcriptional regulation of MMP-2 versus the other MMPs in SKOV3 cells. Additionally, ERK inhibition was more effective at reducing MMP gene expression than AP-1 inhibition. This may be due to the inhibition of additional transcription factors activated downstream of ERK that have promoter elements in the MMP -1, -2, -9 and -12 genes, such as the Ets family member, PEA3. Interestingly, PEA3 expression is also elevated in advanced metastatic ovarian cancer [36]. Some MMP genes are also transcriptionally activated by NF- κ B; however, our previous studies indicate that MLK3 does not promote NF- κ B activation, and therefore it is not likely a major contributor to MLK3-dependent activation of MMP gene expression [37].

Collectively, our results demonstrate aberrant levels of active and total MLK3 protein in ovarian cancer cells. MLK3 is required for MMP -1, -2, -9 and -12 gene expression, MMP-2 and MMP-9 activities, and cell invasion in SKOV3 and HEY1B ovarian cancer cells. Furthermore, MMP-1, -2, -9 and -12 gene expression and invasion in SKOV3 cells are dependent, at least in part, on AP-1. Hence, targeting MLK3 may be a novel approach for the development of therapies to treat ovarian cancer.

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Highlights

- Ovarian cancer cell lines have high levels of total and phosphorylated MLK3.
- MLK3 is required for MMP expression and activity in ovarian cancer cells.
- MLK3 is required for invasion of SKOV3 and HEY1B ovarian cancer cells.
- MLK3-dependent regulation of MMP-2 and MMP-9 activities requires ERK and JNK.

Fig. 1.

Total and p-MLK3 levels in human ovarian tumor cell lines. (A) Cell lysates were prepared from immortalized ovarian epithelial T29, T80 cells and ovarian cancer SKOV3, HEY1B and HEY cells and immunoblotted with p-MLK3, MLK3 and β -Actin antibodies. 3-Actin was used as an internal control for loading. The MLK3 band intensity for each cell line was first normalized to β -Actin, and expressed as a fold-increase over the band intensity for T29. (B) T29 and SKOV3 cells were seeded at a density of 5×10^5 cells per 10 cm dish, and cultured for 24, 48, and 72 hours. Cell lysates were prepared at each time point and samples were immunoblotted with p-MLK3, MLK3 and β -Actin antibodies.

Fig. 2.

Impaired cell invasion in MLK3 knockdown SKOV3 and HEY1B cells. SKOV3 (A) and HEY1B (B) cells were transfected with nonspecific (NS) or MLK3 siRNA oligo 1 or 2. Cell invasion was analyzed using Transwell chambers containing Matrigel (left panel). Cells that traversed the membrane were stained and counted. Values are the means $\pm$ SD, * $p < 0.01$ relative to NS control. Cell extracts were prepared from a portion of the transfected cells and subjected to immunoblotting with MLK3 and β -Actin antibodies (right panel).

Fig. 3.

Reduced MMP expression and activity in MLK3 knockdown SKOV3 and HEY1B cells. (A) Total RNA was isolated from T29 and SKOV3 cells and RT-PCR was performed with human GAPDH, MMP-1, -2, -9 and -12 primers. MMP mRNA levels were normalized to GAPDH mRNA levels in each sample, and SKOV3 MMP mRNA levels were expressed as a fold-increase over T29. Values represent the means $\pm$ SD, * p < 0.01 relative to T29. (B) Total RNA was isolated from SKOV3 (upper panel) and HEY1B (lower panel) cells that were transfected with nonspecific (NS) or MLK3 siRNA, and RT-PCR was performed with human GAPDH, MMP-1, -2, -9 and -12 primers. MMP mRNA levels were normalized to GAPDH mRNA levels in each sample and expressed as a percent of the nonspecific siRNA control. Values represent the means $\pm$ SD, * p < 0.05 relative to the NS siRNA control. (C) SKOV3 and HEY1B cells were transfected with nonspecific (NS) or MLK3 siRNA oligo 1 or 2. Cell culture medium was collected and subjected to gelatin zymography analysis (upper panel) and cell extracts were immunoblotted with MLK3 and β -Actin antibodies (lower panel).

Fig. 4.

Effect of ERK and JNK inhibitors on MMP expression and activity in SKOV3 cells. (A) SKOV3 cells were untransfected or transfected with nonspecific (NS) or MLK3 siRNA, and cell extracts were immunoblotted with MLK3, p-ERK, p-JNK, ERK, JNK and β -Actin antibodies. (B) SKOV3 cells were treated with DMSO, U0126, or SP600125. Total RNA was isolated and RT-PCR was performed with human GAPDH, MMP-1, -2, -9 and -12 primers (left panel). MMP expression levels were normalized to GAPDH expression levels in each sample and expressed as a percent of the DMSO control. Values are the means $\pm$ SD, * $p < 0.01$ relative to the DMSO control. Cell extracts were prepared and subjected to immunoblotting with p-ERK, ERK, p-JNK and JNK and β -Actin antibodies (right panel).

Fig. 5. ERK and JNK are required for MLK3-dependent activation of MMP-2 and MMP-9 gene expression. (A) SKOV3 and HEY1B cells were transfected with pCMV5 vector or FLAG-MLK3, and cell extracts were immunoblotted with MLK3, p-ERK, p-JNK, ERK, JNK antibodies. (B) SKOV3 and HEY1B cells were transfected with pCMV5 vector or FLAG-MLK3. Cells expressing FLAG-MLK3 were treated with U0126 or SP600125. Cell culture medium was subjected to gelatin zymography analysis (upper two panels). Cell extracts were immunoblotted with anti-FLAG antibody (lower panel).

Fig. 6.

AP-1 inhibition reduces invasion and MMP expression in SKOV3 cells. (A) SKOV3 cells were transfected with empty vector or c-Jun (Δ 1-245). Cell invasion was analyzed using Transwell chambers containing Matrigel (left panel). Cells that traversed the membrane were stained and counted. Values are the means $\pm$ SD, * p < 0.01 relative to the empty vector control. Cell extracts were immunoblotted with anti-c-Jun antibody to determine c-Jun (Δ 1-245) expression (right panel). (B) Total RNA was isolated from SKOV3 cells that were treated with empty vector or c-Jun (Δ 1-245) as described in A, and RT-PCR was performed with human GAPDH, MMP-1, -2, -9 and -12 primers. MMP expression levels were normalized to GAPDH expression levels in each sample. Values are means $\pm$ SD, * p < 0.01 relative to the empty vector control.