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TITLE: Evaluation of the Role of the Metastasis-Suppressor Gene
MKK4/SEK1 in Transgenic Models of Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) Metastasis-suppressor genes suppress the growth of metastases without affecting tumor growth. We have been studying the role of inactivation of one such metastasis suppressor gene, Map Kinase Kinase 4 (MKK4) in the process of metastatic colonization. Work proposed in this application is aimed at extending our ongoing studies in the AT6.1 model system into established transgenic models of prostate cancer and the use of transgenic approaches to test further MKK4's metastasis suppressor activity. In the past year we have applied the tools generated in the preceding year and found a need for changing our experimental design. As described in this report, we are establishing new collaborations to address these issues. We are also obtaining additional frozen samples to strengthen our correlative studies				
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1. Introduction

Despite improvements in the detection and treatment of primary tumors, metastatic disease remains refractory to current therapeutic interventions. Thus, presence of detectable metastases remains the hallmark of incurable disease. Metastasis is a dynamic process that requires cell from a primary tumor to: 1) invade and migrate through primary tumor stroma, 2) intravasate into vascular structures at the primary site, 3) survive transport through the vasculature to a discontinuous site, 4) extravasate from the vasculature at a secondary site, 5) survive and ultimately proliferate at a secondary site. Disseminated cells which do not complete all of these steps are not metastases.

A wealth of studies have shown that the process of metastasis requires a cancer cell(s) to have the ability to carry out a complex set of cellular functions (i.e. alterations in cellular motility, adhesion, deformability, resistance to extracellular stress, etc.) as it makes its way from a primary tumor to distant sites within the host. While establishing a correlation between 'metastatic ability' and molecular and cellular parameters (e.g. changes in tumor pathology, mutation of cancer-related genes or gene expression profiles, etc.) has been relatively straightforward, demonstrating a functional relationship between these specific alterations and specific steps in metastasis has posed a significant challenge. For more than two decades the majority of metastasis studies focused on events related to escape of cells from the primary tumor. The focus on invasion was logical given that both clinical and experimental observations indicated that escape of cells from the primary tumor was the rate-limiting event determining metastatic efficiency. Recently, however, the development and application of new technologies has enabled studies which have resulted in a re-examination of the steps within the metastatic cascade.

During the past decade clinical correlative experiments using RT-PCR and immunohistochemistry have indicated that cells may disseminate from the primary tumor very early in the course of disease and often lie dormant at secondary sites as clinically undetectable microscopic metastases at the time of cancer diagnosis. Such data corroborate studies that demonstrate that even after successful surgery for localized tumors; cells can still be detected in the circulation and at distant sites. Simultaneously, development of improved techniques for detection and imaging tumor cells within intact tissues (i.e. fluorescence microscopy, intravital video-microscopy, cell tracking with quantum dots, etc.) are enabling investigators to visualize and quantify the steps of metastasis in real-time. These studies using well-characterized animal models of metastasis have yielded experimental data which supports clinical findings. Taken together these findings suggest that single disseminated cancer cell(s) which survive at secondary sites are not by definition autonomous; they remain subject to growth controls in their immediate environment. Thus, metastatic colonization must be considered a potential rate-limiting step of metastasis formation.

The observation that disseminated cancer cells may persist for significant periods of time without forming metastases has raised many questions regarding dormancy of disseminated cells at metastatic sites. Dormancy is broadly-defined term used to describe behavior of disseminated cells which are present at secondary sites but have not yet given rise to overt metastases. While the concept of dormancy was brought to the fore by the findings in angiogenesis research, it is becoming clear that steps prior to the induction of angiogenesis also contribute to the dormant phenotype. The working hypothesis is that disseminated cells exist either as single cells or stable, non-proliferating microscopic metastases for extended periods of time.

The direct clinical relevance of the study of factors regulating dormancy is emphasized by studies demonstrating disseminated, viable prostate cancer cells in the bone marrow of patients' years after successful treatment for their primary tumors. Such cells are considered dormant, because they have *not yet and may never* cause clinically relevant disease. The importance of *persistent dormant cells* and the mechanisms by which these cells can *break dormancy* and give rise to lethal metastases is being debated. Detection of disseminated cancer cells raises new clinical questions. How can we distinguish disseminated cells that will remain dormant 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 work began more than ten years ago with the hypothesis that identification of genes that encode proteins which specifically suppress metastasis *in vivo* could lead to the identification of both biological and biochemical pathways that control specific steps of metastasis. Metastasis suppressor genes (MSGs) were operationally defined as genes that encode proteins that suppress the formation of overt metastases while exerting no measurable effect on primary tumor growth. Since then a wealth of data has been generated that shows that in addition to alterations in oncogenes and tumor suppressor genes, the acquisition of metastatic ability requires additional genetic and epigenetic changes. Functional studies as well as clinical correlative studies have identified metastasis suppressor functions for both novel and known genes (proteins) including NM23, MKK4, Brms1, Kiss1 and KAI1 and RKIP. It is interesting to note that when we initiated these studies we never anticipated that genes/proteins thus identified would play a role in regulation of metastatic colonization. Indeed our work on MKK4 and our colleagues' work on NM23 and Brms1 have identified a novel role for these proteins in regulation of metastatic colonization and potentially dormancy of disseminated cancer cells.

2. Body

We have previously identified a role for the stress-activated protein kinase (SAPK) signal transduction pathway in the suppression of metastatic colonization. Specifically, our laboratory identified the mitogen-activated protein kinase kinase 4/c-Jun NH2-terminal kinase-activating kinase/stress-activated protein/Erk kinase 1 (hereafter referred to as *MKK4*), as a metastasis-suppressor gene encoded by human chromosome 17p11.2 [1]. Ectopic expression of MKK4 in highly metastatic Dunning AT6.1 rat prostatic cancer cells suppressed the metastatic ability of the cells by approximately 77%. Using biologic approaches we have recently shown that equal numbers of cells escape from the primary tumor and reach secondary sites further supporting our initial observation that MKK4 metastasis-suppressed cells complete all early steps in the metastatic cascade, but are growth-inhibited at the secondary site. More recently our laboratory has shown that suppression of colonization is dependent on MKK4's kinase activity. In addition, MKK4 appears to signal through the JNK MAPK. While we initially thought that MKK4's activity induced apoptosis, our recent studies suggest that at solitary disseminated cells expressing MKK4 are growth suppressed, but viable. Kim *et al.* found that MKK4 expression was down regulated in clinical prostatic tumors with increased metastatic potential. In collaboration with Yamamada and her colleagues we found similar results in ovarian cancer. Specifically down regulated in clinical ovarian cancer metastases and MKK4 expression suppresses metastatic colonization in xenograft models of ovarian cancer.. These findings support a role for MKK4 as a metastasis suppressor gene in clinical cancers.

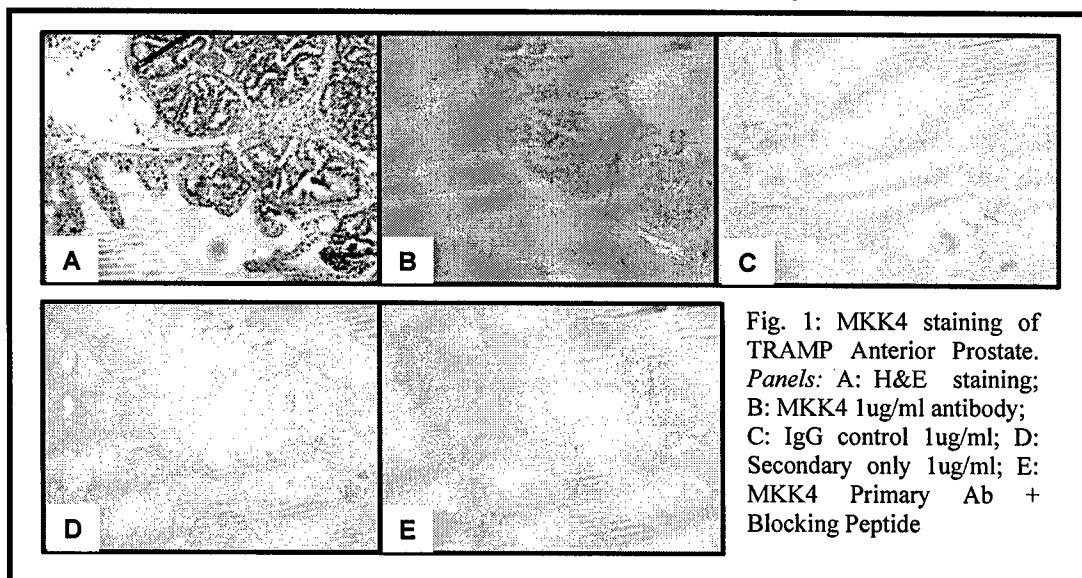
The purpose of the work proposed in this DOD Idea Award was to extend our findings from clinical materials and xenograft models into additional prostate cancer models. This has been a difficult task for us and for other researchers focused on dissecting the function(s) of metastasis suppressor proteins. Indeed, our colleagues studying Brms1 and NM23 have not successfully conducted studies analogous to the ones that we proposed. The overall objectives of our work continue to be correlative studies of MKK4 expression in transgenic models of prostate cancer and to test the ability of MKK4 down regulation to promote metastasis in transgenic models of prostate cancer. Based on our previous studies we hypothesize the expression of MKK4 protein will be down regulated in high grade primary tumors that give rise to metastases and metastatic lesions.

3. Key Research Accomplishments

- We used our immunohistochemical detection technique to evaluate expression of MKK4 in a series of rodent tissues.
- We evaluated our gene targeting construct for generation of Floxed MKK4 mice.

4. Reportable Outcomes

- **Immunohistochemical detection of MKK4.** We used our optimized protocol to evaluate expression of MKK4 in a series of rodent tissues. As anticipated we observed high level staining in normal prostate tissues (N=20). Representative immunohistochemical staining is shown in Figure 1. We saw decreased staining in primary tumors which showed a trend in toward correlation between absence of staining and aggressiveness of the primary tumor (N=20). At this point the data cannot be evaluated appropriately for statistical significance because of the small sample number. We have been able to stain a limited number of metastases (N=10) and also saw low level staining in many samples. In order to reach power sufficient for appropriate analysis we will need to increase the sample size and, it is hoped, to also collect metastatic tissues from the same animals. Having matched primary tumors and metastases would significantly strengthen the analysis. When this study was originally designed the prevailing view was that metastasis formation was a common event in TRAMP mice. Since then this notion has been shown to be incorrect. Metastases are infrequent and those that arise are often neuroendocrine in nature. This has presented a significant problem for accumulating tissues for meaningful analysis. An additional complication that has become apparent is that the background on which the Tag expressed has a profound effect on the extent and type of metastases observed. The method we proposed to generate metastatic tissues (in our original application) will not yield adequate or appropriate tissues for the study.



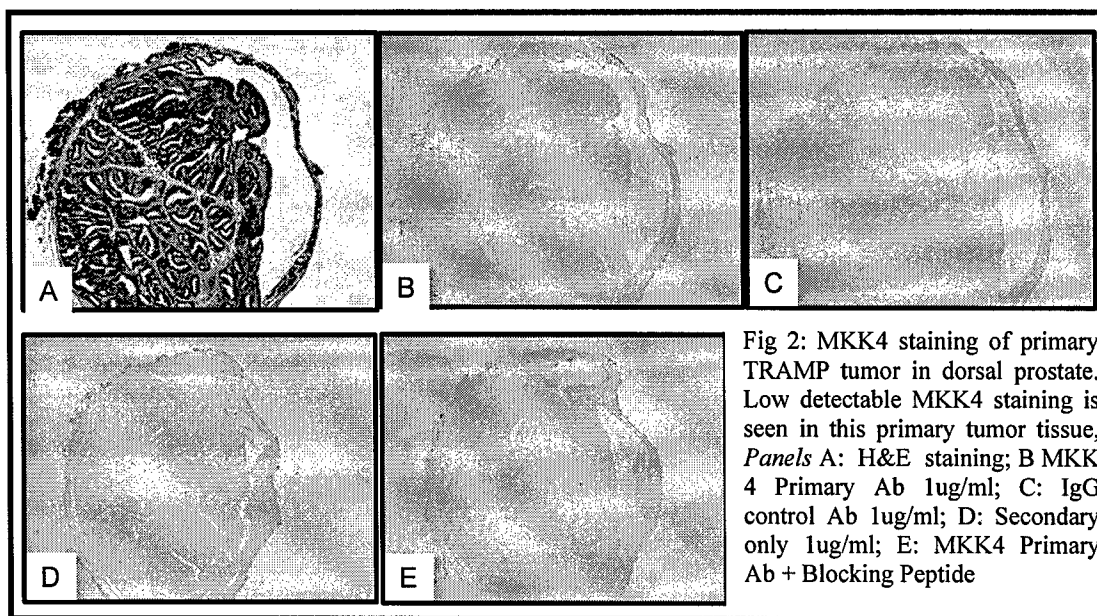


Fig 2: MKK4 staining of primary TRAMP tumor in dorsal prostate. Low detectable MKK4 staining is seen in this primary tumor tissue, *Panels A: H&E staining; B MKK 4 Primary Ab 1ug/ml; C: IgG control Ab 1ug/ml; D: Secondary only 1ug/ml; E: MKK4 Primary Ab + Blocking Peptide*

In order to address these two issues we are initiating collaborations with Dr. Barbara Foster (Roswell Park Cancer Center) and Dr. Kent Hunter (NCI). Dr. Foster has significant expertise in the TRAMP model and a repository of tissues that should ensure sufficient power to detect significant differences in MKK4 expression. She also routinely breeds the TRAMP mice onto different genetic backgrounds in sufficient numbers to develop additional tissues if needed. (See letter of Collaboration). Dr. Hunter is a mouse geneticist whose specific interest is the affect of host genetic background on metastasis. We believe that these interactions will enable us to solve both the experimental and technical problems faced in work in this aim.

- Construction of MKK4/SEK1 targeting vectors.** We developed a targeting vector that would enable us to insert Flox sites for the excision of the tissue-specific excision of MKK4 when mice were crossed with probasin Cre mice. Design of these targeting constructs, shown in Figure 3, was previously described. These constructs were completed and ready for use when, upon further evaluation of these constructs, we determined that there an there was a flaw in their design and their use generated a hypomorph phenotype. This would result in a partial loss of MKK4/SEK1 protein function that would confound our

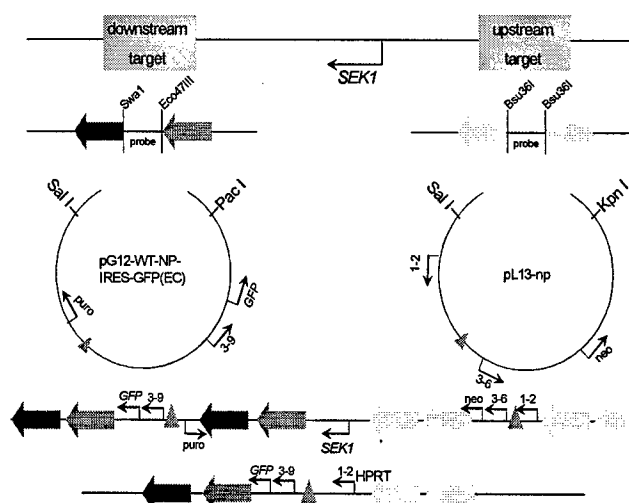


Fig 3. Revised MKK4/SEK1 Targeting Scheme. Further analysis of this strategy showed that it would result in a hypomorph phenotype which would confound our studies

studies. While the targeting vectors may still be used as a tool for some gene dosage studies, it does not meet the needs of our experimental design. In addition, because of the technology available at the time of the initial proposal, the original design did allow us to track and identify disseminated cells at metastatic sites. We now need to redesign the targeting approach to take into account both of these issues. Because of difficulties we have faced in the design and implementation of a targeted scheme for the construction of the transgenic mice for our studies, we spent a significant amount of time looking for an additional collaborator who can help us with these issues. In addition to consulting with Drs. Foster and Hunter, are recruiting Dr. Kay Macleod to help us in the design and implementation of these redesigned vectors. Dr. Macleod is an outstanding biochemist with significant expertise in transgenic model design. She helped us to uncover the hypomorph mutation and has agreed to work with us on the design and construction of vectors for a new targeting strategy. We anticipate that this will be a significant improvement over our initial design and will be a useful reagent for many experiments testing MKK4 function in metastasis.

5. Conclusions

We have identified flaws in our experimental design and deficits in our scientific team and have found ways to solve these issues by initiating new collaborations with truly outstanding collaborators. We are holding a meeting with these collaborators in the near future to lay out specific plans to address our experimental and technical needs. We hope to complete the IH evaluation of normal tissues, primary tumors and metastases from transgenic models of prostate cancer during the no cost extension of the proposal. We also hope to have completed the construction of the new targeting vector.

6. References

7. Appendices



The future is at Roswell.

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September 15, 2005

Carrie Rinker-Schaeffer
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Dear Carrie,

I am pleased to be a collaborator on your project for the Department of Defense on "**Evaluation of the role of the metastasis-suppressor gene MKK4/SEK1 in transgenic models of prostate cancer**". I am excited about your research exploring MKK4/SEK1 in metastasis of prostate cancer. This is an important area of research that should be explored further given the strong data implicating a role of signaling molecule in metastasis and the clinical importance of metastasis in the lethal phenotype of prostate cancer. The TRAMP model may be an ideal model for exploration of MKK1/SEK1 in prostate cancer progression since the prostate develops normally and at sexual maturity the transgene (SV40 T antigen) is expressed in the prostatic epithelium. I Understand the problems that your group has faced in your studies and I am delighted to join your effort to test MKK4's potential function in this model system. I have extensive experience working with the TRAMP model and used it to identify molecular changes associated with prostate cancer progression. With my collaborators we have tested immune based therapies (CTLA-4 blockade) for treatment of localized and metastatic prostate cancer in the model. My lab currently utilizes the TRAMP model to evaluate new therapies for the prevention and treatment of prostate cancer. We have a homozygous colony of TRAMP animals that can be used to breed the experimental animals needed for these studies. Furthermore we have established a tumor tissue bank for TRAMP tumors through progression of the disease including metastatic lesion. We can provide the experimental TRAMP animals and TRAMP tissues for the studies outlined in your proposal. I am willing to score all of the TRAMP tumors using the grading system established for TRAMP. In addition, I will be happy to lend my expertise with regard to any other issues that may arise during the course of these studies. I am very excited about our research. This line of research could lead to new approaches for the treatment of prostate cancer. I look forward to our continued collaborations and the results using the TRAMP model.

Best wishes,

Barbara A. Foster, PhD