Mitogen-activated Protein Kinase Kinase 4 (M KK4) Acts as a Metastasis Suppressor Gene in Human Ovarian Carcinoma

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INTRODUCTION

Ovarian cancer causes more deaths than any other gynecologic malignancy despite improved therapeutic regimens, such as aggressive surgical cytoreduction and the combination of paclitaxel and platinum-based chemotherapy. In the year 2002, it is estimated there will be 23,300 new cases of ovarian cancer and 13,900 deaths (1). The significant mortality associated with ovarian cancer is the result of advanced stage disease at the time of diagnosis because of lack of early, recognizable symptoms, and the development of peritoneal and extraperitoneal metastases that become chemotherapy-resistant.

The acquisition of metastatic ability requires the coordinated expression of metastasis-promoting genes and the down-regulation of metastasis-suppressing genes. In recent years, a number of metastasis-suppressor genes have been identified in various organ systems (2–8). Metastasis-suppressor genes have been shown to suppress the growth of metastases without affecting the growth of the primary tumor (9). These genes are distinct from tumor suppressor genes, which suppress primary tumor growth. Particularly important in this process is the concept of metastatic colonization whereby disseminated cancer cells lodge and grow at a secondary site. Metastatic colonization requires disseminated cells to initiate context-dependent signaling cascades that allow them to survive, enter the cell cycle, and divide to become microscopic metastases, and subsequently, overt metastases (10). Growth control mechanisms at these secondary sites of disease govern whether cells will form clinically significant metastases or not (10, 11). Although a plethora of data exists in other organ systems, little specific information is available on metastasis suppression gene expression and function in ovarian cancer (12–16). Such information would be of considerable value in identifying the molecular alterations, which regulate the dissemination of ovarian cancer cells from the primary tumor and subsequent colonization of extra-ovarian sites.

We identified recently the MKK4/JNK1/SEK1 (hereafter referred to as MKK4), which is located on chromosome 17p11.2, as a metastasis suppressor gene in prostate cancer. MKK4 is a member of the SAPK signaling pathway that ultimately controls transcription factors such as c-jun (17). In this way, MKK4 plays a role in such processes as cellular proliferation, differentiation, and apoptosis. In highly metastatic AT6.1 prostate cancer cells, ectopic expression of MKK4 reduces overt lung metastases by ~80% without affecting primary tumor growth (18). An association between decreased MKK4 expression and increasing Gleason grade, a predictor of metastatic potential, supports a role for MKK4 down-regulation in clinical prostate disease (19). Although the metastatic patterns of spread in prostate and ovarian cancer are clinically different, we speculated that fundamental mechanisms governing metastatic colonization would be conserved. Of interest is the observation that loss of 17p is a frequent event in ovarian cancers. Therefore, we hypothesized that MKK4, or members of its signaling pathway, would be down-regulated in clinical metastases and that ectopic expression of MKK4 would suppress metastatic colonization in a model of ovarian cancer.

To begin to test this hypothesis, the expression of MKK4 in normal and metastatic ovarian tissues was examined. In the simplest scenario, if MKK4 inactivation played a role in the development of metastases, then the level of MKK4 expression in ovarian cancer metastases would be decreased as compared with normal ovarian epithelial cells. To test the ability of MKK4 to function as a metastasis suppressor in vivo, MKK4 was ectopically expressed in the highly metastatic...
SKOV3ip.1 human ovarian cancer cell line to determine the effect on metastatic colonization.

**MATERIALS AND METHODS**

**Evaluation of MKK4 Staining in Clinical Samples**

Normal and cancerous ovary samples were identified from the Department of Pathology database at our institution. In accordance with a protocol approved by the Institutional Review Board at the University of Chicago, 34 normal ovary specimens from patients undergoing surgery for benign conditions that specifically contained normal surface epithelium or epithelial inclusion cysts containing normal epithelial cells were identified. Metastatic tumor specimens from 34 patients undergoing primary cytoreductive surgery for ovarian cancer were similarly identified.

Specimen preparation and immunohistochemical studies were conducted as described previously (19). In brief, slides were prepared for immunostaining and incubated overnight at 4°C using rabbit anti-MKK4/MEK4 (5 μg/ml; Santa Cruz Biotechnology, Inc., or isotype control (5 μg/ml; whole rabbit IgG) prepared in protein blocking solution (5% horse serum + 1% goat serum in PBS). Slides were subsequently incubated for 3 h at 37°C with goat anti-rabbit biotin-conjugated IgG (3 μg/ml; Santa Cruz Biotechnology, Inc.) followed by incubation with ABC Elite Vectastain kit (Vector Labs). Immunostaining was detected using a 3.3′-diaminobenzidine peroxidase substrate kit (Vector Laboratories), and slides were counterstained with Fast Green (Fisher Scientific). Controls for sensitivity and specificity of the immunostaining included an epitope control (peptide competition using 5-fold excess of peptide; Santa Cruz Biotechnologies, Inc.), secondary antibody alone, and avidin/biotin alone (described in detail in Ref. 19).

A semiquantitative evaluation scale was developed based on intensity of MKK4 staining. A numerical scale of 0, 1, 2, 3 or 4 was assigned with 4 designated for those epithelial areas that did not stain for MKK4 and 3 assigned to epithelial cells that stained with greatest intensity for MKK4. Samples were interpreted and scored by two independent reviewers, a pathologist with dedicated expertise in gynecologic pathology and a gynecologic oncologist (A. M. and S. D. Y., respectively).

**Human Ovarian Cancer Cell Lines and Control Cell Lines**

Caov-3 was obtained from American Type Culture Collection and grown in DMEM supplemented with 8% FCS, 1% penicillin, and 1% streptomycin. SKOV-3, SKOV3ip.1 and HEY-A8 (the generous gifts of Dr. Gordon Mills, M.D. Anderson Cancer Center, Houston TX) were grown in DMEM supplemented with 5% FCS, 1× sodium pyruvate, 1× nonessential amino acids, 1× t-glutamine, and 2× vitamin solution (Life Technologies, Inc.). ASCP-1, a pancreatic cell line, which contains a homozygous deletion from D17S969 to MKK4 exon C, served as a negative control and was obtained from American Type Tissue Collection, with permission of Dr. Gary Nolan, Stanford University, Palo Alto, CA) using an Effectene transfection reagent per manufacturer’s instructions (Qiagen, Santa Clarita, CA). This approach routinely yields high efficiency transfer of the target DNA into recipient cells. Transfection medium was collected 48 h later. SKOV3ip.1 cells (2×10⁶) were plated 24 h before infection, and incubated with transfection medium and Polybrevin for 8 h. Transfection medium was replaced with fresh medium, and the cells were then diluted in growth medium containing G418 (500 μg/ml; Sigma). Cells were split for selection and establishment of clonal cell lines.

HA-MKK4 expression was verified by immunoblotting using an anti-HA.11 monoclonal antibody (1:500 dilution; Covance Inc.), a horseradish peroxidase-conjugated goat antimouse secondary antibody (1:2000 dilution; Sigma) followed by detection with an enhanced chemiluminescence kit (Sigma). The positive and negative controls used were AT6.1-SR3-3-HA-JNK1, the AT6.1 prostate cancer cells transiently transfected with HA-MKK4 (JNK1) using the Sr3 plasmid (hereafter referred to as AT6.1-SR3-3-HA-MKK4), and the parental SKOV3ip.1 cell line, respectively.

**Metastasis Assays.** Parental SKOV3ip.1 cells were injected i.p. (1×10⁶ cells) into female SCID mice (Frederick Laboratories) to determine the time course for metastasis formation, as well as the location and number of metastases. A similar model has been used by other investigators (20, 21). On the basis of our preliminary experiments in which animals survived an average of 40 days, 7 SKOV3ip.1-HA-MKK4 clones and 5 vector control clones, SKOV3ip.1-pLNCX2, were injected i.p. (1×10⁶ cells/mouse) into 60 different mice (5 mice/clone). The number and extent of overt metastases were quantitated 20 days after injection. This time point was chosen because it gave a reproducible number of overt metastases without significant morbidity. Metastases were defined as visible tumor implants >1 mm in diameter. Two independent studies were conducted. To determine total survival time, each clonal cell line was also injected into an additional 5 mice/clone and the mice followed until moribund.

**In Vivo Characteristics of SKOV3ip.1-HA-MKK4 Clones and SKOV3ip.1-pLNCX2 Clones**

**Cell Proliferation.** To determine whether the introduction of MKK4 into the SKOV3ip.1 cells affected inherent growth rate, each clonal cell line (15,000 cells/well) was plated in triplicate in a six-well plate and allowed to grow under standard tissue culture conditions. A Coulter counter was used to count cells at interval time points of 0, 72, 96, 120, 144, and 240 h after plating. At the final time point, cells were 95–100% confluent.

**DAP1 Staining.** To assess whether ectopic MKK4 expression induced apoptosis, DAPI staining and flow cytometry were performed on SKOV3ip.1-HA-MKK4 clones and results compared with those obtained from SKOV3ip.1-pLNCX2 clones. In brief, each clonal cell line was plated in a six-well plate and allowed to grow under standard tissue culture conditions until 70–80% confluent. Cells were fixed for 30 min at room temperature in 3.7% paraformaldehyde (Sigma). The medium was aspirated and the plates allowed to dry for 1 h. Cells were stained with 1 μg/ml of DAPI (Sigma) solution for 5 min. The solution was aspirated and Gel/Count (Biomeda) added. Cells were viewed by microscopy at a magnification of ×400.

**Flow Cytometry.** The fractions of apoptotic and proliferating cells were also assessed by flow cytometry. Each clonal cell line was plated in a six-well plate and allowed to grow under standard tissue culture conditions until 70–80% confluent. Cells were trypsinized and added to supernatant, spun at 1200 rpm, and washed two times in FACS buffer (0.2% BSA/0.01% NaN₃, in 1× PBS). Cells were resuspended in 250 μl of FACS buffer. Ten μl of 100 μg/ml propidium iodide (Molecular Probes) was added to each tube and immediately analyzed via flow cytometry.

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Statistical Analyses

Immunohistochemistry. For the expression of MKK4 in the clinical specimens, the proportion of normal ovary specimens that demonstrated MKK4 staining intensity ≥2 was compared with the proportion of metastatic samples demonstrating MKK4 staining intensity ≥2. A power calculation indicated that a sample size of 28 normal specimens and 28 cancer specimens would provide >90% power to detect a true reduction from 95% of the normal ovaries staining at the designated intensity level to 55% of the cancer specimens staining at the designated intensity level. A continuity corrected χ² analysis was used to compare the observed proportions in the two groups. A Wilcoxon rank sum test was also used to compare the full set of ordinal responses (intensity score 0, 1, 2, or 3) in the different groups (22). P < 0.05 was considered statistically significant.

Metastasis Assays. The mean number of metastases at 20 days after injection in the 5 SKOV3ip.1-pLNCX2 clones was compared with the mean number of metastases in the 7 SKOV3ip.1-HA-MKK4 clones using a mixed model ANOVA treating each clone as a random effect because observations within each clone were correlated (23). To allow for the within-clone correlation, survival times were averaged across mice within the same clone and the method of Kaplan and Meier (24). To stabilize the variance, the data were considered statistically significant.

RESULTS

MKK4 Is Down-Regulated in Clinical Ovarian Cancer Specimens. To test the potential role of MKK4 down-regulation in metastasis, we compared intensity of MKK4 expression in the epithelial component of normal ovary samples to that in metastatic ovarian cancer specimens. Of the 34 patients with metastatic ovarian cancer, 3 had stage II disease, 19 had stage III disease, and 12 had stage IV disease. A scoring system was devised to assign a staining intensity score for MKK4 expression ranging from 0 (no expression) to 3 (highest intensity staining) in the epithelial components. Fig. 1, A and B, show MKK4 staining in the normal epithelium corresponding to level 3 staining (Fig. 1A) and its corresponding negative control (Fig. 1B). In contrast, Fig. 1, C and D show level 1 staining in the metastatic epithelial component and no staining in the corresponding IgG control. Table 1 shows the distribution of MKK4 staining intensity scores among the normal ovary and metastatic cancer specimens. The majority of normal ovary specimens (31 of 34, 91%) stained for MKK4 at an intensity level ≥2 with 20 (58%) samples staining at the highest intensity level, 3. The staining was uniform and epithelial-specific, with relatively little stromal staining noted. In contrast, only 11 of 34 (32%) metastatic samples stained at an intensity level ≥2, and none of these samples stained at the highest intensity level, 3 (P < 0.0001). The Wilcoxon test also yielded highly significant results (P < 0.0001). These data support a role for MKK4 down-regulation in ovarian cancer cells that have acquired the ability to metastasize.

Metastatic Human Ovarian Cancer Cell Lines Lack MKK4 Expression but Retain Other Components of the SAPK Pathway. To test the hypothesis that ectopic expression of MKK4 would suppress metastatic colonization in vivo, we needed to identify an appropriate model system. The expression of endogenous MKK4 and components of the SAPK signaling cascade (Fig. 2A) were evaluated in four human ovarian cancer cell lines. ASPC-1 cells, which have a homozygous deletion in the MKK4 gene and do not express the protein, were used as a negative control, whereas rat brain, which expresses high levels of MKK4, was used as a positive control (Fig. 2B). HEY-A8, SKOV-3ip.1, and SKOV-3, which have been shown previously to form metastases in vivo (20), had undetectable levels of MKK4 expression even when 100 μg of protein was loaded per sample (Fig. 2B). MEKK1, which is known to phosphorylate MKK4 in response to cellular stimuli, was expressed in all four of the cell lines as were the MAP kinases JNK1, JNK2, and p38, and the transcription factor, c-jun. Of the MAP kinase kinases, MKK3 was detected in all four of the cell lines. Representative data are shown in
pressed HA-MKK4 (e.g., was confirmed by immunoblotting. AT6.1 cells that transiently expressed MKK4 protein was present; however, other MAP kinase upstream (MEKK1) and downstream (JNK and c-jun) constituents of the MKK4 signaling pathway. In addition to MKK4, p38, JNK, and c-jun were present, as was MKK3, MKK6, MKK7, and p38. SKOV-3) human ovarian cancer cell lines lacked detectable levels of MKK4 expression even when 100 μg protein (B, top) was resolved. MEKK1 (data not shown) and the downstream components of the MKK4 pathway (JNK and c-jun) were present, as was p38. The Caov-3 cell line, MKK4 protein was present; however, other MAP kinase kinases, MKK6 and MKK7, were undetectable.

Fig. 2B. Interestingly, MKK6 and MKK7, known to primarily activate p38 and JNK, respectively, were expressed in all of the cell lines except Caov-3, the only cell line that retained MKK4 expression. The SKOV3ip.1 cell line was chosen for functional evaluation of the MKK4 metastasis suppressor activity based on the absence of detectable MKK4 expression and the reproducible number of overt i.p. metastases formed by 20 days after injection.

### Ectopic MKK4 Expression Suppresses Metastatic Ability in Vivo

To test the ability of MKK4 protein to suppress metastatic colonization in vivo, 5 SKOV3ip.1-pLNCX2 clonal vector-only control cell lines (Fig. 3, left) and 7 SKOV3ip.1-HA-MKK4 clonal cell lines (Fig. 3, right) were established. HA-MKK4 transgene expression was confirmed by immunoblotting. AT6.1 cells that transiently expressed HA-MKK4 (e.g., AT6.1-SRα-HA-MKK4) were used as a positive control, whereas parental SKOV3ip.1 cells were used as a negative control. The effect of ectopic MKK4 expression on metastatic colonization was assessed by injection of 1 × 10^6 cells i.p. into female SCID mice. Twenty days after injection, mice were sacrificed and the number of overt (i.e., > 1 mm diameter) surface metastases counted (Fig. 4). Animals injected with SKOV3ip.1-pLNCX2 vector-only control cell lines had overt metastatic implants on the liver, the small bowel, and near the stomach and spleen (Fig. 4, A and B). On histological section, the metastatic implants retained the papillary serous histology that is characteristic of the parental cell line and the majority of clinical human ovarian cancers (Fig. 4C). The data from two independent experiments were tabulated and are summarized in Table 2. Ectopic expression of MKK4 suppressed the number of overt metastases by 88% (Fig. 4D). The mean number of metastases in mice injected with the vector control clones was 27.2 ± 14.8 as compared with the mean number in the mice injected with the SKOV3ip.1-HA-MKK4 clones, which was 3 ± 4.6 (Table 2, left).

**Ectopic MKK4 Expression Does Not Affect Proliferation or Induce Apoptosis in Vitro.** To determine whether ectopic HA-MKK4 expression affected cell proliferation, thereby reducing the inherent proliferative rate of metastatic implants, the in vitro doubling time of the cell lines were determined (Table 2, right). Mean doubling time for the vector control clones of 34.7 h (95% confidence interval, 27.2–42.1) was not significantly different when compared with that of the HA-MKK4 transfectants, 37.8 h (95% confidence interval, 31.3–44.2; P = 0.44).

To test the possibility that ectopic expression of MKK4 increased the number of cells undergoing apoptosis, two independent methods, DAPI and flow cytometry, were used to quantitate the fraction of apoptotic cells. The percentage of apoptotic cells was determined in three representative SKOV3ip.1-pLNCX2 clones and four representative SKOV3ip.1-HA-MKK4 clones used in the in vivo experiments described above. Experiments were performed in duplicate and triplicate for DAPI staining and flow cytometry, respectively, and the mean number of apoptotic cells in the control clones and MKK4 containing clones compared. By DAPI staining, the majority of cells in both groups were not apoptotic: 97.4 ± 0.93% in the vector controls versus 97.5 ± 1.13% in the SKOV3ip.1-HA-MKK4 clones (P = 0.94). These results were similar when flow cytometry was used with 95.4 ± 2.4% of the vector control cells and 94.7 ± 4.0% of the MKK4-expressing clones being nonapoptotic (P = 0.30). The 95% confidence intervals for the true difference (MKK4-control) were −1.3% to 1.4% and −3.0% to 1.0%, respectively. Taken together, results of these in vitro studies suggest that ectopic expression of MKK4 does not alter the inherent growth properties of SKOV3ip.1
cells. Future studies will be conducted to examine these parameters in vivo.

**Ectopic MKK4 Expression Prolongs Survival.** To test the effect of MKK4-mediated metastasis suppression on animal survival, additional mice \((n = 5/clone)\) injected with SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4 clones were followed until moribund and sacrificed (Table 2, middle). Overall mean survival was significantly increased by 70\%, from 37 days in the mice injected with vector control clones to 63 days in mice injected with the SKOV3ip.1-HA-MKK4 clones (Wilcoxon \(P = 0.0045\); see also Fig. 5).

**DISCUSSION**

Ovarian cancer is, by far, the most lethal of the gynecologic malignancies. Screening techniques for early detection of disease and an adequate understanding of the molecular mechanisms contributing to development of disease are ineffective and lacking, respectively. Given that the majority of women present with metastatic disease, the greatest impact on reducing the death rate from ovarian cancer lies particularly important to defining the critical steps that regulate metastasis.

MKK4 is a central mediator in the stress activated protein kinase signaling pathway that responds to a number of cellular and environmental stressors (17). By phosphorylating MAP kinases such as JNK, MKK4 can ultimately transmit stress signals to nuclear transcription factors that mediate various processes including proliferation, apoptosis, and differentiation. In recent years, distinct biological functions have been identified for MKK4 including a role in development, hepatogenesis, and metastasis suppression (18, 19, 25). The characterization of MKK4 as a prostate cancer metastasis suppressor gene is supported by work from both animal model and clinical correlative studies (18, 19). These studies have shown that MKK4 expressing prostate cancer cells are capable of reaching remote sites, yet, at these sites, are growth arrested in comparison to control cells. A comprehensive understanding of MKK4-mediated growth regulation is particularly important to defining the critical steps that regulate metastatic colonization.

In the current study, we have demonstrated that the normal surface epithelium, from which 90% of epithelial ovarian cancers originate, express high levels of MKK4. In contrast, the majority of metastatic ovarian cancer specimens show significantly decreased expression suggesting that MKK4 protein levels are down-regulated in clinical

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**Table 2. In vitro and in vivo characteristics of SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4 clones.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean number of metastases on day 20 ± SD(^a)</th>
<th>Mean survival time ± SD (days)(^b)</th>
<th>In vitro doubling time (h)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3ip.1-pLNCX2 cl 1</td>
<td>22.6 ± 3.9</td>
<td>43.8 ± 5.5</td>
<td>35.8</td>
</tr>
<tr>
<td>SKOV3ip.1-pLNCX2 cl 2</td>
<td>33.7 ± 8.1</td>
<td>38.4 ± 7.5</td>
<td>26.4</td>
</tr>
<tr>
<td>SKOV3ip.1-pLNCX2 cl 3</td>
<td>41.3 ± 23.0</td>
<td>32.6 ± 8.0</td>
<td>41.4</td>
</tr>
<tr>
<td>SKOV3ip.1-pLNCX2 cl 4</td>
<td>19.4 ± 8.7</td>
<td>43.4 ± 0.9</td>
<td>31.2</td>
</tr>
<tr>
<td>SKOV3ip.1-pLNCX2 cl 5</td>
<td>20.3 ± 7.7</td>
<td>27.6 ± 0.9</td>
<td>38.9</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 1</td>
<td>2.6 ± 3.5</td>
<td>92.4 ± 1.3</td>
<td>34.7</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 2</td>
<td>2.0 ± 1.5</td>
<td>56.8 ± 2.7</td>
<td>45.5</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 3</td>
<td>0.4 ± 0.5</td>
<td>62.0 ± 2.3</td>
<td>29.8</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 4</td>
<td>0.8 ± 1.3</td>
<td>73.0 ± 4.8</td>
<td>49.9</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 5</td>
<td>1.5 ± 1.7</td>
<td>60.8 ± 8.8</td>
<td>34.7</td>
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<tr>
<td>SKOV3ip.1-HA-MKK4 cl 6</td>
<td>0.8 ± 0.8</td>
<td>68.4 ± 2.8</td>
<td>34.3</td>
</tr>
<tr>
<td>SKOV3ip.1-HA-MKK4 cl 7</td>
<td>13.1 ± 3.1</td>
<td>44.2 ± 6.6</td>
<td>36.0</td>
</tr>
</tbody>
</table>

\(^a\) \(P < 0.0001\) as determined by mixed model analysis of variance.

\(^b\) \(P < 0.0045\) as determined by Wilcoxon rank-sum test.

\(^c\) \(P = 0.4104\) as determined by two-sample t test.

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**Fig. 4. Metastases in female SCID mice injected i.p. with a representative SKOV3ip.1-pLNCX2 clone or SKOV3ip.1-HA-MKK4 clone and sacrificed 20 days after injection. A, representative female SCID mouse injected i.p. with \(1 \times 10^6\) SKOV3ip.1-pLNCX2 cells. A large tumor implant is present near the stomach (arrowhead). Numerous 1-mm metastases are present along the small bowel surfaces (box) and the liver (yellow arrows). B, inset of A. Magnified view of small bowel implants (small arrows). C, histological section of a small bowel implant taken from a SCID mouse injected with a representative SKOV3ip.1-pLNCX2 clone. The implant retains a papillary serous histology (arrowhead) and is seen attached to the small bowel (small arrows). D, representative female SCID mouse injected i.p. with \(1 \times 10^6\) SKOV3ip.1-HA-MKK4 cells. Significantly fewer tumor implants are present at 20 days after injection. A single implant is shown on the liver (arrowhead).**

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**Fig. 5. Kaplan-Meier curve summarizing survival of mice injected with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK4 clones. Female SCID mice \((n = 5\) mice/clone) were injected i.p. with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK4 clones \((1 \times 10^6\) cells/mouse) and followed until moribund.**
disease when cells acquire the ability to grow at a metastatic site. Sufficient numbers of early stage ovarian cancers were not available to address the question of whether MKK4 expression inversely correlated with advancing stages of disease. Analysis of a number of human ovarian cancer cell lines revealed that MKK4 expression was undetectable in three cell lines (SKOV3.ip1, SKOV-3, and HEY-A8) known to be metastatic in vivo. Lack of significant MKK4 expression appeared to be an isolated occurrence as other key constituents of the MKK4 signaling module including MKK1, MKK7, JNK, and c-jun were present in these same cell lines. In addition, key members of the p38 pathway were also present including MKK6, MKK3, and p38. Interestingly, the Caov-3 cell line, which was the only one to demonstrate significant MKK4 expression, lacked other MAP kinase kinases (MKK6 and MKK7). These data implicate dysregulation of the SAPK signaling cascade in ovarian cancer metastasis formation. Expressing the MKK4 transgene in the SKOV3.ip1 cell line in vivo significantly reduced the number of metastatic implants by nearly 90%, which translated into a significant improvement in survival. Taken together, these data support our hypothesis that MKK4 regulates metastatic colonization in ovarian cancer.

Our clinical and experimental data support the notion that ectopic expression of MKK4 compliments a signaling defect in the SKOV3.ip1 cells, thus restoring the ability of disseminated SKOV3.ip-HA-MKK4 cells to respond to stress stimuli resulting in inhibition of metastatic colonization. The undetectable expression of MKK4 in three human cell lines and the undetectable expression of MKK7 in the Caov-3 cell line, suggest a model in which MKK4 signaling through JNK causes context-dependent effects, which suppresses metastatic colonization. How this effect on metastatic colonization is mediated and whether JNK plays a vital role in this process is the subject of ongoing study. In vitro, MKK4 expression does not affect inherent proliferation or increase cell death. These findings are consistent with previous studies, which show that MK4 exerts its effect in a context-dependent manner in vivo. Future studies will examine the biochemical and biological mechanisms by which MKK4 suppresses colonization in vivo.

Numerous lines of evidence, specifically targeting ovarian cancer cells, implicate the importance of the MKK4 signaling module in ovarian cancer growth and apoptosis. For instance, BRCA1, a tumor suppressor gene responsible for the majority of hereditary ovarian cancers, has been shown to mediate apoptosis after serum withdrawal in the OV177 cell line via the Ras/MKK4/JNK pathway (26). While it is tempting to speculate that reconstitution of the MKK4-JNK pathway may affect metastatic colonization via apoptosis, a known downstream effect of JNK, it is possible that MKK4 may be mediating other processes vital to metastatic colonization such as adherence, invasion, and context-dependent proliferation.

From a translational perspective, our finding that ectopic MKK4 expression increased animal life span by 70% is particularly exciting. This suggests that modulation of the MKK4 pathway, either by restoration of MKK4 function alone or in combination with therapeutic agents such as chemotherapy, could have a clinical benefit. After surgical cytoreduction, paclitaxel is used as front-line chemotherapy for the adjuvant treatment of ovarian cancer. Recently, the MKK4 signaling module has been recognized as mediating the apoptotic effects of agents including paclitaxel in ovarian cancer models. Paclitaxel has been shown to activate the SAPK signaling cascade and promote apoptosis, an effect which is abrogated by use of a dominant negative MKK4 (27–29). Additional mechanistic studies will be needed to test the possibility that MKK4 expression reconstitutes a stress-signaling pathway that suppresses metastatic colonization and potentiates the effect of chemotherapy.

In conclusion, these data provide a clinical and biological link between down-regulation of MKK4 and capacity for metastasis formation. Further study to elucidate mechanisms of action will be required before appropriate therapeutic interventions can be formulated, but, certainly, a first step will be to analyze in vivo the combined effects of MKK4 reconstitution with standard therapies such as chemotherapy. Our ultimate goal is to identify targets to improve ovarian cancer treatment. To this end, we find the effect on animal survival encouraging. As we better understand the role the MKK4 signaling pathway plays in metastatic colonization, perhaps we will be able to identify novel modulators of this pathway that may act as targets for treatment and ultimately, improved patient survival.

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REFERENCES


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