The p38 Kinases MKK4 and MKK6 Suppress Metastatic Colonization in Human Ovarian Carcinoma

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Abstract

Despite considerable efforts to improve early detection of ovarian cancer, the majority of women at time of diagnosis will have metastatic disease. Understanding and targeting the molecular underpinnings of metastasis continues to be the principal challenge in the clinical management of ovarian cancer. Whereas the multistep process of metastasis development has been well established in both clinical and experimental models, the molecular factors and signaling pathways involved in successful colonization of a secondary site by disseminated cancer cells are not well defined. We have previously identified mitogen-activated protein kinase (MAPK) kinase 4/c-Jun NH2-terminal kinase (JNK)-activating kinase (MKK4/JNK1/SEK1, hereafter referred to as MKK4) as a metastasis suppressor protein in ovarian carcinoma. In this study, we elucidate key mechanisms of MKK4-mediated metastasis suppression. Through the use of a kinase-inactive mutant, we show that MKK4 kinase activity is essential for metastasis suppression and prolongation of animal survival. Because MKK4 can activate either of two MAPKs, p38 or JNK, we expressed MKK6 or MKK7, specific activators of these MAPKs, respectively, to delineate which MAPK signaling module was involved in MKK4-mediated metastasis suppression. We observed that MKK6 expression suppressed metastatic colonization whereas MKK7 had no effect. Our finding that MKK4 and MKK6 both suppress metastasis points to the p38 pathway as an important regulatory pathway for metastatic colonization in ovarian cancer. (Cancer Res 2006; 66(4): 2264-70)

Introduction

An estimated 22,220 women will be diagnosed with ovarian cancer in the United States in 2005 (1). Ovarian cancer has the highest mortality rate of all the gynecologic malignancies, with nearly 16,210 women expected to succumb to this disease this year, largely as a result of metastatic, chemoresistant disease (1). The identification of hereditary forms of ovarian cancer and their corresponding genetic susceptibility genes, BRCA1, BRCA2, and the DNA mismatch repair genes, have highlighted the need for genetic testing and the benefit of early prophylactic salpingo-oophorectomy. However, for the majority of patients in which ovarian cancer is not hereditary (90%) and not diagnosed early (70%), metastatic disease remains the primary clinical problem that must be tackled and better understood if we are to impact on the lethality of this disease (1).

In clinical and experimental models, metastasis formation requires completion of a series of distinct events, commonly referred to as the metastatic cascade (2). These steps include escape of malignant cells from the primary tumor, dissemination and lodging at a discontinuous secondary site(s), and finally, the survival and growth into clinically detectable overt metastases, a process termed metastatic colonization. Whereas it is becoming increasingly apparent that disseminated cancer cell behavior, before overt growth, can be modulated by context and microenvironment (3, 4), the molecular factors and signaling pathways that specifically regulate metastatic colonization of disseminated cancer cells are not well defined.

Identification of proteins and signaling pathways necessary for regulation of metastasis formation (5–8) in individual disease processes will significantly augment our understanding of the metastatic process and, more importantly, will help us to identify unique targets for therapy. One such protein is mitogen-activated protein kinase (MAPK) kinase 4/c-Jun NH2-terminal kinase (JNK)-activating kinase (MKK4/JNK1/SEK1, hereafter referred to as MKK4), which was originally identified as a metastasis suppressor protein in prostate cancer using the well-characterized Dunning AT6.1 spontaneous metastasis model (9–11). We have since identified MKK4 as a metastasis suppressor protein in ovarian carcinoma (12). We have shown that clinical specimens of normal ovarian surface epithelium express high levels of MKK4 whereas ovarian cancer omental metastases express significantly lower levels of MKK4. In addition, using an i.p. model of metastatic colonization (13–17), we have shown that ectopic expression of MKK4 in SKOV3ip.1, a highly metastatic human ovarian cancer cell line which lacks significant endogenous levels of MKK4, suppresses metastatic colonization by 90% and prolongs animal survival by 70% (12).

MKK4 is known to play a central role in the stress-activated protein kinase (SAPK) signaling pathway (Fig. 1), which has been well characterized biochemically. The SAPK pathway, which consists of the p38 and JNK MAPK signaling modules, relays signals to the nucleus in response to environmental stresses and extracellular stimuli. MKK4 is capable of signaling through either the p38 or JNK pathway, which ultimately results in activation of transcription factors such as c-jun and activating transcription factor 1. Activation of these transcription factors regulates such cellular processes as proliferation, differentiation, and apoptosis.

Based on this biochemical knowledge of the SAPK pathway, we hypothesized that modulation of the appropriate MAPK signaling module (p38 or JNK) would suppress metastasis in our ovarian
The human ovarian carcinoma cell line SKOV3ip.1 (13), a generous gift from Dr. Gordon Mills (M.D. Anderson Cancer Center, Houston, TX), was cultured in DMEM containing l-glutamine (304 mg/L) and glucose (4.5 g/L; Mediatech, Herndon, VA), supplemented with 5% FCS (Atlanta Biologicals, Norcross, GA), 1% penicillin (100 units/mL)/streptomycin (100 µg/mL) streptomycin (100 µg/mL) mixture, 10 mmol/L sodium pyruvate, 1% nonessential amino acids, and 2 × MEM vitamin solution (all from Mediatech). For selection and establishment of stable SKOV3ip.1 clones, the above medium was additionally supplemented with 500 µg/mL (active concentration) of G418 sulfate (Mediatech). RetroPack PT67 Packaging cells were kindly provided by Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA). Stable SKOV3ip.1 clonal cell lines were isolated as previously described (11, 12). Briefly, hemagglutinin (HA)-tagged MKK4, MKK4(KR), MKK6, or MKK7 cDNAs was subcloned into the retroviral vector pLNCX2 (Clontech). Infectious, replication-incompetent retroviruses was produced by transient transfection of retroviral constructs into RetroPack PT67 cells using an Effectene transfection reagent per instructions of the manufacturer (Qiagen, Santa Clarita, CA). Viral medium was collected 48 hours later and passed through a 0.2-µm filter. SKOV3ip.1 cells (2 × 10⁵) were plated 24 hours before infection and incubated for 24 hours with infected virus containing 8 mg/mL Polybrene (SpecialtyMedia, Phillipsburg, NJ). Cells were subsequently split 1:10 in growth medium containing 500 µg/mL (active concentration) G418 for selection and establishment of clonal cell lines. Transgene expression was confirmed in clonal cell lines by immunoblotting for expression of the HA epitope–tagged protein.

**Materials and Methods**

**Cell culture and cell lines.** The human ovarian carcinoma cell line SKOV3ip.1 (13), a generous gift from Dr. Gordon Mills (M.D. Anderson Cancer Center, Houston, TX), was cultured in DMEM containing l-glutamine (304 mg/L) and glucose (4.5 g/L; Mediatech, Herndon, VA), supplemented with 5% FCS (Atlanta Biologicals, Norcross, GA), 1% penicillin (100 units/mL)/streptomycin (100 µg/mL) streptomycin (100 µg/mL) mixture, 10 mmol/L sodium pyruvate, 1% nonessential amino acids, and 2 × MEM vitamin solution (all from Mediatech). For selection and establishment of stable SKOV3ip.1 clones, the above medium was additionally supplemented with 500 µg/mL (active concentration) of G418 sulfate (Mediatech). RetroPack PT67 Packaging cells were grown to 80% confluence (3.5 × 10⁵ cells/cm²), washed twice in ice-cold PBS, and protein lysates prepared using lysis buffer [10 mmol/L Tris (pH 7.5), 1 mmol/L β-glycerophosphate, 2 mmol/L DTT, 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5 mmol/L NaF, 2 mmol/L NaVO₃, 0.1% NP40, 10 µmol/L phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 (w/v), 70 units/mL aprotinin, and one Complete Protease Inhibitor Cocktail tablet (Roche, Basel, Switzerland)]. Cells were collected, passed through a 27-gauge needle, placed on ice for 10 minutes, and subsequently centrifuged at 14,000 rpm (19,000 × g) for 10 minutes at 4°C. Overt metastases were excised rapidly, washed in ice-cold PBS, minced, and homogenized manually using a 1-mL tissue grinder (Fisher Scientific, Hanover Park, IL) with chilled cell lysis buffer. The homogenate was centrifuged at 14,000 rpm (19,000 × g) for 10 minutes at 4°C and the supernatant fraction was transferred to a new tube via a 27-gauge needle. Protein concentrations were determined using a bicinchoninic acid assay reduction kit (Pierce, Rockford, IL). Thirty micrograms of total protein from each cell lysate were resolved by SDS-PAGE (10% acrylamide) and transferred to a Hybrid enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked at 4°C overnight in TBS-Tween 20 plus 5% nonfat dry milk (w/v).

Primary antibodies were diluted in blocking solution and incubated with the membrane for 1 hour. The primary antibodies and dilutions used are as follows: HA.11 (1:1,000; Covance, Princeton, NJ), MKK4 (1:1,000; Cell Signaling Technologies, Danvers, MA), MKK6 (1:2,000; Lab Vision, Fremont, CA), MKK7 (1:1,000; Zymed Laboratories, Carlsbad, CA), JNK (1:1,000; Cell Signaling Technologies), and p38 (1:1,000; Cell Signaling Technologies). Following a 10-minute wash in blocking solution and six washes for 5 minutes each in TBS-Tween 20, the membrane was incubated with a horseradish peroxidase (HRP)–conjugated immunoglobulin G secondary antibody for 1 hour per instructions of the manufacturer. After an identical series of washes, the HRP-conjugated secondary antibody was detected using the SuperSignal West Fermo Maximum Sensitivity Chemiluminescence Substrate (Pierce). Probed membranes were stripped using Restore Western blot Stripping Buffer (Pierce), washed, and blocked overnight before reprobing. As a loading control, membranes were probed for actin (1:10,000; Calbiochem, San Diego, CA) followed by incubation with a goat anti-mouse immunoglobulin M-peroxidase conjugated secondary antibody (1:10,000; Calbiochem).

**Immunoprecipitation.** For each immunoprecipitation, 1.0 mg of total protein from each cell line was brought to a final volume of 600 µL with ice-cold immunoprecipitation buffer [20 mmol/L Tris (pH 7.6), 2 mmol/L/
In vivo kinase assay. Following immunoprecipitation, beads were resuspended in chilled kinase reaction buffer [10 mmol/L β-glycerophosphate, 3 mmol/L DTT, 30 mmol/L HEPES, 30 mmol/L MgCl₂, 100 mmol/L NaVO₄, 0.5 mmol/L ATP]. To each sample, 0.1 μg of inactive purified JNK or p38 substrate (Upstate, Charlottesville, VA) and 1 μCi of [α-32P]ATP (Amersham Biosciences) were added. Reactions were incubated for 1 hour at 30°C with gentle mixing every 15 minutes. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and radioactively labeled substrate was detected by autoradiography. As a loading control, membranes were immunoblotted for either JNK or p38 as described above.

In vitro metastasis assay. All stable SKOV3ip.1 clones were cultured as described above and were not grown beyond 80% confluence before preparation for mouse injection. Cells (1 x 10⁶; 500 μL of a 2 x 10⁶ cells/mL suspension) were injected through a 25-gauge needle i.p. into 4- to 6-week-old female severe combined immunodeficient mice as previously described (12). At the experimental end point (either 20 or 30 days postinjection), mice were euthanized by CO₂ overdose and the number of overt metastases was counted by two independent investigators (J.A.H. and S.D.Y.). Overt metastases are defined as visible implants counted in vivo.

Statistical analyses. The mean number of metastases 30 days postinjection in mice injected with SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4, or SKOV3ip.1-HA-MKK4(KR) was compared using a mixed-effect model ANOVA with heterogeneous variances in which each clone was treated as random effect because observations within each clone were correlated. The Tukey multiple comparison procedure was used to adjust for multiple pairwise testing. SEs of variable estimates were adjusted for small sample size using a method detailed by Kenward and Roger (21) and Satterthwaite-type degrees of freedom were computed based on this adjustment. Similarly, the mean number of metastases 20 days postinjection in mice injected with SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK7, or SKOV3ip.1-HA-MKK6 clones was compared using a mixed model ANOVA based on this hypothesis, we used MKK4(KR), which contains a mutation of a crucial ATP-binding residue (K118R) that renders MKK4 catalytically inactive. Multiple independent SKOV3ip.1-pLNCX2 clones were derived and characterized. As shown in Fig. 2A, representative SKOV3ip.1-HA-MKK4(KR) clones all showed expression of a HA-tagged protein. SKOV3ip.1-HA-MKK4 and SKOV3ip.1-pLNCX2 clones served as positive and negative controls, respectively. All clones had similar endogenous levels of p38 and JNK.

To confirm that ectopically expressed HA-MKK4 and HA-MKK4(KR) proteins were biochemically functional, serum-starved cells were treated with anisomycin, a known activator of the SAPK/JNK and p38 pathways, and protein lysates were subjected to HA immunoprecipitation. Immunoprecipitates were either immunoblotted for MKK4 to validate the identity of the HA-tagged protein or subjected to a kinase assay (Fig. 2B). HA-MKK4 that was activated in vitro phosphorylated purified both JNK (His-JNK) and p38 substrates whereas the HA-MKK4(KR) from anisomycin-treated cells did not. These results confirm that ectopically expressed HA-MKK4 can be activated and phosphorylate both downstream MAPKs whereas ectopically expressed HA-MKK4(KR) is enzymatically inactive. SKOV3ip.1-HA-MKK4(KR) clones were then tested for in vivo metastasis formation in vivo using our standard experimental metastasis assay (12). SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4 clonal cell lines served as positive and negative controls, respectively. Briefly, 1 x 10⁶ cells were injected i.p. into female

**Results**

M KK4 mediates metastasis suppression and its effect on animal survival is kinase dependent. We have previously reported that ectopic expression of HA-MKK4 in SKOV3ip.1 cells suppresses metastasis by nearly 90% in vivo (12). Based on the published biochemical functions of MKK4, we predicted metastasis suppression would be dependent on activation of the downstream targets of MKK4, JNK, and/or p38 MAPKs (Fig. 1; ref. 23). To test this hypothesis, we used MKK4(KR), which contains a mutation of a crucial ATP-binding residue (K118R) that renders MKK4 catalytically inactive. Multiple independent SKOV3ip.1-pLNCX2 (vector control), SKOV3ip.1-HA-MKK4, and SKOV3ip.1-HA-MKK4(KR) clonal cell lines were derived and characterized. As shown in Fig. 2A, representative SKOV3ip.1-HA-MKK4(KR) clones all showed expression of a HA-tagged protein. SKOV3ip.1-HA-MKK4 and SKOV3ip.1-pLNCX2 clones served as positive and negative controls, respectively. All clones had similar endogenous levels of p38 and JNK.

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Severe combined immunodeficient mice with the number of metastases formed in mice (\( A \), MKK4 requires its kinase activity to suppress SKOV3ip.1 metastasis.

Figure 3. MKK4 requires its kinase activity to suppress SKOV3ip.1 metastasis. A, number of metastases formed in mice (\( n = 5 \) per clone) injected with SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4, or SKOV3ip.1-HA-MKK4(KR) clones at the experimental end point. Pairwise comparisons were determined using a mixed-effect model ANOVA with heterogeneous variance: SKOV3ip.1-pLNCX2 versus SKOV3ip.1-HA-MKK4, \( P = 0.026 \); SKOV3ip.1-HA-MKK4(KR) versus SKOV3ip.1-HA-MKK4, \( P = 0.017 \); SKOV3ip.1-pLNCX2 versus SKOV3ip.1-HA-MKK4(KR), \( P = 0.85 \). B, metastases from mice injected with SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4, or SKOV3ip.1-HA-MKK4(KR) clones were collected. Protein lysates were prepared and immunoblotted for HA. Actin served as a loading control.

Figure 4. Kaplan-Meier curve summarizing survival of mice injected with SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4, or SKOV3ip.1-HA-MKK4(KR) clones. Female severe combined immunodeficient mice (\( n = 15 \) per clone) were injected i.p. with three SKOV3ip.1-pLNCX2, four SKOV3ip.1-HA-MKK4, or three SKOV3ip.1-HA-MKK4(KR) clones and followed until moribund. Mean survival time of SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4(KR)–injected mice was 38 and 30 days, respectively (\( P = 0.34 \)); SKOV3ip.1-HA-MKK4–injected mice had an increased average survival time of 63 days when compared with both SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4(KR)–injected mice (\( P < 0.001 \)).

We have previously reported that ectopic expression of HA-MKK4 dramatically reduced the number of metastases when compared with pLNCX2 controls (3.0 ± 2.0 versus 23.2 ± 17.3, \( P = 0.026 \)). Mice injected with SKOV3ip.1-HA-MKK4(KR) clones had significantly more metastases when compared with mice injected with SKOV3ip.1-HA-MKK4 (19.5 ± 13.7 versus 3.0 ± 2.0, \( P = 0.017 \)) whereas there was no significant difference in the number of metastases in SKOV3ip.1-pLNCX2 compared with SKOV3ip.1-HA-MKK4(KR) (23.2 ± 17.3 versus 19.5 ± 13.7, \( P = 0.83 \)). Lysates of SKOV3ip.1-HA-MKK4 and SKOV3ip.1-HA-MKK4(KR) metastases obtained from the in vivo experiment retained expression of the HA-tagged protein (Fig. 3B), indicating that the observed number of metastases was not the result of a loss of ectopic protein expression.

We have previously reported that ectopic expression of HA-MKK4 dramatically prolongs animal survival by >70% (12). As a biological validation of the HA-MKK4(KR) findings shown above, we followed additional mice (\( n = 15 \) mice per clone) injected with multiple independent SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4(KR), or SKOV3ip.1-HA-MKK4 clones until moribund to determine the effect of HA-MKK4(KR) or HA-MKK4 expression on survival (Fig. 4). As previously observed, overall mean survival time was significantly prolonged from 38 days in mice injected with SKOV3ip.1-pLNCX2 clones to 63 days in mice injected with SKOV3ip.1-HA-MKK4(KR) clones (\( P < 0.001 \)). Mice injected with SKOV3ip.1-HA-MKK4(KR) clones had a mean survival of 30 days that was similar to mice injected with SKOV3ip.1-pLNCX2 clones (mean survival 38 days, \( P = 0.34 \)) and significantly different from mice injected with SKOV3ip.1-HA-MKK4 clones (mean survival 63 days, \( P < 0.001 \)). Taken together, these studies indicate that the kinase activity of MKK4 is essential for regulating metastasis suppression of SKOV3ip.1 cells and prolonging survival time in vivo.

Ectopic expression of MKK7 does not affect metastatic colonization in vivo. Because MKK4 can activate two MAPKs, p38 and JNK (Fig. 1), MKK4-mediated metastasis suppression may be the result of activating the p38 pathway, the JNK pathway, or both (20). To determine which signaling module MKK4 activates, we first tested the ability of MKK7, a specific activator of JNK, to suppress metastasis (24, 25). Multiple independent SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK7 clones were generated and characterized (Fig. 5A). All clonal cell lines had comparable endogenous levels of p38 and JNK as determined by immunoblotting, with actin serving as the loading control. Anisomycin-treated SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK7 cell lysates were HA immunoprecipitated and either immunoblotted for MKK7 to validate the identity of the HA-tagged protein or subjected to a kinase assay (Fig. 5B). Activated HA-MKK7 showed specific phosphorylation of a purified JNK substrate but not of a p38 substrate, consistent with its known biochemical function.

In our standard in vivo assay, no significant differences in the number of metastases were observed in mice injected with SKOV3ip.1-pLNCX2 clones (24.0 ± 8.6) as compared with SKOV3ip.1-HA-MKK7 clones (19.6 ± 8.6; \( n = 5 \) mice per clone, \( P = 0.43 \)); Fig. 5C). Additionally, SKOV3ip.1-HA-MKK7 metastases retained expression of the HA-tagged protein (data not shown). Thus, stable expression of HA-MKK7 in SKOV3ip.1 cells does not suppress metastasis formation, suggesting that activation of the JNK module is not sufficient for suppression of metastatic colonization in the SKOV3ip.1 ovarian carcinoma model.

Ectopic MKK6 expression suppresses metastatic colonization in vivo. In contrast to MKK7, MKK6 is a specific activator of
independent of the JNK signaling pathway (26). Whereas MKK3 can also activate p38, we chose MKK6 over MKK3 because of its ability to activate all known p38 isoforms (27). Multiple SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK6 clones were derived and characterized. All clonal cell lines had comparable endogenous levels of p38 and JNK as determined by immunoblotting, with actin serving as the loading control (Fig. 6A). Anisomycin-treated SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK6 cell lysates were HA immunoprecipitated and either immunoblotted for MKK6 to validate the identity of the HA-tagged protein or subjected to a kinase assay (Fig. 6B). In vitro stimulation of SKOV3ip.1-HA-MKK6 clones resulted in activated HA-MKK6 that specifically phosphorylated a purified p38 substrate but not a JNK substrate.

We observed a significant reduction in metastases in mice injected with SKOV3ip.1-HA-MKK6 clones (6.7 ± 2.6) as compared with SKOV3ip.1-pLNCX2 clones (22.5 ± 9.2; n = 15 mice per clone, $P = 0.0082$; Fig. 6C). Additionally, SKOV3ip.1-HA-MKK6 metastases retained expression of the HA-tagged protein (data not shown).

Figure 5. Ectopic expression of HA-MKK7, a specific JNK kinase, does not affect SKOV3ip.1 metastasis. A, lysates of three SKOV3ip.1-pLNCX2 clones and five SKOV3ip.1-HA-MKK7 clones were immunoblotted for the expression of a HA-tagged protein, p38, and JNK. Actin was used as a loading control. B, in vitro kinase activity of HA-MKK7. Protein lysates from anisomycin-treated clones were subjected to HA immunoprecipitation. Immunoprecipitates were either immunoblotted for MKK7 or subjected to a kinase assay using purified p38 or JNK. As a positive control (PC), a stimulated SKOV3ip.1-HA-MKK4 clone was used. Immunoblotting for total p38 and JNK served as a loading control. C, number of metastases formed in mice ($n = 5$ per clone) injected with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK7 clones at the experimental end point. A pairwise comparison was determined using a mixed-effect model ANOVA: SKOV3ip.1-pLNCX2 versus SKOV3ip.1-HA-MKK7, $P = 0.43$.

Figure 6. Ectopic expression of HA-MKK6, a specific p38 kinase, suppresses SKOV3ip.1 metastasis. A, lysates of three SKOV3ip.1-pLNCX2 clones and three SKOV3ip.1-HA-MKK6 clones were immunoblotted for the expression of a HA-tagged protein, p38, and JNK. Actin was used as a loading control. B, in vitro kinase activity of HA-MKK6. Protein lysates from anisomycin-treated clones were subjected to HA immunoprecipitation. Immunoprecipitates were either immunoblotted for MKK6 or subjected to a kinase assay using purified p38 or JNK. As a positive control, a stimulated SKOV3ip.1-HA-MKK4 clone was used. Immunoblotting for total p38 and JNK served as a loading control. C, number of metastases formed in mice ($n = 15$ per clone) injected with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK6 clones at the experimental end point. A pairwise comparison was determined using a mixed-effect model ANOVA: SKOV3ip.1-pLNCX2 versus SKOV3ip.1-HA-MKK6, $P = 0.0082$. 

p38, independent of the JNK signaling pathway (26). Whereas MKK3 can also activate p38, we chose MKK6 over MKK3 because of its ability to activate all known p38 isoforms (27).
These observations identify a new role for MKK6 as a metastasis suppressor protein in our SKOV3ip.1 ovarian cancer model and implicate the p38 signaling module in metastasis suppression.

Discussion

Ovarian cancer has the highest mortality rate of all cancers of the female reproductive system due, in part, to lack of early symptoms and effective ovarian cancer screening tests to detect organ-confined disease. Thus, in ~70% of patients, ovarian cancer is diagnosed when the cancer has metastasized to the peritoneal cavity. Five-year survival for patients diagnosed with stage III and IV disease remains poor at 13% to 29% (28). This statistic emphasizes the critical need to better understand the mechanisms that allow for survival of disseminated, neoplastic cells and how to regulate these processes. While it has long been known that the ability of disseminated tumor cells to survive and proliferate in a specific secondary microenvironment must be controlled by molecular and/or signaling events, the genes and signaling pathways involved in regulating metastatic colonization have remained largely undefined.

The extensive characterization of the SAPK pathway and its regulatory components allowed us to conduct a biochemically rigorous evaluation of the effect of MKK4 on metastasis regulation using the SKOV3ip.1 experimental metastasis model. In this report, we observe that MKK4 suppresses metastasis in a kinase-dependent manner and not through a novel interaction. Because MKK4 can activate both the p38 and JNK MAPKs, MKK4-mediated metastasis suppression may be the result of activating the p38 pathway, the JNK pathway, or both. Activation of either p38 or JNK has a well-established role in the processes of inflammation, apoptosis, and tumorigenesis in a tissue-specific manner (26, 27). Using ectopic expression of either MKK6 or MKK7, it was possible to ascertain which MAPK mediated MKK4-induced metastasis suppression. We observed that whereas expression of MKK7 had no effect on metastasis formation, expression of MKK6 significantly reduced the number of in vivo metastases.

Our observations reported here contrast with recent findings by Vander Griend et al. (11) using the Dunning AT6.1 rat prostate cancer cell line. In this spontaneous metastasis model, MKK4 suppressed lung metastasis in a kinase-dependent manner as well. However, ectopic expression of MKK7 suppressed metastatic colonization whereas MKK6 expression had no effect on metastasis. It would seem that in the prostate cancer model, the JNK signaling module plays a vital role in suppression of metastatic colonization whereas in this ovarian cancer model, the p38 pathway plays the primary role in mediating metastasis suppression. One possible reason for this discrepancy between model systems may be the different physical stresses and environmental stimuli cells encounter in hematogenous spread to the lung in the AT6.1 model versus i.p. dissemination in the SKOV3ip.1 model. Regardless, the difference in these results highlights the context dependency of MAPK pathway activation and the fact that findings about in vivo MAPK regulation cannot be extrapolated from one organ system to another.

MKK6 has recently been implicated in regulating tumorigenesis. However, our report is the first to describe a role for MKK6 in metastatic colonization. Brancho et al. (29) showed a dramatic increase in tumor burden when SV40 large T antigen immortalized Mkk3−/− Mkk6−/− fibroblasts were injected s.c. in athymic nude mice. Furthermore, Timofeev et al. (30) reported that a subtle activation of p38 MAPK is sufficient to suppress tumorigenesis as measured by the ability to form tumors when MKK6-inducible cells were explanted into nude mice. Of note, E-cadherin, a transmembrane glycoprotein responsible for Ca2+-dependent cell-cell adhesion that has been identified as a metastasis suppressor in breast, colon, and pancreatic cancer (6), when expressed in immortalized ovarian surface epithelial cells, induces MKK6 expression and increases p38 expression (31). These findings potentially link two metastasis suppressor proteins and the role of the p38 signaling pathway in the modulation of neoplastic growth.

Our findings that MKK4 and MKK6 suppress SKOV3ip.1 metastasis implicate the p38 pathway as a key regulatory pathway for metastatic colonization. Studies by Aguirre-Ghiso et al. (32–34) support such a role for p38. Using the human epidermoid carcinoma HEp3 cell line, the authors isolated two populations that were either dormant (D-HEp3) or tumorigenic (T-HEp3) when inoculated in a chorioallantoic membrane assay. They then developed a novel reporter system in which extracellular signal-regulated kinase (ERK) or p38 activation regulates green fluorescent protein expression through the activation of a GAL4-Elk or GAL4-CHOP transactivator, respectively. Using this system, they observed that metastatic cells undergoing dormancy exhibited elevated p38 activity whereas those that went on to form overt metastases had elevated ERK activity. Thus, a model was proposed where the balance between p38 and ERK activation regulates whether the cell will be dormant or metastatic. Our in vivo findings support the hypothesis that p38 activation can result in suppression of metastatic colonization. Additional studies are under way to investigate the role of p38 and ERK activation in the proliferation of disseminated cells in our model system.

Determining the biochemical and biological mechanisms responsible for regulating metastatic colonization has direct clinical implications. Both the platinum and taxane chemotherapy agents, frequently used in treatment of ovarian cancer, have been shown to activate the JNK and p38 MAPK pathways in ovarian cancer cell lines (35–38). Restoration of key upstream kinase activators could be used to therapeutic advantage. For instance, if p38 activation regulates dormancy of disseminated cells, modulation of this pathway may enhance the effect of adjuvant or consolidation chemotherapy after surgical debulking, when tumor volume is lowest. Future studies are needed to determine which step(s) of metastatic colonization is impaired by expression of MKK4 and/or MKK6 if we are to understand what type of targeted therapies to use and when to administer them. It is clear that further study of metastatic colonization of ovarian cancer using well-characterized models of metastasis will increase our understanding of the biochemical mechanisms specifically regulating metastatic growth, as well as provide novel targets for antimetastatic therapies.

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