Targeted Deletion of M KK4 in Cancer Cells: A Detrimental Phenotype Manifests as Decreased Experimental Metastasis and Suggests a Counterweight to the Evolution of Tumor-Suppressor Loss

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Abstract

Tumor-suppressors have commanded attention due to the selection for their inactivating mutations in human tumors. However, relatively little is understood about the inverse, namely, that tumors do not select for a large proportion of seemingly favorable mutations in tumor-suppressor genes. This could be explained by a detrimental phenotype accruing in a cell type–specific manner to most cells experiencing a biallelic loss. For example, M KK4, a tumor suppressor gene distinguished by a remarkably consistent mutational rate across diverse tumor types and an unusually high rate of loss of heterozygosity, has the surprisingly low rate of genetic inactivation of only ~5%. To explore this incongruity, we engineered a somatic gene knockout of M KK4 in human cancer cells. Although the null cells resembled the wild-type cells regarding in vitro viability and proliferation in plastic dishes, there was a marked difference in a more relevant in vivo model of experimental metastasis and tumorigenesis. M KK4−/− clones injected i.v. produced fewer lung metastases than syngeneic M KK4-competent cells (P = 0.0034). These findings show how cell type-specific detrimental phenotypes can offer a paradoxical and yet key counterweight to the selective advantage attained by cells as they experiment with genetic null states during tumorigenesis, the resultant balance then determining the observed biallelic mutation rate for a given tumor-suppressor gene. (Cancer Res 2006; 66(11): 5560-4)

Introduction

M KK4 (J NKK1, MAP2K4, and SEK1) is a dual-specificity kinase gene on chromosome 17p11. M KK4 protein is activated by >10 kinases, and active M KK4 cooperates with M KK7 to phosphorylate and thereby activate Jun NH2-terminal kinase (JNK) in the stress-activated cascade (1, 2). On exposure to cellular stressors, activated JNK phosphorylates several targets, such as the transcription factor Jun, which in turn associates with other activator protein (AP-1) components (e.g., Jun, Fos, and Fra-1) to form an active AP-1 dimer to control gene transcription. These events are dependent on M KK4 status (3).

Loss-of-function mutations in M KK4 have been found at a fairly consistent rate (~5%) in primary cancers of the pancreas, bile ducts, breast, colon, lung, and testis (4–6), distinguishing M KK4 as the only distal member of the mitogen- or stress-activated protein kinase family known to be mutated. By virtue of selection for inactivating mutations, M KK4 should be classified as either a tumor-suppressor or genome-maintenance gene (7). Conflicting data nevertheless exist: Wang et al., for instance, recently reported evidence for M KK4 having pro-oncogenic activity in an experimental setting (8). Such incongruities suggest that the biological role of M KK4 in experimental systems is cell type–specific. A rational extension of this cell type–specificity to human tumors is supported by the observation that nearly all tumors potentially experimenting with M KK4-null states do not select for a M KK4-null state, suggesting the possibility of a deleterious null phenotype in vivo. Such a phenotype could account for the observation that despite a consistency of mutational rate across diverse tumor types and an unusually high rate of 17p loss of heterozygosity, M KK4 has the surprisingly low rate of genetic inactivation of only ~5%. The goal of the present work was to design a more physiologic M KK4 model to explore the inconsistencies in the M KK4 literature and the possibilities of a deleterious or advantageous phenotype.

Materials and Methods

Targeted disruption of M KK4. The M KK4 gene was disrupted as described previously (9). Briefly, the pA AAV, pBRC, and pJDPer plasmids were cotransfected into PL5 (Panc 04.08) cells using LipofectAMINE (Invitrogen, Carlsbad, CA), and neomycin-resistant clones were selected. PCR screening identified clones having homologous integration events. Correctly targeted clones were confirmed using four different primer sets. The targeted clones were infected with an adenovirus expressing Cre recombinase, diluted to single cells, screened for loss of the selection cassette, and used when targeting the second allele.

PCR, reverse transcription-PCR, and direct sequencing. Cellular genomic DNA was prepared using Lyse-N-Go (Pierce, Rockford, IL). RNA was extracted using RNeasy (Qiagen, Valencia, CA). cDNA was made using Superscript II (Invitrogen). PCR products were resolved on 1% agarose gels using sodium boric acid electrophoresis (Faster Better Media LLC, Hunt Valley, MD). Automated sequencing of amplified fragments was done at the Johns Hopkins DNA Sequencing Facility.

Northern blot analysis. Northern blot analysis using 20 µg RNA was done using standard protocols. Oligomers complementary to the 18S rRNA or M KK4 mRNAs were end-labeled using [α-32P]dATP and terminal transferase. Hybridized probes were visualized and quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting. Immunoblotting was done using standard protocols. Antibodies recognizing phosphorylated JNK (Promega, Madison, WI), M KK4 (K-18, Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated p38 (Cell Signaling, Danvers, MA) were used. Equal protein loading

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Scott E. Kern, Department of Oncology, Sidney Kimmel Cancer Research Center, Johns Hopkins University School of Medicine, Cancer Research Building 464, 1650 Orleans Street, Baltimore, MD 21231. Phone: 410-614-3314; Fax: 443-287-4653; E-mail: sk@jhmi.edu.

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was confirmed by glyceraldehyde-3-phosphate dehydrogenase immunodetection or staining of total protein with Fast Green (Sigma, St. Louis, MO).

**Cell survival assays.** See Supplementary Data.

**Cell proliferation assays.** MKK4-knockout clones and parental cells were plated at low confluency. For cell counting, several counts were made of two separate aliquots of trypsinized cells at each time point using trypan blue exclusion. Six independent experiments were done. For Picogreen assays, DNA content was assayed as above. Two independent experiments were done.

**Xenografts.** Guidelines for the in vivo study of metastasis were followed for all experiments (10). Female athymic nude mice of ages 6 to 8 weeks were used (Harlan, Indianapolis, IN). For s.c. injections, $3.5 \times 10^6$ cells

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Targeted disruption of MKK4. A, schematic targeting procedure. OF+OR, forward and reverse primers outside the deleted sequence; IF+IR, forward and reverse primers inside the deleted sequence; SF+SR and SF2+SR2, screening primers; LHA+RHA, left and right homology arms. A, inset, By PCR, a band corresponding to the size of the wild-type (WT) sequence was detected in parental and heterozygote cells, whereas a smaller-fragment band, corresponding to the deleted Cre-recombinant sequence, was found in heterozygote and homozygote cells. B, top to bottom, Northern blot, RT-PCR, and Western blot analyses confirmed heterozygous and homozygous gene disruption. PT-PCR primers were designed to distinguish the MKK4 gene from the pseudogene. C, direct cell counting ($N = 6$ independent experiments) and D, assaying DNA content ($N = 2$ independent experiments, except for the 6- and 7-hour time points where $N = 1$) showed that all cells had similar proliferation rates regardless of MKK4 status.
Results

Targeted disruption of MKK4. We disrupted MKK4 in the pancreatic cancer cell line PL 5 (Panc 4.03) by deleting exon 2 (Fig. 1A), thereby disrupting the reading frame to create a premature stop codon 9 bp downstream of the exon 1/3 junction. This produced five MKK4+/− clones (K1-K5) and two control MKK4−/− clones (H1 and H2). Reverse transcription-PCR (RT-PCR) and Northern and Western blot analyses were done to confirm heterozygous and homozygous gene disruption (Fig. 1B). All of our clones and parental cells, regardless of MKK4 status, had similar growth rates in vitro (Fig. 1C and D). Furthermore, functional confirmation was observed as a loss of active JNK in null clones detected by Western blot analysis after UVC treatment (N = 2 independent experiments; data below).

Survival after stress in vitro. Because of the known role of the MKK4-JNK pathway in mediating stress signals and the likelihood that significant cellular stresses are present during tumorigenesis and propagation, we assayed the survival of parental cells, one or two MKK4+/− clones, and three to five MKK4−/− clones following exposure to a panel of different cellular stressors or pharmacologic treatments, and, in all but one stress treatment, no difference was observed (Supplementary Fig. S1): following serum starvation, MKK4+/− cells survived better than MKK4+/− or MKK4−/− clones. This result agrees with studies using small interfering RNA that found MKK4 knockdown cells to be more sensitive to serum starvation than control cells (8). This in vitro survival difference did not seem to be attributable to differing apoptotic rates as assessed by Annexin V assay (data not shown). We next transitioned our somatic cell knock-out system into the more relevant in vivo models, injecting our syngeneic cells i.v., i.p., and s.c. into athymic mice.

In vivo growth: i.v. injections. Consistent with our working hypothesis that the MKK4-null state might often be deleterious in vivo, we observed more lung lesions in the mice receiving i.v. injections of MKK4+/− and MKK4−/− cells compared with three different clones of MKK4−/− cells (Fig. 2A) evaluated in three independent experiments (P = 0.0034). The lungs of the mice with MKK4-proficient cells were burdened with metastases, so numerous as to become nearly confluent. Mice with MKK4-null cells, by contrast, had few to no lung metastases and maintained their body weight, whereas the mice with tumor-burdened lungs lost 10% to 20% of their body weight.

Lungs from mice with and without grossly detectable metastases were randomly sectioned to confirm the presence of metastatic cancer (Fig. 2B). Microscopic foci of viable cancer cells were detected in the grossly tumor-free lungs of mice receiving MKK4+/− cells, suggesting that a failure to become grossly and clinically detectable, not the failure of the cells to arrive intact and viable to the lungs, accounted for the observed differences.

No gross lesions were detected in other organs, including liver, spleen, and intestines. Liver and spleen sections were also randomly sampled, and microscopic foci of cancer cells were not detected (data not shown).

In vivo growth: i.p. injections. In separate sets of independent experiments, MKK4+/−, MKK4−/−, and MKK4−/+ cells were injected i.p. Using similar end points, mice were euthanized, and i.p. lesions were counted, the number of which did not depend on MKK4 status in either of two experiments. In experiment 1, three mice injected with MKK4+/− cells had 51.7 ± 24.2 (SE) lesions per mouse compared with five MKK4−/−...
cells (solid line; parental+/+ cells in four mice and H3 cells in five mice) and 10 mice receiving MKK4-null cells (dashed line; K1 cells in four mice, K2 cells in two mice, and K3 cells in four mice).

mice having 25.2 ± 5.2 lesions ($P = 0.07$). In experiment 2, three mice injected with MKK4+/− cells had 24.7 ± 9.9 lesions compared with five MKK4−/− mice having 62.6 ± 13.9 lesions ($P = 0.28$).

**In vivo growth: s.c. injections.** We also injected MKK4+/+, MKK4+/−, and three clones of MKK4−/− cells s.c. into the flanks of athymic nude mice. Animal weights and tumor volumes were measured every 2 to 3 days. Injection nearly always produced a tumor. Despite a considerable variation among the mice of each group and among the individual MKK4−/− clones, a clear MKK4-dependent difference was observed: tumors in the MKK4+/+ and MKK4+/− groups behaved similarly, with an average volume-doubling rate of 3.9 days during the phase of exponential growth, whereas the rate of MKK4−/− tumors was 5.1 days during this phase (Fig. 3).

**JNK and p38 activation.** To investigate the role of downstream kinases in mediating stress signals dependent on the MKK4 pathway, we treated our cells with UV light (UVC) and assayed for protein activation. JNK was robustly activated after UVC treatment in the MKK4+/+ and MKK4+/− cells compared with markedly reduced responses among three MKK4−/− clones. p38 responses were similar irrespective of MKK4 status (Fig. 4).

**Discussion**

MKK4 is one of the most consistently mutated tumor-suppressors, but the reason that only 5% of diverse tumor types select for its genetic inactivation is poorly understood. Perhaps, even less well understood is the inverse that despite MKK4 being located on the most frequently lost arm among the human chromosomes (17p), 95% of tumors do not inactivate MKK4. This observation, together with an incongruous MKK4 literature (4, 5, 8), prompted us to do a somatic knockout of the MKK4 gene, open to the possibility of finding either a deleterious or advantageous phenotype.

Empirical data from human studies indicate that tumors experimenting with MKK4-null states will encounter a growth advantage adequate to present itself through the clonal selection process in ~10% of cancers having 17p loss. We infer from these data that the vast majority of tumor cells seem not to experience the MKK4-null state as providing a critical growth advantage. We suggest that we have modeled this majority of cells, providing a rationale for why our MKK4-null cells had a detrimental in vivo phenotype. Interestingly, this growth disadvantage was not evident in culture but required the in vivo setting to be observed.

Currently, it is unknown how best to model MKK4 functions in vivo. Endogenous targeting of MKK4 has the advantage of isolating MKK4 as the agent of study, without potential artifacts caused by models that use the nonphysiologic overexpression of exogenous kinases. Experimental metastasis assays using our model produced results that, when seen in consideration of the previous literature on MKK4 and metastasis (11, 12), were paradoxical.

After the finding of MKK4 mutations in primary tumors, MKK4 overexpression models were developed and suggested that MKK4 is metastasis-suppressing (11, 12). These observations, although prima facie intuitive, are essentially tangential to the observation that MKK4 is mutated clonally in the primary tumors of humans. These mutations simply imply a role in causing the primary tumor. To date, no metastasis-specific mutations have been reported from human cancer patients to suggest that the loss of MKK4 is specifically metastasis-enabling. This observation is consistent with the demonstration that the metastasis occurs with probabilities indicative of a nongenetic mechanism (13). Furthermore, a conceivable role for epigenetic inactivation in metastasis is not supported on the observation that loss of MKK4 protein expression

**Figure 3.** S.c. xenografts. Using 20 mice, cells were injected into the flank of each mouse producing 19 tumors. Nine mice receiving MKK4-competent cells (solid line; parental+/+ cells in four mice and H3 cells in five mice) and 10 mice receiving MKK4-null cells (dashed line; K1 cells in four mice, K2 cells in two mice, and K3 cells in four mice).

**Figure 4.** Stress activation of JNK and p38 after targeted deletion of MKK4. Forty minutes after treatment with UVC, cells were lysed. JNK and p38 activation was assayed by detection of phosphorylated JNK and phosphorylated p38, respectively. Representative of two independent experiments.
is seen at the same rate as the rate of genetic inactivation (~5%) when large numbers of human cancers and metastases are assayed with genetically-calibrated immunolabeling assays (14).

Other instances of paradoxes can be considered. An example relevant to the current studies is the observation that transforming growth factor β signaling is tumor-suppressive in primary mouse mammary cancer while promoting pulmonary metastasis in the same transgenic mice (15). Given that tumors select for the genetic inactivation of MKK4, the gene seems to have a tumor-suppressive function in humans. Yet, in our in vivo model, using cells that model the natural genetic loss of MKK4 in human tumors, we found that disruption of both alleles produced not a growth advantage but a less aggressive (a "detrimental") null state. Cell type specificity may be an important consideration for this paradox because our model of MKK4 loss differs from the naturally occurring MKK4 loss in a significant feature; namely, we have imposed the loss onto cells, whereas the naturally occurring loss is selected from a competitive environment of cells. This is an advantage of an engineered genetic model. For several carcinogenic mutations in human cancers, it remains possible that the aggressively growing mutated cells represent a less common phenotype, brought to attention only through tumor emergence.

Cell-type- or model-specific differences have produced previously paradoxical findings, such as the opposing effects on the development of colorectal cancers in phosphatidylinositol 3-kinase-γ-null mice engineered in different laboratories (16). Similarly, when the tumor-suppressive ability of MKK4 is assayed in different cellular environments, different results are obtained due to cellular influences that may be unrelated directly to metastatic ability. In a recently reported model of spontaneous metastasis, for example, overexpression of MKK4 was associated with decreased lung metastasis but an equivalent growth rate of primary tumors (12). It is possible, however, that overexpression of MKK4 was deleterious in the lungs, where MKK4 was shown to be enzymatically active, but not in the primary tumor, where it was found to be inactive in that study.

Paradoxes have been observed also for oncogenes. For example, the overexpression of the RAS oncogene leads to increased apoptosis and cytotoxicity. More recently, the oncogene AKT1 was found to have possible antimetastatic functions (17). The genome-maintenance genes, such as BRCAl2 (18) and ATR (19), also likely have a detrimental null state after somatic cell knockouts. Our MKK4-null model may be the first demonstration of such a paradoxical phenomenon after somatic disruption of a tumor-suppressor gene, but other examples should be forthcoming.

The literature is similarly divided about the role of p38 in mediating MKK4 stress signals. Hickson et al. (20) recently reported data suggesting the p38 pathway as an important regulatory pathway for metastatic colonization, but Vander Griend et al. (12), using a similar system, reported that the p38-specific kinase MKK6, as compared with the opposite effect of MKK4, had no effect on the rate of metastasis. We found no effect of MKK4 loss on p38 activation (Fig. 4), suggesting that JNK and not p38 is likely responsible for mediating MKK4 signals in our model. Indeed, the role of JNK in tumor development and metastasis is considered controversial, with some evidence for dual tumor-suppressive and oncogenic roles of JNK (21). Such a dual role, however, speaks well to the observed counterweights that balance protumorigenic and antitumorigenic effects experienced by neoplastic cells when they acquire new mutations in central regulatory genes.

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