# Inhibition of MAP Kinase Kinase Causes Morphological Reversion and Dissociation between Soft Agar Growth and *in Vivo* Tumorigenesis in Angiosarcoma Cells

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**Activated ras causes increased activity of several signal transduction systems, including the mitogen-activated protein kinase kinase (MAPKK) pathway and the phosphoinositol-3-kinase (PI-3-K) pathway. We have previously shown that the PI-3-K pathway plays a major role in regulation of ras-mediated tumor angiogenesis in angiosarcoma cells. However, the contribution of the MAPKK pathway to tumorigenesis and angiogenesis is not fully understood. Overexpression of constitutively active forms of MAPKK has previously been shown to transform nonmalignant NIH3T3 fibroblasts, but the effect of down-regulation of MAPKK on tumorigenesis and angiogenesis in a well established tumor has not been fully explored. We introduced a dominant negative MAPKK gene into SVR murine angiosarcoma cells. Introduction of a dominant negative MAPKK causes a significant decrease in proliferation rate *in vitro* and morphological reversion. Cells expressing the dominant negative MAPKK have a greatly decreased ability to form colonies in soft agar compared with wild-type cells. Despite the decreased cell growth *in vitro* and inability to grow in soft agar, the cells were equally tumorigenic in nude mice. Our results suggest that the MAPKK pathway is required for soft agar growth of angiosarcoma cells, and separates the phenotypes of soft agar growth *versus in vivo* tumorigenicity. These findings have implications in the development of signal transduction modulators as potential antineoplastic agents. (Am J Pathol 2000, 157:1937–1945)**

Tumorigenesis due to activation of ras family oncogenes is a common event in solid tumors. Ras is known to activate several signal transduction pathways, including phosphoinositol-3-kinase (PI-3-kinase) and mitogen-activated protein kinase pathways.[1–3] Both of these pathways have been implicated in tumorigenesis and angiogenesis in transformation assays employing NIH3T3 cells.[4,5] Overexpression of PI-3 kinase has been shown to confer resistance to apoptosis in NIH3T3 cells that overexpress the c-*myc* oncogene and rescue epithelial cells from anoikis.[6,7] Expression of activated raf or constitutively active MAP kinase kinase (MAPKK) has been shown to convert NIH3T3 cells into aggressive fibrosarcomas.[4,5,8] In addition, transfection of activated ras and raf into NIH3T3 cells has been shown to cause increased expression of the potent angiogenic chemoattractant, vascular endothelial growth factor (VEGF).[9,10] However, the contribution of these pathways to the maintenance of angiogenesis and tumorigenesis in tumors other than transformed NIH3T3 cells has not been extensively studied.

We have established a two-step model for the development of malignant endothelial tumors by the sequential introduction of a temperature-sensitive SV40 large T antigen and activated H-ras into murine endothelial cells. The addition of activated H-ras causes a switch from cells that produce small quantities of angiogenic mediators to aggressive angiosarcomas, which produce high levels of angiogenic mediators. We have previously shown that treatment with wortmannin, a potent inhibitor of the PI-3-kinase pathway, resulted in down-regulation of the angiogenic mediators VEGF and matrix metalloproteinases, and decreased *in vivo* tumor size. However, inhibition of PI-3-kinase had no effect on the angiogenic antagonist tissue inhibitors of matrix metalloproteinases (TIMPs).[11]

In this report, we describe the effect of inhibition of the MAP kinase signal transduction pathway by both introduction of a dominant negative MAPKK gene into angiosarcoma cells that express SV40 large T antigen and H-ras, and treatment of angiosarcoma cells with a chemical inhibitor of MAPKK, PD98059. These studies show that inhibition of the MAP kinase pathway leads to decreased proliferation and morphological reversion to the untransformed phenotype, as well as greatly decreased growth in soft agar, yet cells remain highly tumorigenic *in vivo*. These results point to a role of the MAP kinase pathway in mediating soft agar growth and a tissue-specific effect of the MAP kinase pathway on tumorigenesis.

## Materials and Methods

### Generation of Cell Lines

MS1 and SVR cells are derived from primary murine endothelial cells and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum.[11] SVR cells were infected with retroviruses encoding β-galactosidase (F. Boyce, Massachusetts General Hospital, Boston, MA) or a dominant negative MAPKK mutant A221 (C. Marshall, Institute of Cancer Research, London, UK). Both vectors encode puromycin resistance, and cells were selected in 2 μg/ml puromycin. β-Galactosidase expression was confirmed by histochemical staining with x-gal,[12] and expression of the dominant negative MAPKK gene was confirmed by growing individual clones and performing Western blot analysis with an antibody specific to rabbit MAPKK and phosphorylated MAPK. Protein extracts were prepared as described in Arbiser et al.[11] Two clones with reduced expression of phosphorylated MAP kinase were selected for further study and were named SVRA221a and SVRA221b. Expression of the dominant negative MAPKK was confirmed using antibody 177 to rabbit MAPKK (C. Marshall). This antibody cross-reacts with endogenous murine MAPKK, so an authentic clone that expresses the gene would be expected to have elevated levels of total MAPKK (rabbit transgene and endogenous murine MAPKK), but diminished phosphorylated MAP kinase. A clone expressing β-galactosidase was named SVRbag4 and was found to have identical expression of phosphorylated MAP kinase as parental SVR cells, and high level expression compared with MS1 cells, which do not contain activated H-ras. Both SVRA221a cells, which express the lowest quantity of phosphorylated MAP kinase, and SVRbag4 cells have been submitted to the American Type Culture Collection (Manassas, VA) for distribution.

### In Vitro *Proliferation Assays*

We plated 10,000 cells of each cell type in 24-well dishes. The next day, the medium was replaced with fresh medium containing the inhibitors or vehicle controls. Cells were incubated at 37°C for 72 hours, and cell number was determined in triplicate using a Coulter Counter (Hialeah, FL). PD98059[13] and LY294002[14] were obtained

from Calbiochem (San Diego, CA) and were reconstituted in dimethylsulfoxide (DMSO) to a final concentration of 5 mg/ml stocks.

### Western Blotting

Cells were lysed in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% (v/v) Triton X-100, 10% glycerol, 1 mmol/L EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L $Na_3VO_4$.

Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard. Samples were treated with Laemmli sample buffer and heated to 90°C for 5 minutes before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (National Diagnostics, Atlanta, GA) and transfer to nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in TBST and subsequently incubated with the appropriate antibody for immunoblotting. Anti-Erk2 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-MAP kinase polyclonal antibody was from Promega (Madison, WI).

### Assays of Matrix Metalloproteinase Bioactivity

Cells were grown to approximately 75% confluence in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. After washing with phosphate buffered saline, medium was replaced with Cellgro Serumless medium (Mediatech, Herndon, VA). Wild-type cells were incubated in serum containing medium supplemented with 5 μg/ml PD98059 or an equal volume of DMSO before shifting into Cellgro medium containing either PD98059 or DMSO vehicle. In the case of SVRA221a or SVRbag4 cell lines, medium was shifted to Cellgro serumless medium for 24 hours before harvest.

Substrate gel electrophoresis (zymography) was conducted as described by us previously.[11] Briefly, Type 1 gelatin was added at a concentration of 1 mg/ml to the standard Laemmli acrylamide polymerization mixture. Conditioned medium from equivalent numbers of cells was diluted 3:1 with sample buffer (10% sodium dodecyl sulfate, 4% sucrose, 0.25 mol/L Tris (pH 6.8), 0.1% bromphenol blue) and electrophoresed as previously described. At the end of electrophoresis, gels were rinsed in 2.5% Triton X-100 for 30 minutes, then incubated overnight in substrate buffer (50 mmol/L Tris, pH 8, 5 mmol/L $CaCl_2$, 0.02% $NaN_3$). The gels were stained with 0.5% Coomassie blue R-250 in acetic acid:isopropyl alcohol: $H_2O$ (1:3:6) and destained in the same buffer in which the Coomassie blue was prepared. Densitometry of destained areas was quantified using a Datascopy GS Plus scanner connected to Macintosh II computer with Macimage software (Xerox Imaging Systems).

### Assay of TIMP Bioactivity

TIMP bioactivity was quantified using conditioned media from equal numbers of cells treated with either PD98059

or vehicle control, or equal numbers of stable cells ex-pressing the dominant negative MAPKK (SVRA221a) or vector control (SVRbag4). A solid collagen film assay was performed using $C^{14}$-labeled collagen as previously de-scribed by us.[11]

## Assay of Anchorage-Independent Growth

Anchorage-independent growth was determined by add-ing equal numbers of viable cells to a plate containing 0.35% Noble agar in 10% fetal bovine serum containing media at a density of either $5 \times 10^4$ or $5 \times 10^5$ cells per 6-cm$^2$ plate. The number of colonies formed in agar was recorded 2 weeks after plating the cells.[15] The effect of the MAP kinase inhibitor PD98059 on soft agar formation of SVRbag4 cells was assessed by continuous treatment of SVRbag4 cells with either 50 $\mu$mol/L PD98059 or DMSO vehicle control for 4 days before trypsinization. Once trypsinized, the cells were placed into growth me-dium containing either 50 $\mu$mol/L PD98059 or DMSO vehicle for the entire soft agar assay.

## In Vivo *Tumorigenesis*

SVRbag4 and SVRA221a,b ($1 \times 10^6$) cell lines were injected into the flank of 6-week-old nude male mice obtained from Massachusetts General Hospital. Three weeks after tumors appeared, they were excised and fixed in both formalin and Carnoy's fixative for hematox-ylin and eosin staining. Tumor volume was measured using the formula (width $\times$ width $\times$ length) $\times$ 0.52, where width represents the shortest dimension.[11]

To determine whether the inability to grow is soft agar was lost after *in vivo* growth, tumors of SVRbag4 and SVRA221a,b were explanted into tissue culture; once confluent, cells were assayed for their ability to grow in soft agar as described above. Cells were analyzed for growth in soft agar and expression of MAPKK by Western blot once explanted from mice.

## Cell Cycle Analysis

Nuclei from cultured cells were isolated and stained with propidium iodide using the CycleTest Plus DNA Reagent Kit (Becton Dickinson, San Jose, CA). Data acquisition and analysis were performed on a FACSort flow cytome-ter (Becton Dickinson) using ModFitLT version 2.0 soft-ware (Becton Dickinson).

## Results

### Generation of Cells Expressing a Dominant Negative MAPKK

SVR cells were infected with recombinant retroviruses encoding either (i) a dominant negative MAPKK mutated at allele Ser-221 in the catalytic domain or (ii) $\beta$-galacto-
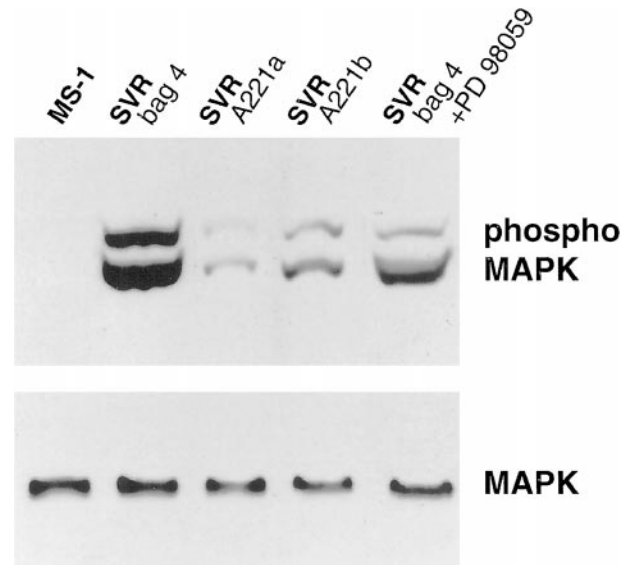


**Figure 1.** Analysis of phospho MAP kinase and total MAP kinase expression in immortalized (MS1) and transformed endothelium (SVRbag4) in the pres-ence and absence of a chemical MAPKK inhibitor (PD98059), and cell lines (SVRA221a,b,c) expressing a dominant negative MAPKK gene. The top gel represents an immunoblot with an antibody specific for phosphorylated MAP kinase, and the bottom gel represents an immunoblot with an antibody for total MAP kinase.

sidase, and selected in puromycin. Resistant clones were screened by Western blot analysis for expression of phosphorylated MAP kinase and for expression of the rabbit MAPKK allele using antibody 177, which is specific for rabbit MAPKK.[16] Two clones expressing decreased levels of phosphorylated MAP kinase were selected for further study. A $\beta$-galactosidase-expressing clone was selected as a control and showed high levels of phos-phorylated MAP kinase expression (Figure 1). This clone had identical expression of phosphorylated MAP kinase compared with parental SVR cells (data not shown).

### Introduction of Dominant Negative MAPKK into SVR Cells Induced Morphological Change

SVR cells have a spindle-type morphology, which is re-tained on introduction of $\beta$-galactosidase (Figure 2B), but treatment of parental SVR cells with PD98059 led to in-creased cellular size and width (Figure 2C). Similar mor-phological changes were seen with introduction of the MAPKK mutant Ser-221 (Figure 2D). These morphologi-cal changes are similar to endothelial cells (MS1 cells), which lack activated ras activity (Figure 2A). Treatment of cells with the phosphoinositol-3-kinase inhibitor LY294002 had no noticeable morphological effect on SVR or SVR-bag4 cells.

### Effect of Signal Transduction Inhibitors on Proliferation of SVRbag4 and SVRA221a Cells

Both cell lines were grown in the presence of a MAPKK inhibitor, PD98059, and a phosphatidylinositol-3-kinase inhibitor LY294002 in doses ranging from 0 to 10 $\mu$g/ml,

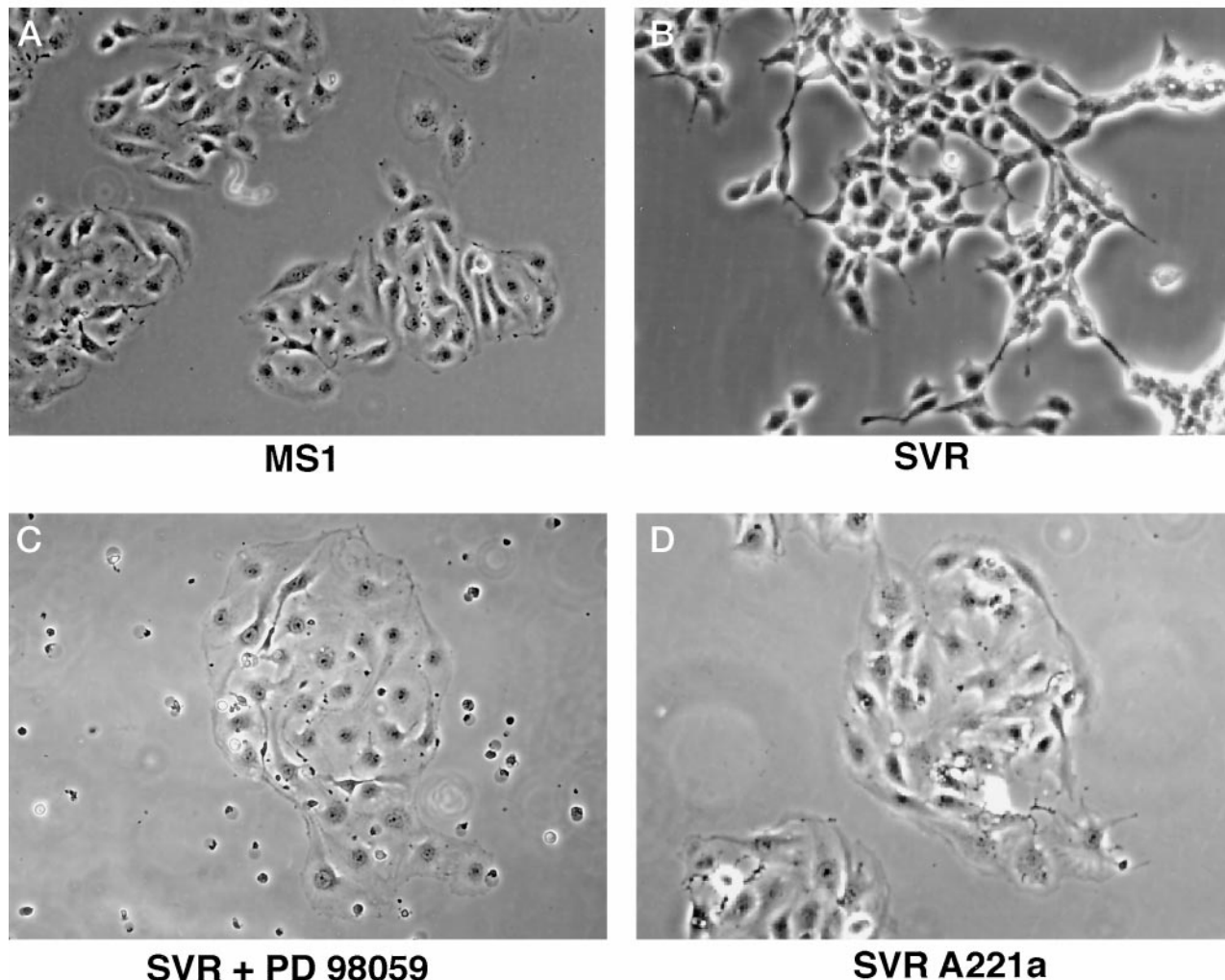## Effect of MAPKK inhibition on cell morphology



**Figure 2.** Effect of inhibition of MAPKK by a dominant negative MAPKK gene or by the chemical inhibitor PD98059 on morphology of endothelial cells. MS1 represents endothelial cells containing only SV40 large T antigen; SVR represents MS1 cells transformed with ras; SVR+ PD98059 represents SVR cells treated with PD98059 (5 $\mu$g/ml); and SVRA221a represents cells stably expressing the dominant negative A221 allele of MAPKK. The morphology of SVRAnd SVRbag4 cells are identical. Original magnifications, ×40.

and cell counts were performed 72 hours later. Introduction of the 221 allele of MAPKK led to a decrease of nearly 75% in growth rate. Both cell types were susceptible to growth inhibition by LY294002, the phosphatidylinositol-3-kinase inhibitor, in a dose-dependent fashion, but the SVRA2221a cells were relatively resistant to growth inhibition by PD98059. In contrast, growth of control SVR-bag4 cells was highly inhibited by PD98059 (Figure 3). No cytotoxicity was observed as a result of treatment with either PD98059 or LY294002.

Total RNA was extracted from SVRA221a cells, which stably express the dominant negative A221 allele of MAPKK, or SVRbag4, which serve as wild-type control. No significant differences were observed in VEGF mRNA expression (data not shown).

### Effect of MAPKK Inhibition on MMP and TIMP Bioactivity

Treatment of SVR cells with the low molecular weight inhibitor PD98059 caused a modest increase in gelatinase activity in conditioned media (Figure 4). A similar pattern of enhancement of MMP bioactivity was observed in SVRA221a, which expresses the dominant negative MAPKK gene, compared with the vector control cells SVRbag4 (Figure 4).

Conditioned media from equivalent numbers of SVRA221a and control SVRbag4 cells were tested in a radiometric enzyme assay for TIMP activity. As shown in Figure 5, acute down-regulation of MAPKK by PD98059 resulted in up-regulation of TIMP bioactivity, whereas
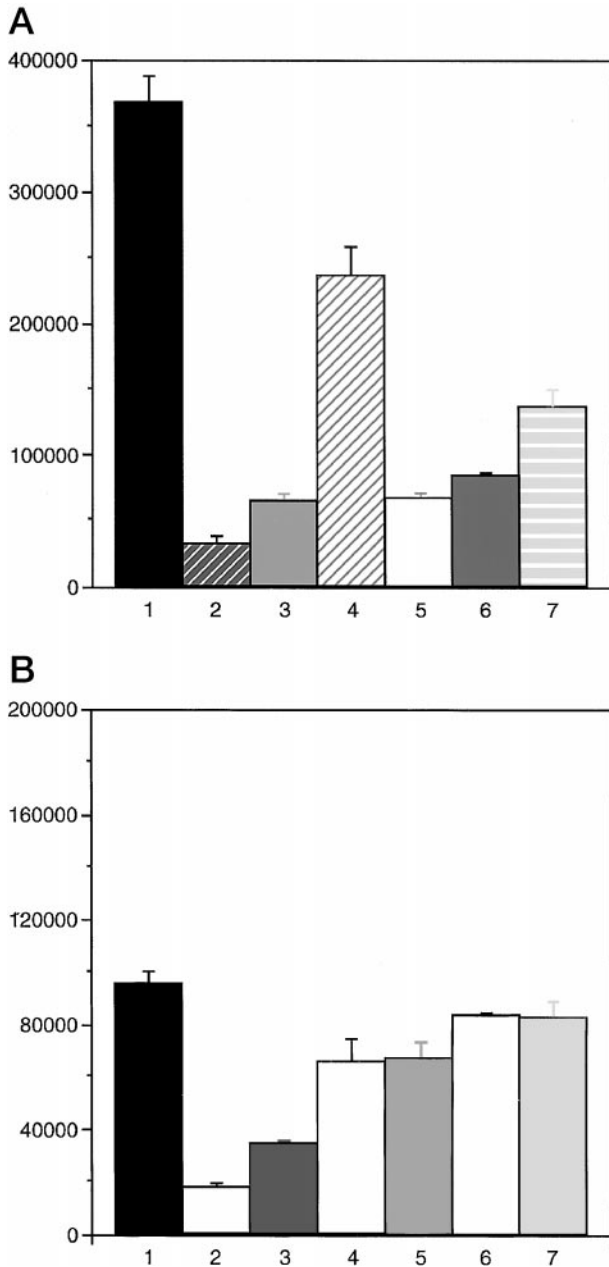
**Figure 4.** Effect of MAPKK inhibition on metalloproteinase bioactivity in SVR-conditioned media. Conditioned media from equal numbers of cells were electrophoresed through an acrylamide gel containing gelatin. White bands represent gelatinolysis. The first two lanes represent SVR cells treated with vehicle (DMSO) or PD98059; the second two lanes compare a vector control (SVRbag4) with a clone expressing a dominant negative (SVRA221a). Experiments were repeated in triplicate.

stable down-regulation of MAPKK resulted in no change in TIMP bioactivity (Figure 5).

## Effect of MAPKK Inhibition on Anchorage-Independent Growth

The overexpression of a dominant negative mutant form of MAPKK suppressed ras-induced anchorage-independent growth in angiosarcoma cells. MS1 endothelial cells transformed by H-ras and containing a vector control (SVRbag4) display anchorage-independent growth as measured by their ability to form colonies in soft agar (Figure 6 and Table 1). Ectopic expression of a dominant negative mutant form of MAPKK in the ras-transformed angiosarcoma cell lines (SVRA221a,b) dramatically decreased the number of colonies observed in soft agar, as compared with SVRbag4 (Figure 6 and Table 1). Similarly, treatment of wild-type SVR cells with PD98059 suppressed the ability of these cells to grow in soft agar. The



**Figure 3. A:** Dose response of SVRbag4 cells to the MAPKK inhibitor PD98059 and the PI-3-K inhibitor LY294002. The first lane represents cell counts of untreated cells; the second lane, cells treated with 10 $\mu$g/ml LY294002; the third lane, 5 $\mu$g/ml LY294002; the fourth lane, 1 $\mu$g/ml LY294002; the fifth lane, 10 $\mu$g/ml PD98059; the sixth lane, 5 $\mu$g/ml PD98059; the seventh lane, 1 $\mu$g/ml PD98059. Cells were treated with inhibitor for 72 hours, and cells were counted. All experiments were repeated in triplicate, and the error bar represents SEM. All doses of inhibitors resulted in significant inhibition compared with untreated cells, $P < 0.05$. **B:** Dose response of SVRA221a cells to the MAPKK inhibitor PD98059 and the PI-3-K inhibitor LY294002. The lanes are labeled as in **A**. Cells were treated as in **A**. All experiments were performed in triplicate, and the error bar represents SEM, $P < 0.05$. Treatment of SVRA221a cells with the PI-3-K inhibitor led to significant decreases in cell proliferation ($P < 0.05$), but there was no significant decrease in proliferation as a result of treatment with the MAPKK inhibitor PD98059.
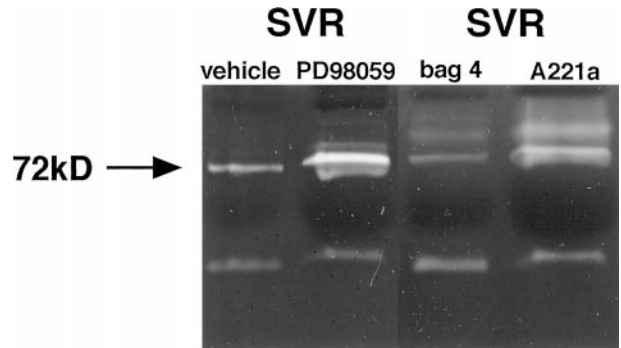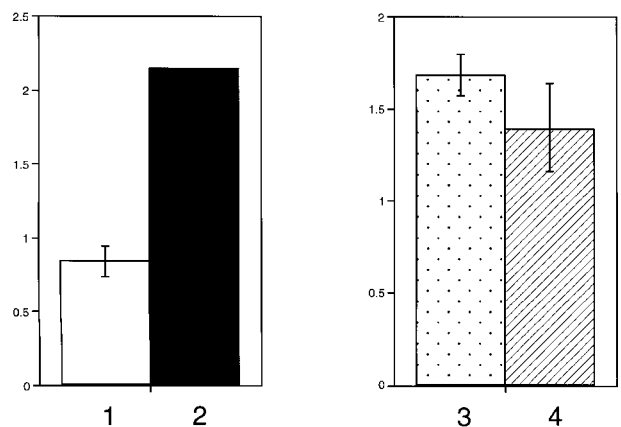


**Figure 5.** Effect of MAPKK inhibition on TIMP bioactivity. The first graph examines the effect of acute inhibition of MAPKK by addition of PD98059 on TIMP bioactivity. This experiment was repeated in triplicate. **Lane 1** represents vehicle-treated cells; **lane 2** represents cells treated with PD98059. The second graph examines the effect of chronic inhibition of MAPKK (SVRA221a) by stable expression of dominant negative MAPKK. **Lane 3** represents SVRbag4 (vector control cells), and **lane 4** represents conditioned media from an equivalent number of SVRA221a cells.
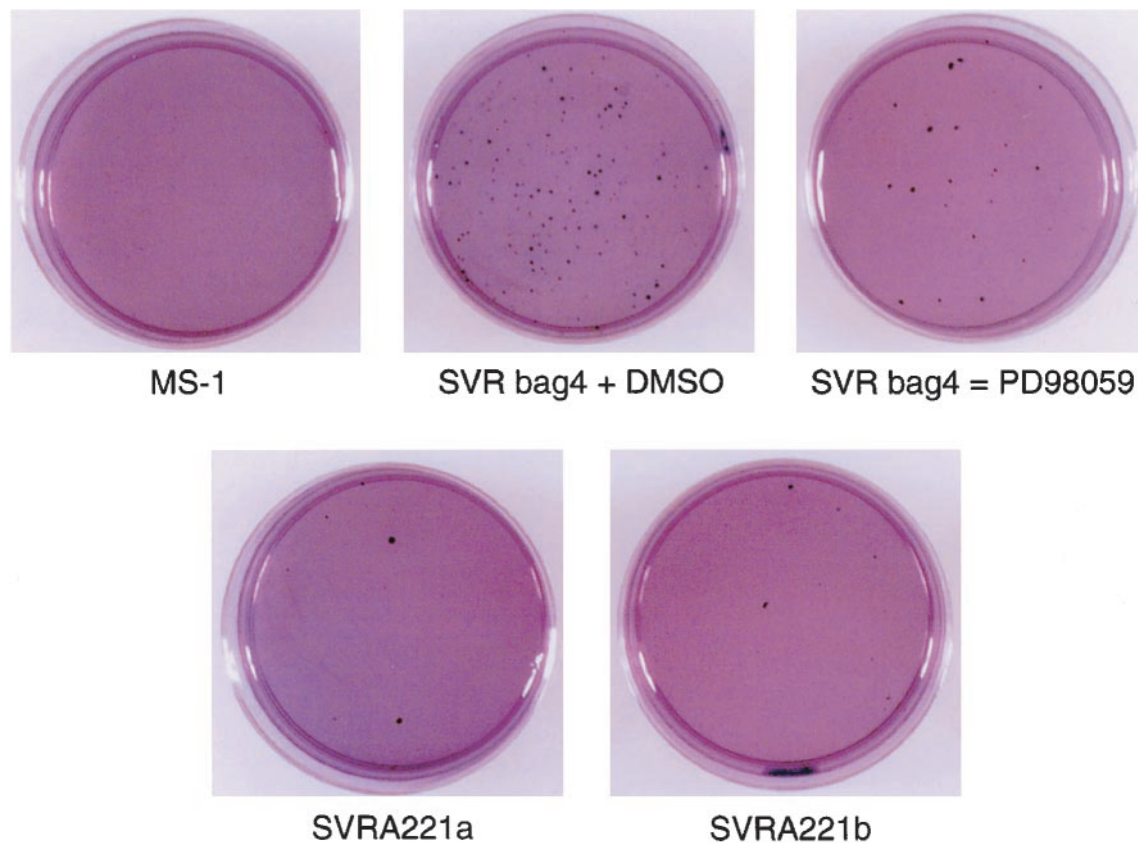
**Figure 6.** A representative soft agar assay stained with 0.2% Giemsa 2 weeks after plating. MS1, SVRbag4 treated with 50 $\mu$mol/L PD98059 or vehicle control, and SVRA221a,b cells were seeded at either $5 \times 10^4$ cells per 6-cm$^2$ dish. Macroscopic colonies were counted 14 days after plating the cells in agar.

ability of the cell lines to grow in soft agar correlated with the expression levels of phosphorylated MAP kinase. These data suggest that activation of MAPKK is required for ras-induced anchorage-independent growth of angiosarcoma cells in soft agar.

### Effect of MAPKK Inhibition on *in Vivo* Tumorigenesis

One million cells of each type were injected subcutaneously into the right flank of nude male mice. The tumors derived from the cells expressing the dominant negative MAPKK gave rise to rapidly growing tumors that did not vary significantly in size from wild-type SVRbag4 tumors (Figure 7). The decreased phosphorylation of MAPKK was also observed after *in vivo* growth, thus ruling out the

possibility that tumor growth in animals was secondary to loss of the transfected gene (data not shown). The finding of equal tumorigenesis of the clones of SVR cells is surprising, in light of the greatly impaired ability of the SVRA221a,b cell lines to form colonies in soft agar. The decreased ability of cells containing the dominant negative MAPKK was preserved after *in vivo* growth, as cells explanted from rapidly growing SVRA221a,b tumors showed diminished growth in soft agar compared with wild-type SVRbag4 cells. The average volume of the SVRbag4 tumors was 1225 mm$^3$, of SVRA221a tumors, 2783 mm$^3$, and of SVRA221b tumors, 1468 mm$^3$. The tumor volume of SVRA221a and SVRA221b did not differ significantly from that of SVRbag4.

### Cell Cycle Analysis

To determine whether the decreased growth rate of SVRA221a cells was due to decreased plating efficiency or was secondary to changes in cell cycle progression, cells were stained with propidium iodide and analyzed by flow cytometry. Wild-type SVRbag4 cells showed the following percentages of cells in G0-G1: 53.6%, G2-M: 4.3%, and S: 42.1%, whereas SVRA221a cells showed G0-G1: 59.4%, G2-M: 15.7%, and S: 24.9%. Thus, SVRA221a cells showed a decreased percentage of cells in S phase, suggesting that the decreased growth *in vitro* is due in part to slowing of cell cycle progression.

**Table 1.**  Cell Line Data

| Cell line | Number of colonies |
|---|---|
| MS1 | 0 |
| SVRbag4 | 2039 ± 74 |
| SVRbag4/PD98059 | 837 ± 66 |
| SVRA221a | 21 ± 3 |
| SVRA221b | 54 ± 11 |

Data represent the mean ± standard error of the number of colonies observed in three independent experiments in cells before injection into animals, each done in triplicate. The difference in colony formation between SVR bag4 and SVR A221a,b are significant ($P < 0.05$).

**Figure 7.** Effect of dominant negative MAPKK expression on *in vivo* tumorigenesis in nude mice; $n = 5$ mice per group. The differences in tumor volume are not significant ($P > 0.05$). The mouse under **1** contains a representative SVRbag4 tumor; the mouse under **2** contains a representative SVRA221a tumor; and the mouse under **3** contains a representative SVRA221b tumor.

## Maintenance of Phenotype after Tumorigenesis in Mice

To determine whether the dissociation of soft agar growth and *in vivo* tumorigenesis was maintained, we explanted tumors from animals bearing SVRbag4 and SVRA221a,b into tissue culture. Cells were cultured from tumors, and the ability of these cells to form colonies in soft agar was analyzed. Consistent with the phenotype before implantation, SVRA221a,b were significantly impaired in their ability to form colonies in soft agar, even after *in vivo* growth (Table 2). In addition, high level expression of transgene was observed in explanted cells, with A221a exhibiting a sixfold increase and A221b showing a twofold increase in total (transgene and endogenous) MAPKK compared with SVRbag4 (endogenous murine MAPKK; Figure 8). This is consistent with maintenance of the phenotype after *in vivo* growth.

## Discussion

Mutations in ras are associated with numerous forms of human malignancy, but the individual contributions of the

**Table 2.** Soft Agar Assay from Cells Derived from Tumors

| Cell line | Number of colonies |
|---|---|
| SVRbag4 | $159 \pm 27$ |
| SVRA221a | $16 \pm 3$ |
| SVRA221b | $111 \pm 12$ |

Data represent the mean ± standard error of the number of colonies observed in two independent experiments in cells explanted from mice bearing tumors, each done in triplicate. The difference in colony formation between SVRbag4 and SVRA221a,b are significant ($P < 0.05$).
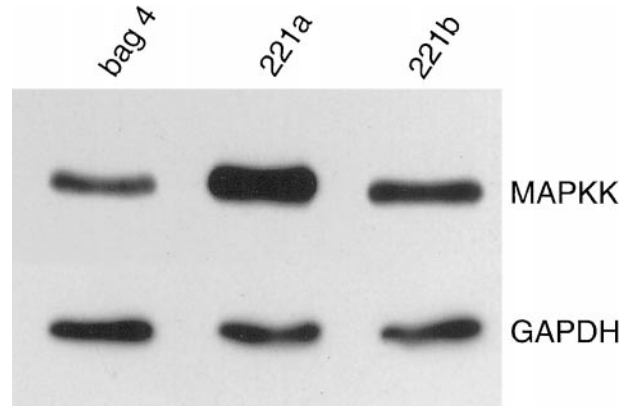


**Figure 8.** Western blot analysis of expression of dominant negative MAPKK after *in vivo* growth. The upper band represents total MAPKK (transgene plus endogenous murine MAPKK), and the bottom lane represents GAPDH, a control for loading. A sixfold increase is noted in SVRA221a and a twofold increase is noted in SVRA221b compared with SVRbag4.

signal transduction pathways activated by ras is less well understood. Ras has been shown to activate multiple signal pathways including MAPKK and PI-3-K. Most of the studies involving ras and MAPKK have used NIH3T3 cells, an immortalized fibroblast cell line deficient in the tumor suppressor gene p16.[17] Introduction of dominant negative MAPKK into ras transformed NIH3T3 fibroblasts causes loss of tumorigenesis, thus MAPKK plays an important role in ras transformation of NIH3T3 cells.[18] However, recent evidence suggests that the findings in NIH3T3 fibroblasts may not be generalized to other cell types. For example, oncogenic raf, which causes activation of MAPKK, is insufficient to transform immortalized intestinal epithelial cells.[19,20] This may be due to either the cell type (intestinal epithelium *versus* fibroblast) or the genetic background of the cell line being transformed by ras. We previously described a two-step system of transformation in endothelial cells to study the contribution of these signal transduction pathways in promoting tumorigenesis and angiogenesis. Introduction of H-ras into immortalized endothelium results in increased *in vivo* proliferation relative to apoptosis and increased production of the angiogenic mediators VEGF and MMP, as well as decreased production of the angiogenesis inhibitor TIMP.[11] We have previously shown that the PI-3-K pathway regulates the production of the angiogenic mediators VEGF and MMP, and treatment of animals with the PI-3-K inhibitor wortmannin resulted in a significant decrease in tumor size. Consistent with our results, expression of the p110 catalytic subunit of PI-3-K caused transformation of chicken endothelium into angiosarcomas.[21] The effect of MAPKK modulation in this system has not been fully explored. Because of this, we initially sought to introduce a constitutively active MAPKK into large T immortalized, nontumorigenic endothelial cells. We were unable to obtain any stable clones overexpressing a constitutively active MAPKK. This result may be secondary to a growth inhibitory effect of raf-MAPKK pathway overexpression observed in certain cell types.[22–24]

Primary animal cells and untransformed cell lines require a solid surface on which to attach, spread, and

multiply, a phenomenon known as anchorage-dependent growth. These cells, if detached from solid supports, undergo apoptosis through a process termed anoikis.[25] Researchers have taken advantage of the property of transformed cells to grow in suspension, using agar, to show the ability of viruses to transform cells in an anchorage-independent assay.[26–29]

A correlation exists between anchorage-independent growth and tumorigenicity in mice.[15,26–29] Shin et al have demonstrated that other cellular phenotypes of transformation, such as cell density and growth in reduced serum are not strongly correlated with neoplastic growth *in vivo*.[29] The ability of cells to grow in the absence of adhesion presumably reflects the ability of transformed cells to survive and grow in inappropriate locations *in vivo*.

In this report, using the various endothelial cell lines, we tested their transforming ability *in vitro* by a colony formation assay in soft agar. These results suggest that the endothelial cell line MS1, which is normally anchorage-dependent, requires the activation of MAPKK downstream of ras to be transformed *in vitro*. In contrast, SVRA221a,b cells, which demonstrate low levels of MAP kinase activation and poor growth in soft agar, are fully tumorigenic *in vivo*. A potential predictor of the effect of signal transduction inhibition in a tumor cell line may be the bioactivity of MMPs, enzymes required for tumor growth and metastasis.[30] Inhibition of MAPKK in our system, by both the small molecular weight inhibitor PD98059 and a dominant negative MAPKK led to an increase in MMP bioactivity. Another MAPKK-related gene, MAPKK-4, has been implicated as a tumor suppressor gene in gastrointestinal malignancy,[31] so pharmacological inhibition of MAPKK may be beneficial in some malignancies, but not in others.

Our findings, that introduction of a dominant negative MAPKK slows growth *in vitro* and inhibits soft agar growth, but not tumorigenesis, are surprising. However, one must take into account the different environments of these assays. Growth *in vitro* is partially a function of the cell to proliferate in two dimensions attached to a plastic surface. Growth in soft agar requires three-dimensional growth without attachment to physiologically relevant basement membrane proteins and host integrins. Growth *in vivo* involves three-dimensional growth in the presence of host basement membrane proteins and integrins. Our results suggest that down-regulation of MAP kinase may play a role in two- and three-dimensional growth in a solid area in the absence of host proteins and integrins, but is not required for *in vivo* growth. Reliance on the soft agar assay alone may lead researchers to overlook potentially important oncogenic events.

Our results have several implications. First, MAPKK is not likely to be the major signal transduction pathway in regulating tumorigenesis and angiogenesis in ras-transformed angiosarcoma. Recent data showing that overexpression of the catalytic subunit of PI-3-kinase results in avian angiosarcomas suggest that PI-3-kinase mediates the angiogenic switch in angiosarcoma.[21] Second, growth rate *in vitro* cannot always be correlated with *in vivo* tumorigenicity. Control SVRbag4 cells grow much more rapidly than MAPKK-deficient SVRA221a cells *in vitro*, but are about equally tumorigenic. Consistent with our studies in mice, we have demonstrated that phosphorylated MAP kinase is elevated in benign endothelial neoplasms (hemangiomas and pyogenic granulomas), but shows very low level expression in malignant angiosarcoma.[32] These findings suggest that high level expression of phosphorylated MAP kinase is not required for human angiosarcoma growth *in vivo*, consistent with the findings of this study. Examination of expression of phosphorylated MAP kinase may prove diagnostically useful in endothelial neoplasms. Finally, prior studies have concluded that anchorage-independent growth is synonymous with tumorigenesis, and, conversely, that loss of soft agar growth means cells are incapable of forming tumors. Our results suggest that this correlation is not perfect, and that *in vivo* studies are required to fully assess tumorigenesis. Knowledge of the effect of MAPKK inhibition on *in vivo* growth will be necessary to determine the type of tumor that will be most responsive to MAPKK inhibition. Finally, MAPKK inhibitors may be pharmacologically useful for some malignancies but may fail to suppress the growth of other malignancies, depending on tissue type.

## Acknowledgments

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