Suppression of Metastatic Colonization by the Context-Dependent Activation of the c-Jun NH2-Terminal Kinase Kinases JNKK1/MKK4 and MKK7

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Abstract

Advances in clinical, translational, and basic studies of metastasis have identified molecular changes associated with specific facets of the metastatic process. Studies of metastasis suppressor gene function are providing a critical mechanistic link between signaling cascades and biological outcomes. We have previously identified c-Jun NH2-terminal kinase (JNK) kinase 1/mitogen-activated protein kinase (MAPK) kinase 4 (JNKK1/MKK4) as a prostate cancer metastasis suppressor gene. The JNKK1/MKK4 protein is a dual-specificity kinase that has been shown to phosphorylate and activate the JNK and p38 MAPKs in response to a variety of extracellular stimuli. In this current study, we show that the kinase activity of JNKK1/MKK4 is required for suppression of overt metastases and is sufficient to prolong animal survival in the AT6.1 model of spontaneous metastasis. Ectopic expression of the JNK-specific kinase MKK7 suppresses the formation of overt metastases, whereas the p38-specific kinase MKK6 has no effect. In vivo studies show that both JNKK1/MKK4 and MKK7 suppress the formation of overt metastases by inhibiting the ability of disseminated cells to colonize the lung (secondary site). Finally, we show that JNKK1/MKK4 and MKK7 from disseminated tumor cells are active in the lung but not in the primary tumor, providing a biochemical explanation for why their expression specifically suppressed metastasis while exerting no effect on the primary tumor. Taken together, these studies contribute to a mechanistic understanding of the context-dependent function of metastasis regulatory proteins.

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Introduction

Cancer metastasis is a complex, dynamic process, which begins with dissemination of cells from the primary tumor and culminates with the formation of clinically detectable, overt metastases at a discontinuous secondary site(s) (1–3). In clinical and experimental models, metastasis formation requires a series of discrete events commonly referred to as the metastatic cascade (2). These include escape of malignant cells from the primary tumor, transport via the bloodstream or lymphatics, lodging at secondary sites, and finally the survival and growth into detectable masses, a process termed “metastatic colonization” (4, 5). A variety of studies have found that disseminated cancer cells, which have hallmarks of malignancy, may lack the ability to complete metastatic colonization (4, 6–10). There is considerable interest in determining why cells that have the ability to disseminate from a primary tumor and lodge at secondary sites would have impaired ability to complete this last, critical step of metastasis formation. Furthermore, the observations that in many cases these disseminated but undetectable cancer cells eventually form overt metastases suggests that solitary cells, or small groups of cells, can resume growth through mechanisms, which have yet to be defined (6). Taken together, these findings imply that cells that have lodged at secondary sites must respond to and interpret extracellular signals in a manner that enables survival and ultimate growth into metastases.

To identify signaling pathways that regulate metastatic colonization, we and others used a strategy that allowed us to functionally identify genes that specifically regulate metastasis formation. By analogy to tumor suppressor genes, we reasoned that identification of genes that specifically suppress metastasis without affecting primary tumor growth would enable us to identify proteins and signaling pathways that specifically regulate metastasis formation (11–14). The well-characterized Dunning AT6.1 spontaneous metastasis model enabled the identification of metastasis suppressor activities on human chromosomes as well as the KAI1 and Drg1 metastasis suppressor genes (14–16). Using this model, our laboratory also identified the mitogen-activated protein kinase (MAPK) kinase 4/e-Jun NH2-terminal kinase (JNK) kinase 1 (MKK4/JNKK1 also known as SEK1, MK4, and MAP2K4, hereafter referred to as JNKK1) as a metastasis suppressor gene (17). Specifically, ectopic expression of JNKK1 in AT6.1 cells suppressed the formation of overt (>1 mm diameter) lung metastases by >80% without affecting primary tumor growth (17). Interestingly, suppression seemed to occur after the cells reached the metastatic site, indicating that JNKK1's signaling pathway may play a role in regulation of metastatic colonization. Subsequent clinical and experimental studies support a role for JNKK1 as a metastasis suppressor in prostate, breast, pancreatic, and ovarian cancers (18–24).

JNKK1 is a MAP2K dual-specificity kinase that has been shown to phosphorylate and activate the JNK and p38 MAPKs in response to a variety of extracellular stimuli (25–27). Likewise, MKK7 (also known as JNKK2) and MKK6 are MAP2Ks that specifically phosphorylate JNK and p38, respectively (28–32). Although a wide variety of studies have addressed the biochemical regulation of the JNK and p38 signaling cascades (27, 33, 34), less is known about their biological function in processes such as metastasis. Several lines of evidence have identified functions for JNK and p38 signaling in cellular events that may play a role in metastatic...
colonization (7, 33, 35–37). Taken together, data suggesting that JNKK1 expression inhibits early steps in metastatic colonization and its known biochemical functions afforded us the unique opportunity to examine the mechanism of JNKK1-mediated metastasis suppression and the potential contribution of JNK and p38 in this process. In this study, we specifically evaluate the functional role of MKK4/JNKK1, MKK7, and MKK6 signaling in metastatic colonization of the lung using the well-characterized AT6.1 model of spontaneous metastasis.

Materials and Methods

Cell lines and culture conditions. AT6.1 Dunning rat prostate carcinoma cells from the laboratory of Dr. John Isaacs (38) were cultured in DMEM with 1-glutamine and high glucose (4.5 g/L; Mediatech, Herndon, VA), supplemented with 8% FCS (Atlanta Biologicals, Norcross, GA), 1% penicillin (100 units/mL)/streptomycin (100 μg/mL)/t-glutamine mixture (Bio Whittaker/Cambrex, Walkersville, MD), 250 mM/L dexamethasone (Sigma, St. Louis, MO), 10 mM/L sodium pyruvate (Mediatech), 1× nonessential amino acids (Mediatech), and 1× MEM vitamin solution (Mediatech). For stable AT6.1 clones, the medium was additionally supplemented with 500 μg/mL active G418 (Cellgro, Herndon, VA). RetroPack PT67 packaging cells (Clontech, Palo Alto, CA) were cultured in DMEM supplemented with 8% FCS, 1% penicillin/streptomycin, sodium pyruvate, nonessential amino acids, and MEM vitamin solution. For in vitro JNK and p38 pathway stimulation, AT6.1 cells were cultured in complete growth medium containing 0.1% FCS for 24 hours and treated with 50 ng/mL anisomycin (Sigma) for 20 minutes.

Plasmid constructs and generating stable clones. The HA-JNKK1, HA-JNKK1(KR), and HA-MKK6 cDNAs were provided by Dr. Roger Davis (University of Massachusetts Medical School). The cDNAs were excised using HindIII and SalI sites and subcloned into the pLNCX2 retroviral vector, which contained a neomycin resistance cassette (Clontech). Before establishing stable expression, sequences of constructs were confirmed.

Transfection of packaging cells and viral collection of retroviruses containing pLNCX2-cDNA constructs were conducted using the RetroPack PT67 cells per the manufacturer’s instructions (Clontech). Briefly, PT67 cells were transfected with 1 μg of DNA using the Qiagen Effectene Transfection Reagent kit (Valencia, CA). After 24 hours, the cells were washed, and complete medium was applied. After an additional 24 hours (48 hours after transfection), 2 mL of filtered viral medium were mixed with 8 mL of complete AT6.1 growth medium and added to target AT6.1 cells along with 8 mg/mL polybrene (Specialty Media, Phillipsburg, NJ). Twenty-four hours later, target cells were washed once with PBS, and complete medium was applied. After 24 additional hours (48 hours after infection), cells were passaged into complete medium containing 500 μg/mL active G418 to select for stable pools. Clonal cell lines were established by limited dilution cloning of stable transgene-expressing pools, and transgene expression was confirmed by immunoblotting for expression of the hemagglutinin (HA) epitope-tagged protein.

Cell lysis and immunoblotting. Monolayer cell cultures were washed in ice-cold PBS, and protein lysates were prepared using chilled Trition 100-X buffer [10 mM/L Tris (pH 7.5), 150 mM/L NaCl, 1 mM/L EDTA, and 1% Triton 100-X (v/v, Sigma)] containing a complete protease inhibitor pellet (Roche, Indianapolis, IN), 70 μL of 10 μg/mL phenylmethylsulfonyl fluoride (PMSF; Sigma), 200 μL aprotinin (Sigma), and 200 μL of 100 mM/L monovalent NaVO₄, added fresh per 7 mL of lysis buffer. Cells were scraped and placed on ice after being passed through a 27-gauge needle and subsequently centrifuged at 14,000 rpm at 4°C for 10 minutes. Tissues from tumor-bearing animals were rapidly excised, washed in ice-cold PBS, minced, and homogenized by hand using a 1-mL tissue grinder (Fisher Scientific, Pittsburgh, PA) with 300 μL of chilled cell lysis buffer containing inhibitors. The homogenate was centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatant was transferred to a new tube via a 27-gauge needle. Protein lysates were quantified using the Pierce Bicinchoninic Acid Protein Assay kit (Rockford, IL); 30 μg of protein were subjected to SDS-PAGE and transferred to a HyBond Enhanced Chemiluminescence nitrocellulose membrane.

For immunoblotting, membranes were blocked at 4°C overnight in TBS-T plus 5% (w/v) Carnation nonfat dry milk. After incubation with each antibody diluted in blocking solution for 1 hour, the membrane was washed for 10 minutes in blocking solution and then washed six times for 5 minutes each in TBS-T. The horseradish peroxidase-conjugated secondary antibody was detected using the Super Signal West Femto Maximum Sensitivity Chemiluminescence Substrate (Pierce) per the manufacturer’s directions. Probed membranes were stripped using Pierce Restore Western Blot Stripping Buffer, washed in TBS-T, and blocked overnight before reprobing. The antibodies and dilutions are as follows: HA.11 (Covance, Princeton, NJ; 1:1,000), JNKK1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000), and p38 (Cell Signaling Technologies, Beverly, MA; 1:2,000). As a loading control, membranes were probed for actin followed by incubation with a goat anti-mouse IgG-peroxidase-conjugated secondary antibody (Oncogene Research, Uniondale, NY; 1:20,000 and 1:40,000 dilutions of primary and secondary antibody, respectively).

Immunoprecipitations and in vitro kinase assays. Two-hundred micrograms of cell lysate or 1.0 mg of tissue lysate were brought to a final volume of 500 μL with cold M2 immunoprecipitation buffer [20 mM/L Tris (pH 7.6), 0.5% NP40, 0.25 mol/L NaCl, 3 mM/L EDTA, and 3 mM/L EGTA] plus protease and phosphatase inhibitors [10 μL PMSF (10 mg/mL), 15 μL monovalent NaVO₄, 30 μL aprotinin, and 2 μL of 1 mol/L DTT per 3 mL of M2 buffer]. As controls, unstimulated lysates were added to 0.01 mg of active JNKK1 (Upstate, Lake Placid, NY). To this mixture, 1.0 μg of antibody and 30 μL of Protein A/G PLUS Agarose Beads (Santa Cruz Biotechnology) were added. The mixture was rotated at 4°C for 16 hours. The beads were centrifuged and washed twice with ice-cold M2 buffer and twice with ice-cold 50 mM/L HEPES buffer. The washed beads were resuspended in 15 μL of cold double-distilled deionized water (d₅H₂O) and mixed with 0.1 μg inactive recombinant JNKK1 or 0.1 μg inactive recombinant p38α (Upstate); 5 μL of ATP mix (0.035 μL of magnesium/ATP mix (Upstate), 0.05 μL of γ-32P-ATP (10 μCi/mL; Amersham, Piscataway, NJ), and 4.92 μL of d₅H₂O per sample), and 5 μL of chilled 6× kinase reaction buffer (200 μL of 1 mol/L HEPES, 200 μL of 1 mol/L MgCl₂, 20 μL of 1 mol/L DTT, 200 μL of 0.4 mol/L [γ]-glycerophosphate, 5 μL of 100 mM/L NaVO₄, and 842 μL of d₅H₂O). The kinase reactions were incubated at 30°C for 1 hour, with gentle mixing every 10 minutes. After an hour, the reaction was subjected to denaturing SDS-PAGE as described above. The gel was transferred to a nitrocellulose membrane for 16 hours and exposed to autoradiography film to detect radioactively labeled JNK or p38. Membrane bands were quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Spontaneous metastasis assays in severe combined immunodeficient mice. Tumor growth and metastasis experiments were conducted following the considerations prodigiously and eloquently stated in Welch et al. (3) and were approved by the Institutional Animal Care and Use Committee of The University of Chicago. All stable AT6.1 clones were maintained below passage 10 and, before preparation for mouse injection, were cultured on tissue culture plates for a minimum of two passages and not grown beyond 80% confluence. For spontaneous metastasis assays, 2 × 10⁵ cells (200 μL of a 1 × 10⁶ cells/mL suspension) were injected through a 25-gauge needle into the right flank of 6- to 8-week-old male severe combined immunodeficient mice. Tri-weekly tumor (length and width) and weight measurements were recorded. Tumor volume in cubic centimeters (cm³) was calculated using the following formula: [(mm length) × (mm width)] × (mm length) × (mm width) × 2.618 × 10⁻³. Tumor doubling time was calculated using the following formula: log₂(t)/slope of [log tumor volume versus time (days)]. For the survival analysis, 1.5 cm³ tumors were surgically excised using isoflurane anesthesia and aseptic surgical technique. Animals were removed from the experiment after a 10% loss in body weight, which is a predictor of metastatic burden. At the experimental end point, mice were euthanized using CO₂ asphyxiation or cervical dislocation. For most assays, the lungs were excised and inflated with the trachea with 10% buffered formalin. Surface lung metastases (≥1 mm in size) were counted, and statistical analyses were conducted.

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In some cases, histologic examination of formalin-fixed lung tissue was conducted on lungs to determine if microscopic metastases were present. Tissue specimens were paraffin embedded and stained with H&E. H&E-stained sections were examined and imaged using a Zeiss AxiosSkop color Axios camera with a x20 NeoFluar (Oberkochen, West Germany) and captured using Zeiss AxioCam software, courtesy of the University of Chicago Ben May Institute Microscope Core Facility.

Whole-lung clonogenic assays. Lungs were excised and minced, and the tissue dissociated by incubating at 250 rpm at 37°C overnight in a solution of DMEM, 8% FCS, 1% penicillin/streptomycin, 2% (w/v) collagenase (Sigma Chemical, St. Louis, MO), 1% CIPRO (Bayet, West Haven, CT), 1X antibiotic/antimycotic (Life Technologies Bethesda Research Laboratories, Grand Island, NY), and 500 μg/mL active G418 (Cellgro). The tissue suspension was centrifuged at 1,100 rpm, washed in PBS, and plated onto 15-cm diameter tissue culture plates in complete medium containing 500 μg/mL active G418. Colonies were allowed to grow for 2 weeks and then washed in PBS and stained using Crystal Violet [0.5% (w/v) crystal violet (Sigma) and 20% (v/v) ethanol (100%, Sigma)]. Stained plates were washed with water and dried at room temperature overnight. Plates were imaged, and the colonies were counted using the Kodak Gel Logic 100 (New Haven, CT). Regions of interest were counted using Kodak 1D Image Analysis Software (Edge Detection, gradient of 20, 20 pixels minimum and 20,000 pixels maximum).

Statistical analysis. Numbers of lung metastases were quantitated and differences between the experimental groups were evaluated using a mixed effects ANOVA model, where the type of clone [vector, JNKK1, JNKK1(KR), MKK6, or MKK7] was included as a fixed effect, and the clone identification was included as a random effect. The response variable was square root transformed. Models were fitted using PROC MIXED in the SAS V.8.1 software (39). Pairwise tests of differences between groups were assessed based on the fitted model, and P values were adjusted for multiple comparisons using the Tukey-Kramer method. Tumor doubling times were analyzed similarly. Symptom-free survival times, defined as time from surgical removal of the primary tumor until the animal showed signs of weight loss, loss of vigor, and unkempt appearance, were estimated using the method of Kaplan-Meier (40) and compared between AT6.1-vector and AT6.1-JNKK1 tumor-bearing mice using the log-rank test. The number of colonies formed in a standard spontaneous metastasis assay (42, 43). Spontaneous metastatic ability is defined as the ability of the cells to disseminate from the primary tumor and form overt metastases at the secondary site (lung). Consistent with previous studies, ectopic expression of HA-JNKK1 reduced the number of metastases by ≥90% compared with controls (Fig. 2A and B). In contrast, AT6.1-HA-JNKK1(KR) and AT6.1-Vector tumor-bearing animals exhibited similar numbers of metastases at the 42-day experimental end point (Fig 2A and B). AT6.1-HA-JNKK1(KR) metastases retained expression of HA-JNKK1(KR), showing that its expression did not affect the formation of overt metastases (data not shown).

As shown in Fig. 2C, ectopic expression of HA-JNKK1 or HA-JNKK1(KR) had no measurable effect on primary tumor growth rate compared with AT6.1-Vector controls. Taken together, these studies show that JNKK1’s kinase activity is necessary for suppression of AT6.1 metastases.

We have previously shown that ectopic expression of JNKK1 could prolong animal survival in a well-characterized model of ovarian cancer metastasis (22). An initial study of AT6.1-Vector and AT6.1-HA-JNKK1 tumor-bearing mice suggested that this was also the case in our model; however, data was confounded by significant morbidity caused by excessive primary tumor burden (data not shown). To more accurately determine the extent and

**Results**

c-jun NH2-terminal kinase kinase 1–mediated metastasis suppression is kinase dependent and prolongs animal survival. Based upon the known biochemical function(s) of JNKK1, we predicted that metastasis suppression would be dependent on the activation of JNK and/or p38 MAPKs. Abrogation of JNKK1 kinase activity by mutation of a critical ATP-binding residue [JNKK1(K118R)] renders JNKK1 catalytically dead (25, 26, 41).

To evaluate the biological effect of ectopic expression of the HA-JNKK1(KR) mutant, we derived and characterized multiple independent AT6.1 clonal cell lines expressing either HA-JNKK1(KR), HA-JNKK1, or vector alone (Fig. 1A). We verified that these cell lines had similar endogenous levels of the downstream MAPKs p38 and JNK [Fig. 1A, IB (p38) and IB (JNK)]. To confirm that the ectopically expressed proteins were functional, cells were activated with anisomycin, proteins were immunoprecipitated, and kinase assays were done. In vitro–activated HA-JNKK1 phosphorylated purified JNK (His-JNK) and p38 (His-p38) substrates, whereas activated HA-JNKK1(KR) did not (Fig. 1B). Thus, ectopically expressed HA-JNKK1 can be activated, and it phosphorylates both JNK and p38 as previously reported, and the K118R mutation renders JNKK1 enzymatically inactive (26).

The clonal cell lines were then tested for metastatic ability in a standard spontaneous metastasis assay (42, 43). Spontaneous metastatic ability is defined as the ability of the cells to disseminate from the primary tumor and form overt metastases at the secondary site (lung). Consistent with previous studies, ectopic expression of HA-JNKK1 reduced the number of metastases by ≥90% compared with controls (Fig. 2A and B). In contrast, AT6.1-HA-JNKK1(KR) and AT6.1-Vector tumor-bearing animals exhibited similar numbers of metastases at the 42-day experimental end point (Fig. 2A and B). AT6.1-HA-JNKK1(KR) metastases retained expression of HA-JNKK1(KR), showing that its expression did not affect the formation of overt metastases (data not shown).

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**Figure 1.** Biochemical characterization of AT6.1-Vector, AT6.1-HA-JNKK1, and AT6.1-HA-JNKK1(KR) clones. A, immunoblotting (IB) of stable AT6.1-Vector, AT6.1-HA-JNKK1, and AT6.1-HA-JNKK1(KR) clones. Expression of HA-JNKK1 and HA-JNKK1(KR) was detected by means of an NH2-terminal HA epitope tag; no noticeable difference in the expression of JNK or p38 was observed. Actin was used as a loading control. B, in vitro kinase activity (KA) of HA-JNKK1 and HA-JNKK1(KR). Stable AT6.1-Vector, AT6.1-HA-JNKK1, and AT6.1-HA-JNKK1(KR) clones were stimulated with 50 ng/mL anisomycin after serum starvation, and protein lysates were subjected to an HA immunoprecipitation (HA-ip), and kinase assays were done using purified His-tagged JNK (His-JNK) or purified GST-tagged p38 (GST-p38). As controls, a lysate from a serum-starved AT6.1-HA-JNKK1 clone was spiked with recombinant active JNK51 (PC) or left untreated (NC). Immunoblotting for total JNK and p38 served as a loading control.
duration of HA-JNKK1-mediated metastasis suppression, tumors were surgically excised when they reached ~1.5 cm³. Previous studies from our laboratory showed that at this tumor volume, AT6.1 cells had escaped from the primary tumor but had not yet formed overt metastases (42, 43). As shown in Fig. 3, stable expression of HA-JNKK1 significantly prolonged animal survival after surgical tumor resection from ~7 to 20 days (P < 0.0001, log-rank test).

Ectopic expression of mitogen-activated protein kinase kinase 7 suppresses the formation of overt metastases, whereas mitogen-activated protein kinase kinase 6 has no effect. JNKK1 can activate both the p38 and JNK MAPKs (26). Thus, suppression of colonization by HA-JNKK1 may be the result of either p38- or JNK-mediated signaling events. To determine the arm (e.g., p38 or JNK) of the signaling cascade through which JNKK1 is signaling, we tested the ability of specific p38 or JNK kinases to suppress spontaneous metastasis. In contrast to JNKK1, MKK6 is a specific activator of p38, which is independent of the JNK signaling pathway (31). Similarly, MKK7 specifically activates JNK (28–30). Thus, wild-type forms of HA-attaged MKK6 and MKK7 were ectopically expressed to activate the p38 and JNK MAPKs, respectively. Multiple independent AT6.1-HA-MKK6 and AT6.1-HA-MKK7 clones were generated and characterized [Fig. 4. A and B (HA)]. AT6.1-Vector, AT6.1-HA-MKK6, and AT6.1-HA-MKK7 clonal cell lines had comparable endogenous levels of the p38 and JNK MAPKs as determined by immunoblotting [Fig. 4. A and B (p38) and B (JNK)].

In vitro stimulation of AT6.1-HA-MKK6 cell lines resulted in activation of HA-MKK6, which specifically phosphorylated a purified p38 substrate (His-p38) but not a JNK substrate (His-JNK; Fig. 4B). In vivo metastasis assays showed that ectopic expression of HA-MKK6 had no effect on either the primary tumor doubling time or the number of overt metastases at the experimental end point (Fig. 5). Biochemical analyses showed that the HA-MKK6 protein was present and enzymatically active in overt AT6.1-HA-MKK6 metastases (Fig. 4C). Thus, stable expression and activity of HA-MKK6 in AT6.1 cells does not suppress metastasis formation.

In AT6.1-HA-MKK7 clonal cell lines, anisomycin-activated HA-MKK7 showed specific phosphorylation of a purified JNK substrate (His-JNK) but not a p38 substrate (His-p38; Fig. 4B). In spontaneous metastasis assays, AT6.1-HA-MKK7 tumor-bearing mice showed a >90% reduction in overt metastases compared with controls (Fig. 5A and B). Ectopic expression of HA-MKK7 did not suppress the growth of the primary tumors; indeed, AT6.10HA-MKK7 tumors grew slightly faster than the AT6.1-vector tumors (Fig. 5C). Taken together, this data identifies a new role for MKK7 as a metastasis suppressor protein.

Figure 2. JNKK1 requires its kinase activity to suppress metastasis. A, number of >1mm diameter surface metastases on the lungs of AT6.1-Vector (clones 3, 7, 10, and A), AT6.1-HA-JNKK1 (clones 2, 3, 4, 5, and 7), and AT6.1-HA-JNKK1(KR) (clones 19, 24, 25, 38, and 40) tumor-bearing mice at 42 d.p.i. A minimum of nine mice were used for each clone (except AT6.1-Vector clone A). Columns, number of overt lung metastases; bars, SE. Pairwise comparisons were based on a mixed effects model: AT6.1-Vector versus AT6.1-HA-JNKK1, P < 0.0001; AT6.1-Vector versus AT6.1-HA-JNKK1(KR), P = 0.1241; AT6.1-HA-JNKK1 versus AT6.1-HA-JNKK1(KR), P < 0.0001. B, representative lungs at 42 d.p.i. showing overt metastases [AT6.1-Vector and AT6.1-HA-JNKK1(KR)] and reduction in metastases (AT6.1-HA-JNKK1). C, tumor doubling times of AT6.1-Vector, AT6.1-HA-JNKK1, and AT6.1-HA-JNKK1(KR) clones. No significant differences were found between AT6.1-vector, AT6.1-HA-JNKK1, and AT6.1-HA-JNKK1(KR). P = 0.2139 (F test).

Figure 3. JNKK1 expression prolongs animal survival. Kaplan-Meier estimates of survival of AT6.1-Vector (n = 8) and AT6.1-HA-JNKK1 mice (n = 12) after surgical removal of 1.5 cm³ s.c. primary tumors. The average post-surgical survival of AT6.1-Vector tumor-bearing mice was 7.3 ± 0.7 days, and AT6.1-HA-JNKK1 tumor-bearing mice survived an average of 19.6 ± 2.1 days (P < 0.0001, log-rank test).
Discussion

A longstanding obstacle of cancer treatment is the development of distant metastases after a seemingly successful treatment of the original tumor (4, 6, 8, 14). Multiple lines of evidence led to the finding that cancer cells can dislodge and disseminate very early within the time course of primary tumor growth (8, 10, 14). Clinical and experimental studies indicate that the lodging and survival of disseminated single cells is a more frequent event than once envisaged (13, 14, 44). However, the cellular and biochemical pathways that regulate steps in metastatic colonization (e.g., the survival, persistence, and ultimate outgrowth of disseminated cells into metastases) have been incompletely characterized. The study of metastasis suppressor proteins is providing information on facets of this final step in metastasis formation (4, 11).

The identification of a metastasis suppressor function for JNK1 provided the unique opportunity to conduct a biochemically rigorous, in-depth analysis of the functional role of its well-characterized signaling pathway in the colonization process (14, 38, 42). We hypothesized that in response to stimuli in the lung, disseminated AT6.1-HA-JNKK1 and AT6.1-HA-MKK7 cells undergo context-specific suppression of metastatic colonization. This hypothesis predicts that comparable numbers of viable cells are present in the lung at early time points in metastatic colonization within this model (Fig. 6D). To test this hypothesis, we quantified the number of clonogenically viable, disseminated cells present in lungs of mice carrying AT6.1-HA-JNKK1(KR), AT6.1-HA-JNKK1, or AT6.1-HA-MKK7 primary tumors (Fig. 6C). There was no significant difference in the number of cells in the lungs of AT6.1-HA-JNKK1(KR), AT6.1-HA-JNKK1, and AT6.1-HA-MKK7 tumor-bearing mice (P = 0.724). These data support our hypothesis that disseminated HA-JNKK1- and HA-MKK7-expressing cells are impaired in their ability to complete the process of metastatic colonization.

c-Jun NH2-terminal kinase 1 and mitogen-activated protein kinase kinase 7 are active in the host lung. Because metastasis-suppressed AT6.1-HA-JNKK1 and AT6.1-HA-MKK7 cells disseminate to the lung in a comparable, temporal, and quantitative manner as metastatic controls, we hypothesized that HA-JNKK1 and HA-MKK7 are activated within the lung environment but not in the primary tumor. This hypothesis predicts that a stimulus, which is present in the lung, is lacking or insufficient to activate HA-JNKK1 and HA-MKK7 in the primary tumor. To test this, we assessed the kinase activity of HA-JNKK1 and HA-MKK7 immunoprecipitated from tumor and lung tissues. As controls, we used tumor and lung tissue from metastatic AT6.1-Vector and AT6.1-JNKK1(KR) controls. In vitro kinase assays showed that HA-JNKK1 and HA-MKK7 immunoprecipitated from lung tissue phosphorylated a purified JNK substrate (Fig. 6D). In contrast, proteins immunoprecipitated from the primary tumor had no detectable JNK kinase activity (Fig. 6D). These data are consistent with findings that ectopically expressed HA-JNKK1 and HA-MKK7 specifically suppresses metastasis formation without affecting primary tumor growth. Furthermore, this data gives a potential biochemical explanation of the mechanisms underlying metastasis suppressor protein function.

Metastasis suppression is not due to decreased numbers of cells lodging in the lung. We have previously shown that metastasis-suppressed AT6-HA-JNKK1 cells are able to form microscopic foci within the mouse lung, suggesting that JNKK1-mediated metastasis suppression is not blocking tumor cell invasion and dissemination but rather suppressing the growth of disseminated cells within the metastatic site (17). Histologic examination of lung sections from AT6.1-HA-MKK7 tumor-bearing mice similarly revealed small, microscopic foci at the experimental end point (Fig. 6D). We hypothesized that in response to stimuli in the lung, disseminated AT6.1-HA-JNKK1 and AT6.1-HA-MKK7 cells undergo context-specific suppression of metastatic colonization. This hypothesis predicts that comparable numbers of viable cells are present in the lung at early time points in metastatic colonization within this model (Fig. 6D). To test this hypothesis, we quantified the number of clonogenically viable, disseminated cells present in lungs of mice carrying AT6.1-HA-JNKK1(KR), AT6.1-HA-JNKK1, or AT6.1-HA-MKK7 primary tumors (Fig. 6C). There was no significant difference in the number of cells in the lungs of AT6.1-HA-JNKK1(KR), AT6.1-HA-JNKK1, and AT6.1-HA-MKK7 tumor-bearing mice (P = 0.724). These data support our hypothesis that disseminated HA-JNKK1- and HA-MKK7-expressing cells are impaired in their ability to complete the process of metastatic colonization.
JNK-mediated signaling events. Activation of p38 has a well-established role in inflammation, cytokine production, and tumorigenesis (27, 45, 46), and MKK6, a specific upstream activator of p38, is thought to serve an important role within these processes (47–50). Furthermore, a series of elegant studies by Aguirre-Ghiso et al. showed a functional role for p38 activity in dormancy (6, 7, 51). Our in vivo studies showed that specific activation of p38 by HA-MKK6 did not suppress formation of overt AT6.1 metastases. Taken together, our findings and those of Aguirre-Ghiso et al. suggest that the biological outcome of p38 activation may be dependent upon cancer cell types, micro-environmental interactions, and/or the activation status of other key signaling pathways within the cancer cell.

We hypothesized that to form overt metastases, disseminated AT6.1 cells modulate or suppress the effects of JNK activity to survive and proliferate. Consistent with our hypothesis is the remarkable finding that specific activation of the JNK kinases (JNKK1 and MKK7) within the lung microenvironment results in suppression of an early step in metastatic colonization. This provocative finding raises several important questions. What is the mechanism responsible for the differences in JNKK1 and MKK7 activity in the primary tumor versus disseminated cells in the lung? What biochemical and biological mechanisms are responsible for suppression of metastatic colonization? JNKK1 and MKK7 can be activated by a variety of stresses associated with metastatic processes, including alterations in matrix detachment, physical and chemical stresses, and stimulation by cytokines (27, 33, 52). Our working hypothesis is that ectopic expression of JNKK1 or MKK7 increases the strength or duration of a signal in the lung microenvironment. The concomitant increase in JNK activity results in either growth arrest (35, 37), cell cycle alterations (52–54), or increased apoptosis (36). We are currently using a combination of in vivo and in vitro approaches to test this hypothesis and determine the biological mechanism of suppression of colonization.

Figure 5. MKK7, a JNK kinase, suppresses the metastasis of AT6.1 tumor-bearing mice, whereas the p38 kinase MKK6 does not. A, number of >1mm diameter surface metastases on the lungs of AT6.1-Vector (clones 3, 7, 10, A, and B), AT6.1-HA-JNKK1 (clone 2), AT6.1-HA-MKK6 (clones 1, 2, 4, 6, and 8), and AT6.1-HA-MKK7 (clones 1, 4, 6, and 9) tumor-bearing mice at 42 d.p.i. Columns, number of overt lung metastases; bars, SE. Pairwise comparisons were based on a mixed effects model: AT6.1-Vector versus AT6.1-HA-JNKK1, \( P < 0.0001 \); AT6.1-Vector versus AT6.1-HA-MKK6, \( P = 0.9996 \); AT6.1-HA-JNKK1 versus AT6.1-HA-MKK6, \( P < 0.0001 \); AT6.1-Vector versus AT6.1-HA-MKK7, \( P < 0.0001 \); AT6.1-HA-JNKK1 versus AT6.1-HA-MKK7, \( P = 0.2711 \). B, representative lungs showing the presence of overt lung metastases in AT6.1-Vector and AT6.1-HA-MKK6 tumor-bearing mice and metastasis suppression of AT6.1-HA-MKK7 tumor-bearing mice. C, tumor doubling times of AT6.1-Vector, AT6.1-HA-JNKK1, AT6.1-HA-MKK6, and AT6.1-HA-MKK7 clones. Mixed effects model showed no statistical difference between the tumor doubling times of AT6.1-Vector and AT6.1-HA-MKK6 (\( P = 0.4582 \)) but a significant difference between AT6.1-Vector and AT6.1-HA-MKK7 clones (\( P = 0.0143 \)). Although the magnitude of the difference is quite small, it should be noted that the average doubling time for AT6.1-HA-MKK7 clones was higher (5.50 days) than for AT6.1-vector clones (5.03 days). Identifies a new role for MKK7 as a metastasis suppressor protein.
Findings reported herein have implications for both basic and clinical studies of metastasis. Although there is a rich literature documenting examples of context-dependent growth, such as the organ-specific growth of metastases, the biochemical mechanisms responsible for such growth are only beginning to be discerned (4, 8, 55–57). From a basic perspective, our studies provide biochemical explanation for why ectopic expression of JNKK1 and MKK7 specifically suppressed metastasis while exerting no effect on the primary tumor. These findings illustrate an important paradigm in cancer biology: depending on the activation status of key signal transduction pathways, disseminated cells that have molecular and phenotypic hallmarks of cancer cells can still be subject to growth regulation within the metastatic site (4, 8, 9, 55, 58, 59).

The clinical importance of persistent cells and the mechanisms by which these cells can initiate growth and give rise to lethal metastases is being debated (10, 14, 60, 61). Detection of disseminated cancer cells raises clinical questions. How can we distinguish disseminated cells that will remain indolent from those that will give rise to clinical metastases? Can we develop improved strategies to assign risk for development of metastatic disease? How and when should high-risk patients be treated? Our findings support the notion that the activation status of key signaling pathways and not gene expression profiling alone is needed to assess the biological

![Figure 6](cancerres.aacrjournals.org)
References


37. Bode JG, Weisowts Livr, and Department of Defense Ovarian Can cer Research grant DAMID-07-03-1-0169 to (D.J. Vander Griend, C.W. Rinker-Schaeffer), grant RO1 CA 85969 (D.J. Vander Griend, W.M. Stadler, and C.W. Rinker-Schaeffer), and Department of Defense Ovarian Cancer Research grant DAMID-07-03-1-0169 to (J.A. Hickson). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Suppression of Metastatic Colonization by the Context-Dependent Activation of the c-Jun NH₂-Terminal Kinase Kinases JNKK1/MKK4 and MKK7

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