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13. ABSTRACT *(Maximum 200 Words)*

Metastasis to the lung is a common occurrence accounting for approximately 60-70% of metastasis. We have developed a transgenic model of lung metastasis using a MMTV-c-myc/MMTV-VEGF bitransgenic mouse system. We are using this system to evaluate the mechanism by which VEGF is able to dramatically increase metastasis to the lung. In spite of technical difficulties that have resulted in delays in the study, we have made significant progress towards generating the samples required to complete the proposed work and have developed a new series of cell lines for the project. Using these reagents we will evaluate the importance of blood vessel morphology and biology, versus tumor behavior in studies of the determinants of primary tumor intravasation and the estravasation of the shed cells at the site of the establishment of new metastatic deposits. Two papers describing our work will shortly be submitted for publication.

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Introduction:

Mice that carry a transgene in which the MMTV (Mouse Mammary Tumor Virus) promoter directs the expression of c-myc to the mammary glands, develop mammary tumors after multiple pregnancies. These tumors are relatively poorly vascularized and typically only form rare, small metastases in the lungs of the mice. We hypothesized that increasing the expression of the angiogenic growth factor VEGF (Vascular Endothelial Growth Factor) in these tumors would result in increased vascularization of the tumors and increased metastatic potential. In order to achieve this goal, we crossed the MMTV-c-myc mice with another transgenic mouse strain which carry a MMTV driven VEGF expression cassette. The frequency and latency of tumor formation in these mice is unchanged, but as hypothesized, the tumors are much more vascular and metastasize more frequently forming larger secondary tumors.

The goal of this project was to evaluate mechanism by which the overexpression of VEGF results in this increased metastatic behavior. To do this, we proposed to: 1) Evaluate the contribution of VEGF to the extravasation of c-Myc/VEGF tumors cells in the secondary site, and 2) Evaluate the contribution of VEGF to facilitate the intravasation from the primary tumor.

Body:

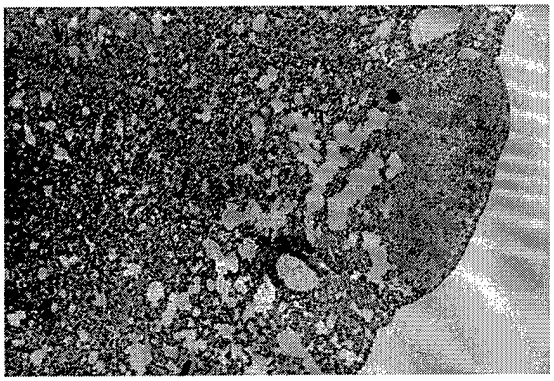
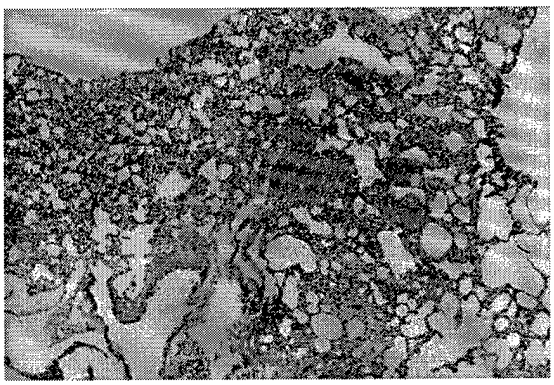
Progress on Aim 1.

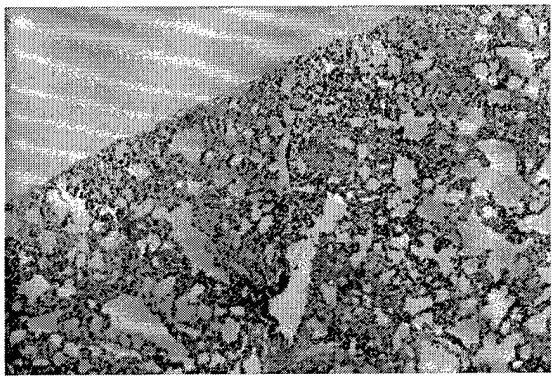
As outlined in the Statement of Work, during the first year of this part of the project we were planning to:

1) Conduct a comprehensive study of the morphology, proliferation, apoptitic index, and growth factor and receptor expression in metastases derived from spontaneous and transplanted c-myc and c-myc/VEGF tumors and

2) Start generating beta-galactosidase transfected cell lines for extravasation experiments to be conducted in the second year of the project.

We have made steady progress towards the completion of these goals. We have continued to generate additional c-myc and c-myc/VEGF bitransgenic mice. Mice are checked daily for palpable tumors. Tumor measurements are taken biweekly and animals were sacrificed prior to tumors reaching 10% of body weight. Gross tumors, lung, liver, kidney, spleen, and brain are harvested and fixed overnight in 10% buffered formalin, embedded in paraffin, and sectioned. Sectioned tissues were stained with hematoxylin and eosin (H&E) for histopathological analysis. Additional tumor tissue is frozen for RNA and protein studies and some samples are frozen viably for tumor transplant studies. The lungs and livers are the main site of metastatic deposits of these tumors and so these tissues are examined for the presence of such deposits and paraffin sections are prepared and stained for further evaluation. Typical sections are shown below:

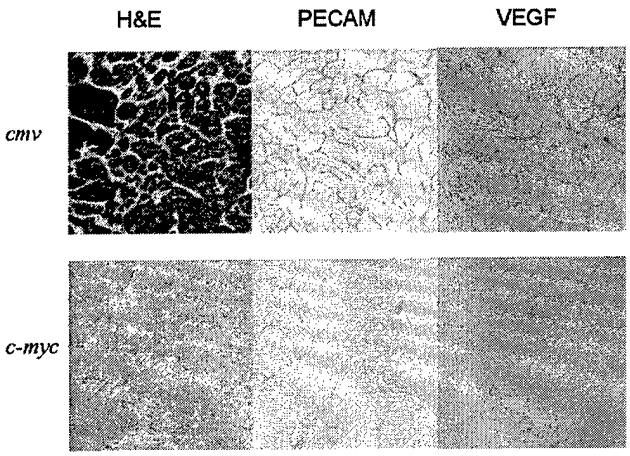


Sections of all tumors and metastases are stained for the expression of VEGF and PECAM – a marker that allows the blood vessels to be visualized – using the following methods:

VEGF Immunohistochemistry: Paraffin embedded tissue sections were baked 1 hour at 55°C, rehydrated, and treated with 2% glycine in PBS for 15 min. Tissue was exposed to a mouse monoclonal antibody to VEGF (Calbiochem Ab-3) for 1h using the Animal Research Kit, (DAKO #K3954). DAB was then utilized for calorimetric detection (Vector). Sections were counter-stained with hematoxylin.

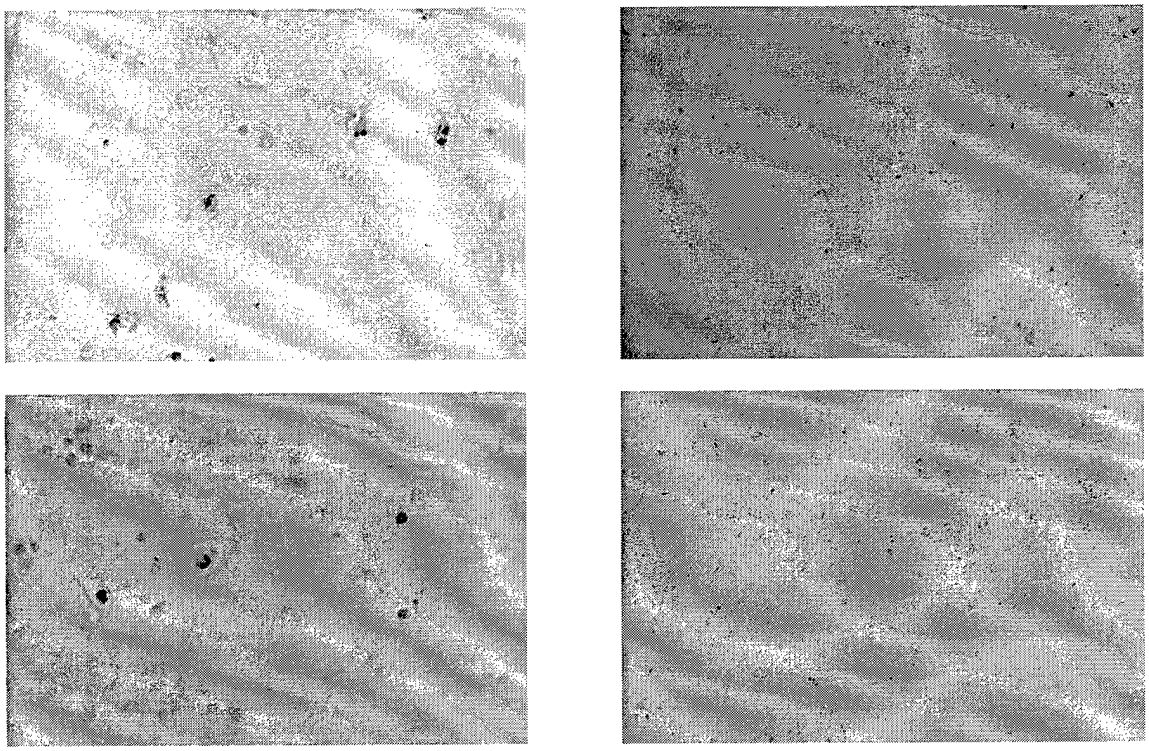
PECAM Staining: Tissue sections were baked for 1 hour at 55°C, rehydrated, and digested with 0.1% trypsin at 37°C for 30 minutes. Tissue was then blocked in 2% normal rabbit serum/5% BSA in PBS, and stained with a rat monoclonal to PECAM (anti-CD31)(MEC 13.3, Pharmingen, San Diego, CA) for two hours. The primary antibody was detected with a biotinylated rabbit anti-rat antibody, preadsorbed with mouse IgG (Vector BA-4001) for 1 hour. DAB was utilized for calorimetric detection (Vector). Sections were counter-stained with methyl green.

Typical results are shown below:



As can be seen, the c-myc/VEGF tumors (cmv) have many more blood vessels and exhibit markedly stronger VEGF staining as would be expected.

Evaluation of the apoptotic index of the tumors has been conducted using TUNEL staining. This method allows the broken ends of the degraded DNA in apoptotic cells to be enzymatically labeled in an IHC based approach. Staining was conducted using a TUNEL kit from Trevigen Inc (Gaithersburg, MD) following the manufacturers instructions. Typical results are shown below:



Two high power (left) and two low power (right) fields are show. The apoptotic cells show up stained brown against a field of light green staining caused by a weak methyl green counter stain. Not all cells that stain brown, however, are apoptotic since the method essentially highlights cells that contain fragmented DNA. Dead cells that have died by non-apoptotic pathways can also be stained and so final scoring is based on a combination of the fact that a cell is stained and the morphology of that cells. Cells that stain and have the classic apoptotic morphology are scored.

In order to evaluate the proliferate index of the tumors we have found that the most reliable method is to take multiple high magnification images of tumor sections and to score them for the number of mitotic figures. Attempts to use bromodeoxy uridine or other methods for labeling cells in which DNA synthesis is going on have not been very useful since so many of the cells end up staining. Counts for mitotic figures, though much more tedious, provides a more discriminating determinate of the number of cells that are in the cell cycle.

We had hoped to have sufficient tumors to complete the analysis of the tumor morphology, proliferation and apoptotic index, by the end of the first year of the project. However, we have had some problems with the animal colonies. The VEGF transgene appears to adversely affect the fecundity of the mice, and so generating additional bitransgenic animals has progressed much more slowly that we had anticipated, which in turn has limited the number of samples we have been able to analyze for these endpoints thus far. In addition we nearly lost our VEGF mouse colony due to these fertility problems, but have now obtained additional animals and have been able to scale up production of the bitransgenic mice. We anticipate that we will be able to generate sufficient tissue to conduct these studies in the next few months. In recognition of the fact that this work is going more slowly than we had anticipated, we will shortly be requesting a no-cost extension of this project for an additional year, which should provide ample time to get the work done.

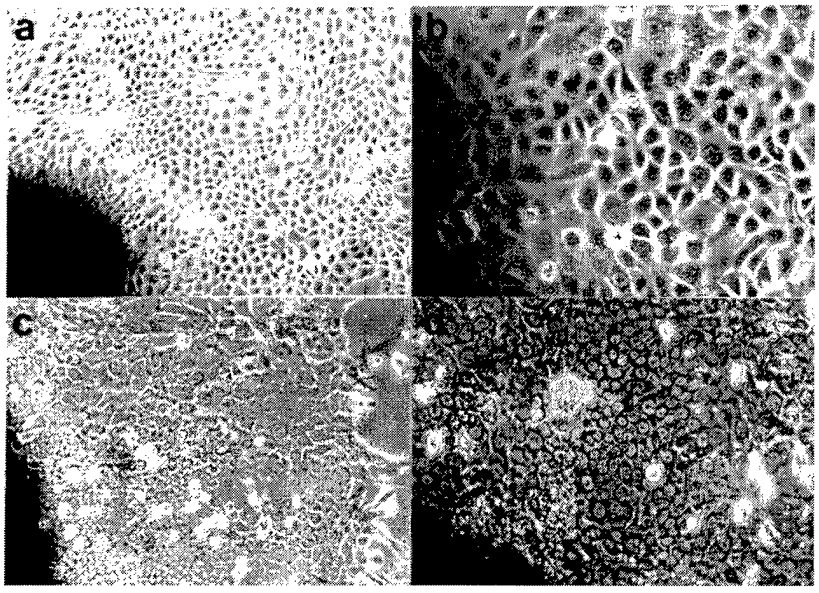
While we have been trying to accrue sufficient animals for the study, to generate the tumor tissue that we need, we have been in the process of working out the IHC assays that we will use to evaluate the tumors for the expression of the various growth factors and receptors that we initially planned to measure. We have also been working on a paper in which we describe the VEGF model and the c-myc/VEGF bitransgenic tumor model – this manuscript is nearing completion and should be accepted for publication by the time of the next annual report.

The second aspect of Aim 1 that we had hoped to have complete by the end of the first year of the project was the generation of c-myc and c-myc/VEGF cell lines with beta-galactosidase. These cells were then to be used in subsequent studies of the processes by which the cells extravisate from the capillaries in the lung to form secondary tumors. However, this aspect of the project has been put on hold whilst we dealt with some issues that arose with the cell lines to be used in these studies. As we reported in the initial proposal, we had generated a series of cell lines that could be used for this part of Aim

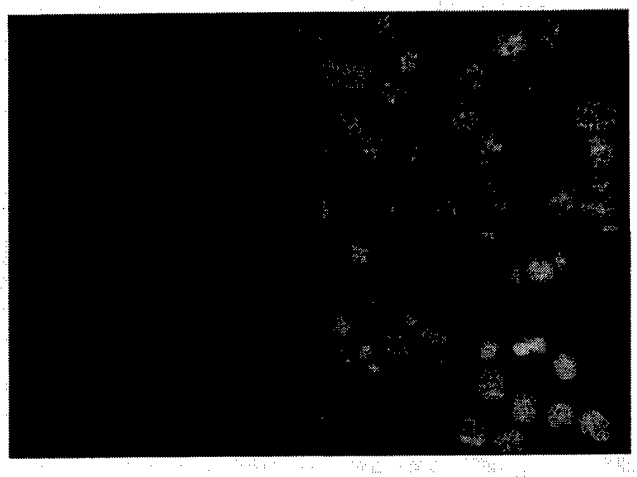
one and for several aspects of Aim 2 described below. However when we conducted experiments to do baseline characterization of these cell lines, we became concerned about their behavior. The myc-VEGF cells were very tumorigenic and formed distant metastasese at high frequencies, but the morphology of the tumors was rather worrying in that our consulting pathologist felt that the morphology was more consistent with salivary gland rather than mammary gland. The MMTV promoter directs transgene expression to the salivary gland in addition to the mammary gland, and it is not uncommon for MMTV driven tumor models to develop salivary tumors. The tumor from which the cell lines in question were derived developed in the #1 mammary gland of the mouse from which it was taken. The #1 gland is situated high on the chest of the mouse and the tail of the fat pad wraps around the neck of the mouse. Thus, it is conceivable that the mass could have in fact been the result of a salivary tumor and not a mammary tumor.

We felt that it was critical that we determine the provenance of this tumor, and hence the cell lines, before starting the proposed studies, since the biology of salivary tumors is quite different to that of mammary tumors. We conducted an extensive series of histological studies on the portions of the original tumor that were preserved in paraffin and tried to evaluate the biochemistry of the cells to determine if they were of mammary or salivary origin. Ultimately, although we are almost certain that the lines are of mammary origin, we could not definitively prove that they were and so we decided to derive a new series of lines from a tumor that was unequivocally of mammary origin.

This work is now complete and we have generated a new series of cell lines for use in Aims 1 and 2. Below is a phase contrast image of two c-myc lines (a, c) and two c-myc/VEGF lines (b, d):



The cells exhibit the typical cobblestone morphology of epithelial cells, and impression that is confirmed by staining for cytokeratins using a pan-keratin type antibody:



We have characterized the growth of these cells in mice and find that they behave as we would expect. That is to say, all lines are highly tumorigenic, but the c-myc/VEGF lines much more frequently form distant metastases to in the lungs and liver and these metastases are much larger, appear to grow more rapidly and have more numerous blood vessels. We have just completed a paper describing the generation of these lines using a modified method which involves propagating the tumor as small explants rather than as completely dissociated cells.

With these cells, we are now again in the position of being able to complete the work planned in the second half of Aims 1 and 2. With the additional time provided by a no-cost extension of the project by one year, we believe we will have no problem completing the proposed work.

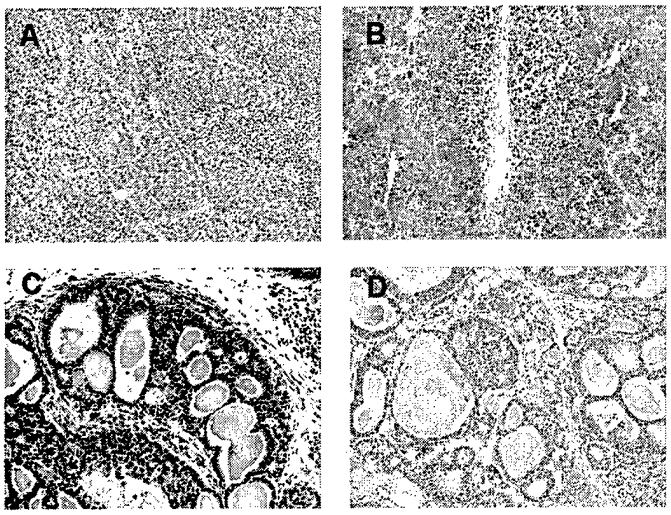
Progress on Aim 2

As outlined in the Statement of Work, in the first year of this project we were planning to

1) Conduct a study of the morphology, proliferation, apoptitic index, and growth factor and receptor expression in spontaneous and transplanted c-myc and c-myc/VEGF tumors and the blood vessels therein, and,

2) Generate stable GFP (Green Fluorescent Protein) and RFP (Red Fluorescent Protein) expressing variants of the c-myc and c-myc/VEGF cell lines.

As will be appreciated from the discussion of progress to date on Aim 1 above, the various technical difficulties we have experienced have slowed down work on Aim 2. However, we have been steadily processing the tumors we have been accruing and typical tumor morphology is shown below:



Primary mammary tumor morphology for MMTV-*c-myc* and MMTV-*c-myc/vegf* mice. A) Representative *c-myc* mammary tumor, 10x; B) Representative *c-myc* mammary tumor, 40x.; C and D) Representative *c-myc/vegf* mammary tumors, 10x

Now that we have generated appropriate cell lines for the studies we will press ahead with their transfection with the GFP and RFP constructs and should be able to complete the studies proposed with the additional time provided by the no-cost extension of the project for one year.

Key Research Accomplishments:

1) We have continued accruing mice and tumors to the study
2) We have established the staining methods to be used in the study
3) We have generated c-myc and c-myc/VEGF lines for use in the study

Reportable Outcomes:

VEGEF-dependent mammary tumor metastasis in a novel bitransgenic model. Nobel et al, in preparation.

Explant-cell culture of primary mammary tumors from MMTV- c-myc and MMTV-c-myc/MMTV-VEGF transgenic mice. Pei et al, in preparation.

Conclusions:

In spite of some un-anticipated technical issues that have hampered progress we have made significant headway on several aspects of the projects. We have all of the resources

that we need to allow us to rapidly complete the planned work. A no-cost extension is to be requested to provide sufficient time for this to be accomplished.

Abbreviations:

βgal	Betagalactosidase
GFP	Green Fluorescent Protein
IHC	Immunohistochemistry
MMTV	Mouse Mammary Tumor Virus
RFP	Red Fluorescent Protein
VEGF	Vascular Endothelial Growth Factor