

AD _____

AWARD NUMBER DAMD17-97-1-7132

TITLE: Function of Maximal Microvessel Density in Breast Tumor Metastasis

PRINCIPAL INVESTIGATOR: Sandra W. McLeskey, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20000829 025

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Function of Maximal Microvessel Density in Breast Tumor Metastasis		5. FUNDING NUMBERS DAMD17-97-1-7132	
6. AUTHOR(S) Sandra W. McLeskey, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command ATTN: MCMR-RMI-S 504 Scott Street Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200 words)</i> Maximum microvessel density (MMVD) in primary tumors is a sensitive prognostic indicator for many solid tumors, including breast. These data imply functional significance for the "hot spots" of microvessels which are quantitated in MMVD determinations. Since prognosis is determined by metastasis, functional significance of the "hot spots" in the process of metastasis is also implied. We hypothesize that the neovascular "hot spots" are the sites of metastasis and that gene expression in the tumor in the vicinity of the "hot spot" is important in the generation of the "hot spot" and in the process of metastasis. In addition to our previous result that metastasis is correlated to tumor size, we have found a relation between metastasis and MMVD. We have developed a method to make sense amplified RNA from 1 pg of RNA. Therefore we have combined amplified RNA production with the Clontech method of template switching and long-distance polymerase chain reaction (PCR). We are able to produce quantities of copy DNA which can be used for analysis of differential gene expression.			
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 18	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

TABLE OF CONTENTS

1. FRONT COVER-----1

2. REPORT DOCUMENTATION PAGE-----2

3. FOREWORD-----3

4. TABLE OF CONTENTS-----4

5. INTRODUCTION-----5

6. BODY-----6

7. CONCLUSION-----12

8. REFERENCES-----13

9. APPENDIX-----16

 List of abbreviations and acronyms-----17

 Meeting abstracts since last report-----18

 Publications since last report-----18

 Manuscripts in preparations-----18

 Personnel receiving pay from this negotiated effort-----18

INTRODUCTION

This proposal combines microvessel density measurements with microdissection in an effort to identify genes differentially expressed by tumor cells in “hot spots” of maximal microvessel density. In order to find the “hottest spot” in each tumor, we screened representative sections from all regions of each tumor. We quantified these spots to see whether a particular area of the tumor has “hotter” spots of microvessels and related these data to the degree of lung metastasis found in the host. Finding the “hottest spot” in each tumor will enable us to microdissect those areas in highly metastatic tumors and compare them to areas not associated with high microvessel density with regard to differential gene expression.

Before we are able to perform expression analysis on our tumor specimens, we first have to be able to purify and amplify RNA from the dissected cells, and make cDNA. Since extremely small amounts of source RNA will be harvested, the cDNA will have to be amplified. As mentioned in the previous report, there are two concerns for any method of amplification: fidelity and yield. Since the last report, we have been working to improve our methods of amplification. This report contains a scheme, which combines the Clontech method of cDNA synthesis and amplification [template switching (1) followed by long-distance PCR(2)] with a new method of amplified RNA, developed in our laboratory.

For differential analysis of gene expression we are currently using the amplified length polymorphism analysis (AFLP) (3) on cDNA in connection with another project in our laboratory. Because of our great experience with this method, we had planned to use it with this project also. However, the recent availability of microarray technology (4) has made differential analysis of gene expression for known genes extremely fast. Therefore we are also considering the use of microarray technology in collaboration with Dr. Yan Su, at the Georgetown University.

BODY

Aim 1. We will identify critical microenvironments in the tumors produced by FGF-1 transfected MCF-7 cells in nude mice by sensitively and accurately correlating the degree of metastasis in the lungs and lymph nodes with maximal microvessel density in the hottest spot in each tumor.

In last year's report we were in the process of scoring the representative PECAM-1 stained sections from all tumors. We had scored most of the 30 tumors for the A1 slice and many of the tumors from other slices. Our goal in this analysis is to identify the "hottest spot" in the most metastatic tumors so as to use those areas for microdissection. Important issues of quantitating the microvessel density (MVD) are: 1.selection of the tissue sample as part of the whole tumor specimen; 2.tissue processing and immunostaining; 3.selection of the area for microvessel enumeration and 4.the technique of MVD evaluation. As described in our previous report, tumors were cut in a standardized way into four transverse and four longitudinal slices, 3-4 mm thick, frozen and sectioned serially onto uncoated slides. Slides were stained for PECAM-1, a specific staining for endothelial cells, described in our previous report.

We used the method of Weidner *et al.* 1991 (5), modified according to Vermeulen *et al.* 1996 (6), which utilizes a Chalkley graticule. Only short, sprouting, vessels without lumens are considered, mostly located in the edge of the tumor. Representative sections from all regions of each tumor were screened under low power (40x) for the area with the highest MVD in the tumor. This MVD was then quantified at 200x magnification. By counting the dots of the graticule coinciding with PECAM positive microvessels, a Chalkley score was determined for each tumor (Table 1). The higher the MVD was for that tumor, the higher the Chalkley score. If more than one candidate area of maximal MVD was identified in the screening step, then the Chalkley score was determined for each and the highest score was used. We were able to find a relation between highest MVD scored in each tumor and the number of lung metastases in the host (Fig.1). Except for one case, we found low MVD related to low number of lung metastases. All tumors with higher numbers of metastases had higher maximal MVD's, but not all tumors with a high MVD had a high number of lung metastases. In the coming year, areas of highest MVD in tumors with highest number of metastasis will be microdissected and

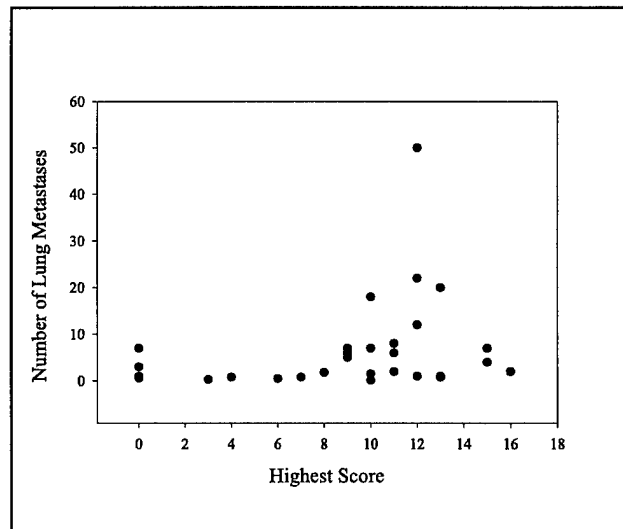


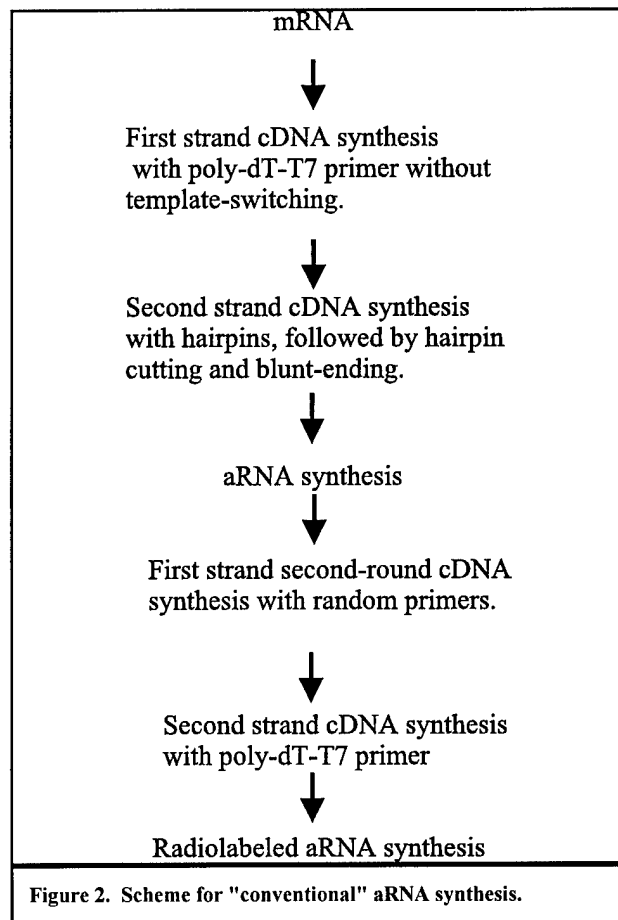
Figure 1. Relationship of lung metastases and Chalkley score. The area of maximal microvessel density for each tumor was quantified with a Chalkley graticule. Lung metastases were quantified by image analysis (see previous report for method).

extracted RNA used to identify genes that may be involved in metastatic processes. These genes would be those differentially expressed in tumor cells adjacent to hot spots when compared to areas of tumor not associated with hot spots.

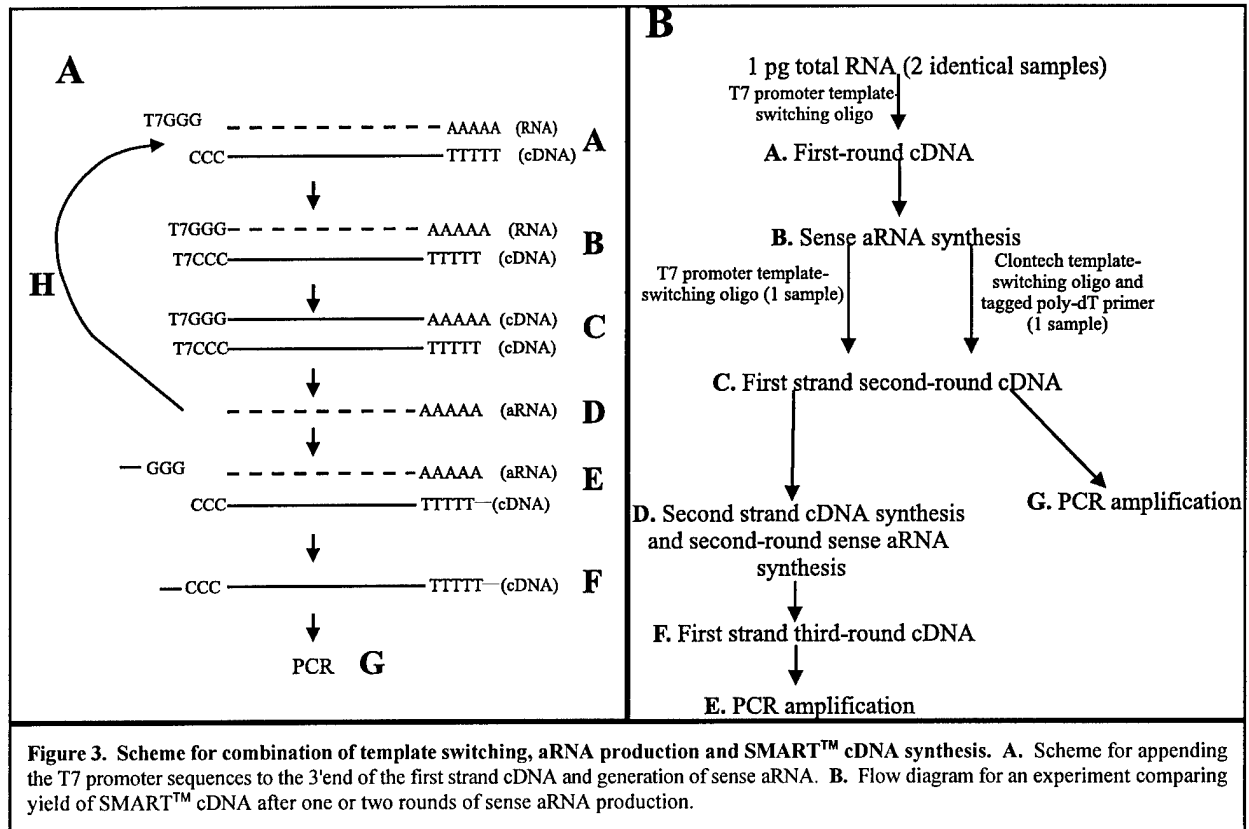
Aim 2. We will analyze differential gene expression in tumor cells in, the area of hot spots, by microdissection followed by differential display PCR.

Laser microdissection will be used to microdissect tumor cells in the area of the hottest spot. A hot spot area is first selected under the microscope at low magnification (100x) and the cells (a spot of $\approx 30 \mu\text{m}$) are "burned" onto a special plastic cap adjusted right on top of this area, by activating the laser beam. As mentioned in the previous report, we have practiced this technique and are able to obtain RNA from it.

For making cDNA from RNA obtained by laser microdissection, we attempted the Clontech method, described in the previous report, utilizing the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). This method combines template switching with long-distance PCR (7). This approach was not successful, possibly due to the small amount of RNA isolated. Therefore we modified the aRNA method described in our previous report, to produce sense rather than antisense aRNA (below).



The "conventional" amplified RNA (aRNA) method as described in the previous report, appends a T7 RNA polymerase promoter sequence to the poly-dT primer used for first strand cDNA synthesis. Problems with the "conventional" aRNA approach as presently implemented include the antisense orientation of the aRNA and the less than full-length species it generates. This product is ideal for use as a probe since it can be easily labeled with radionucleotides at the time of synthesis, but it cannot be used for RT-PCR unless reversed primers (antisense instead of sense and sense instead of antisense) are synthesized. Moreover, although the conditions used to generate the aRNA



favor a high degree of synthesis, information concerning the length of individual species has not been obtained. aRNA can be used to generate second-round cDNA, but because of the antisense orientation, a poly-dT primer cannot be used to prime the reverse transcription reaction. Therefore, random primers are used to prime first strand cDNA synthesis, followed by poly-dT priming of second strand synthesis [Figure 2 and (8,9)]. This guarantees that the second-round cDNA will not be full length.

Using the method of template switching, we were able to make sense aRNA from 1 pg of total RNA. This was accomplished by using a poly-dT primer with a template-switching oligonucleotide incorporating the T7 RNA polymerase promoter for 1st strand cDNA synthesis as described below. We have combined sense aRNA production with the Clontech method of template switching and long-distance PCR to produce quantities of cDNA which can be used for analysis of differential gene expression.

Production of sense aRNA. Total RNA (1 pg) was used as template for a reverse transcription reaction with a poly-dT primer and Superscript II (Life Technologies, Gaithersburg MD) as the RT (step A and B in Figure 2A). The single-stranded template-switching oligonucleotide included in the reaction had four 3' dGs with T7 RNA polymerase promoter sequences appended 5' to the dGs. This same oligonucleotide was used to prime the second strand reaction, yielding double stranded cDNA with an intact T7 RNA polymerase promoter sequence at the 5' end (step C of Figure 3A). Amplified RNA was then synthesized with T7 RNA polymerase, yielding full length sense RNA (step D in Figure 3A). Since the transcription start site for T7 RNA polymerase includes only the terminal 6 nucleotides of the promoter, which in this case would be followed by the 4 Gs used for template switching, the sense aRNA generated by this procedure would have 10 additional nucleotides at the 5' end, when compared to the original mRNA.

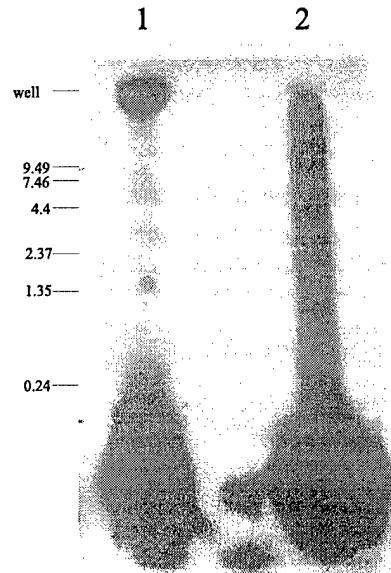
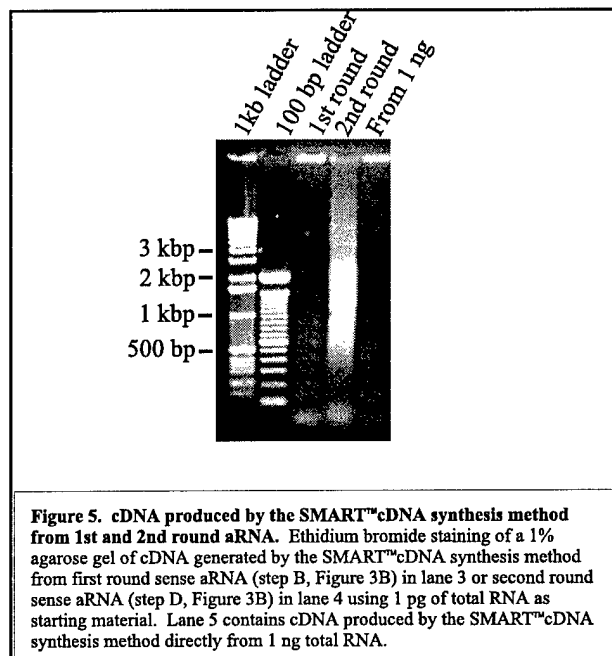


Figure 4. Second-round sense aRNA production. An identical cDNA sample to that produced in Figure 3B, step D was used to produce aRNA incorporating ³²P-labeled UTP. The labeled RNA is shown in lane 2 of the autoradiogram. Lane 1 is the product of a similar aRNA reaction which lacked T7 RNA polymerase.

The first round aRNA generated above was then used for second round cDNA synthesis in 2 ways. First, the first-round aRNA was used with a template-switching, long range PCR protocol to generate PCR-amplified cDNA (Steps E, F and G in Figure 3A). Second, the first-round aRNA was used as a template for reverse transcription followed by second strand synthesis in the same way as the original cDNA was synthesized (Step H in Figure 3A). This produced second-round cDNA, which could be used to produce second-round aRNA. The second-round aRNA was then used as template with the template-switching, long range PCR protocol to generate PCR-amplified cDNA. An overall scheme of the whole experiment is shown in Figure 3B.

Figure 4, lane 2 shows ³²P-labeled UTP incorporation into second-round aRNA produced in this manner (the aRNA was produced in step D, Figure 3B). Scintillation counting of trichloroacetic acid precipitable material from a portion of this sample allowed us to estimate that the reaction produced 1.5 ng of aRNA. If we assume that 5% of total RNA is mRNA, from 1 pg of total RNA, we had 50 fg of starting mRNA, producing a 30,000-fold final amplification. This yield is somewhat short of the million-fold amplification predicted for two rounds of "conventional" aRNA synthesis (9). This may be because our scheme appends the T7 promoter at the 5' end of the first round cDNA,

so shorter than full-length cDNAs will not have the T7 promoter sequences and will not be able to serve as template for aRNA transcription. In contrast, the "conventional" aRNA protocol, which appends the T7 promoter sequence to the poly-dT primer, nearly every product of first strand synthesis, even extremely short ones, will produce aRNA. This selection for short species is increased in second-round aRNA synthesis since random priming of second-round first strand synthesis again produces shorter species. Considering that we have set up our conditions such that incomplete first strands will not give rise to cDNAs which will produce aRNA, a 30-fold reduction in yield is a small price to pay in exchange for longer cDNAs.



Synthesis of cDNA from sense aRNA. Figure 5 shows an ethidium bromide stained agarose gel of cDNA synthesized with the template-switching, long-range PCR method (26 cycles) from aRNA produced from first-round cDNA synthesis in lane 3 (step G in Figure 3B), and from second-round cDNA synthesis in lane 4 (step E in Figure 3B), each produced from 1 pg total RNA as starting material. For comparison, cDNA synthesized directly from 1 ng total RNA (the minimum recommended template is 50 ng) as starting material is shown in the last lane. It should be noted that approximately equal yields of cDNA are obtained from 1 ng of total RNA and from 1 pg total RNA which has undergone one round of aRNA synthesis. Moreover, the intensity of ethidium bromide staining seems to peak around 1.5 kb and extends into very high molecular weights. Therefore, we are optimistic that this cDNA is of high quality and contains long transcripts.

The gel depicted in Figure 5 contains approximately 30% of the cDNA produced in the synthesis reaction. We therefore feel that this method of cDNA synthesis, when applied to RNA purified from microdissected samples, will enable us to perform analysis of differential gene expression as described in the proposal.

Analysis of differential gene expression. As mentioned in the previous report, we are currently performing amplified fragment-length polymorphism analysis (AFLP) on cDNA obtained from tumor-derived endothelial cells in connection with another project in the laboratory. We currently have eight candidate differentially expressed genes resulting from that analysis. Since we are experienced with that method, we could use it in this project also. However, since this proposal was funded, microarray technology has become more universally available. We also have a

McLeskey, Sandra W.
DAMD17-94-J4173

collaborator at Georgetown, Dr. Yan Su, who has worked in the past with Dr. Jeff Trent of NIH, one of the originators of the microarray technique. In addition mouse microarrays are being developed during the next year. As we obtain cDNA from the areas of hot spots, we will be working with Dr. Su to develop a microarray application of this project.

CONCLUSION

Scientific conclusions to date:

1. We are able to find the "hottest spot" in each tumor and quantitate the vessels in a standardized way. In addition to our previous finding that degree of pulmonary micrometastasis is tightly correlated with tumor size, our recent data confirm a relation between degree of pulmonary micrometastasis and highest MVD scored in each breast tumor.
2. We are able to make sense aRNA from 1 pg of RNA, by using the method of template-switching. We have combined sense aRNA production with the Clontech method of template switching and long-distance PCR, to produce quantities of cDNA which can be used for analysis of differential gene expression. We are very optimistic that this cDNA is of high quality and contains long transcripts.

DISCUSSION

As we have mentioned in the previous report, this research is by definition risky, however, we were able to find a relation between microvessel density in the tumor and the degree of lung metastasis in the host. Moreover, we were able to purify RNA and we have modified the methods of RNA amplification and synthesis of cDNA, enabling us to make cDNA from 1 pg of purified RNA. What we have achieved so far brings us closer to our goal to microdissect tumor cells adjacent to hottest spot and compare these with non-hotspot associated tumor cells, by using differential gene expression methods. As the microarray technology is now within our reach, our research, although risky, looks even more promising than before.

REFERENCES

1. McLeskey, S.W., Tobias, C.A., Vezza, P.R., Filie, A.C., Kern, F.G., and Hanfelt, J. Tumor growth of FGF or VEGF transfected MCF-7 breast carcinoma cells correlates with density of specific microvessels independent of the transfected angiogenic factor. *Am. J. Pathol.* 1998.
2. McLeskey, S.W., Kurebayashi, J., Honig, S.F., Zwiebel, J., Lippman, M.E., Dickson, R.B., and Kern, F.G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Res.* 53: 2168-2177, 1993.
3. Kurebayashi, J., McLeskey, S.W., Johnson, M.D., Lippman, M.E., Dickson, R.B., and Kern, F.G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and *LacZ*. *Cancer Res.* 53: 2178-2187, 1993.
4. Zhang, L., Kharbanda, S., Chen, D., Bullocks, J., Miller, D.L., Ding, I.Y.F., Hanfelt, J., McLeskey, S.W., and Kern, F.G. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized metastatic tumors in ovariectomized or tamoxifen-treated nude mice. *Oncogene*, 15: 2093-2108, 1997.
5. Bullocks, J., Zhang, L., Ding, I.Y.F., McLeskey, S.W., Tobias, C.A., Miller, D.L., and Kern, F.G. Overexpression of vascular endothelial cell growth factor (VEGF) in MCF-7 breast carcinoma cells facilitates growth in tamoxifen-treated nude mice and tumor cell dissemination. *Proc. Am. Assoc. Cancer Res.* 38: 3521 1997.
6. McLeskey, S.W., Zhang, L., Kharbanda, S., Kurebayashi, J., Lippman, M.E., Dickson, R.B., and Kern, F.G. Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. *Breast Cancer Res. Treat.* 39: 103-117, 1996.
7. McLeskey, S.W., Zhang, L., El-Ashry, D., Trock, B.J., Lopez, C.A., Kharbanda, S., Tobias, C.A., Lorant, L.A., Hannum, R.S., Dickson, R.B., and Kern, F.G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen, ICI 182,780, and two aromatase inhibitors. *Clin. Cancer Res.* 4: 697-711, 1998.
8. Vecchi, A., Garlanda, C., Lampugnani, M.G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L., Mantovani, A., and Dejana, E. Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.* 63: 247-254, 1994.
9. Melder, R.J., Koenig, G.C., Witwer, B.P., Safabakhsh, N., Munn, L.L., and Jain, R.K. During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium. *Nature Medicine*, 2: 992-997, 1996.
10. Griffioen, A.W., Damen, C.A., Martinotti, S., Blijham, G.H., and Groenewegen, G. Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies: the role of angiogenic factors. *Cancer Res.* 56: 1111-1117, 1996.

11. Deroanne, C.F., Hajitou, A., Calberg-Bacq, C.-M., Nusgens, B.V., and Lapière, C.M. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Res.* 57: 5590-5597, 1997.
12. Hajitou, A., Deroanne, C.F., Noël, A., Colette, J., Nusgens, B.V., Foidart, J.M., and Calberg-Bacq, C.-M. FGF-3 increases tumorigenicity in MCF7 breast cancer cells but FGF-4 effect is more pronounced and associated with VEGF upregulation. *Proc. Am. Assoc. Cancer Res.* 39: 341998.
13. McLeskey, S.W., Zhang, L., Trock, B.J., Kharbanda, S., Liu, Y., Gottardis, M.M., Lippman, M.E., and Kern, F.G. Effects of AGM-1470 and pentosan polysulphate on tumorigenicity and metastasis of FGF-transfected MCF-7 cells. *Br. J. Cancer*, 73: 1053-1062, 1996.
14. Weidner, N., Moore, D.H., II, Ljung, B.-M., Waldman, F.M., Goodson, W.H.I., Mayall, B., Chew, K., and Smith, H.S. Correlation of bromodeoxyuridine (BRDU) labeling of breast carcinoma cells with mitotic figure content and tumor grade. *Am. J. Surg. Path.* 17: 987-994, 1993.
15. Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V., and Lassmann, H. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab. Invest.* 71: 219-225, 1994.
16. Mundle, S.D. and Raza, A. The two *in situ* techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis. *Lab. Invest.* 72: 611-612, 1995.
17. Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V., and Lassmann, H. The two *in situ* techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis --- reply. *Lab. Invest.* 72: 612-613, 1995.
18. Mundle, S., Iftikhar, A., Shetty, V., Dameron, S., Wright-Quinones, V., Marcus, B., Loew, J., Gregory, S., and Raza, A. Novel *in situ* double labeling for simultaneous detection of proliferation and apoptosis. *J. Histochem. Cytochem.* 42: 1533-1537, 1994.
19. Raza, A., Mundle, S., Iftikhar, A., Gregory, S., Marcus, B., Khan, Z., Alvi, S., Shetty, V., Dameron, S., Wright, V., Adler, S., Loew, J.M., Shott, S., Ali, S.N., and Preisler, H. Simultaneous assessment of cell kinetics and programmed cell death in bone marrow biopsies of myelodysplastics reveals extensive apoptosis as the probable basis for ineffective hematopoiesis. *Am. J. Hemat.* 48: 143-154, 1995.
20. Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J. Cell. Physiol.* 169: 522-531, 1996.
21. Maroulakou, I.G., Papas, T.S., and Green, J.E. Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. *Oncogene*, 9: 1551-1565, 1994.
22. Speir, E., Sasse, J., Shrivastav, S., and Casscells, W. Culture-induced increase in acidic and basic fibroblast growth factor activities and their association with the nuclei of vascular endothelial and smooth muscle cells. *J. Cell. Physiol.* 147: 362-373, 1991.

23. Auerbach, R., Auerbach, W., and Polakowski, I. Assays for angiogenesis: A review. *Pharmac. Ther.* 51: 1-11, 1991.
24. Liaw, L. and Schwartz, S.M. Comparison of gene expression in bovine aortic endothelium *in vivo* versus *in vitro*. *Arteriosclerosis & Thrombosis*, 13: 985-993, 1993.
25. Craig, L.E., Spelman, J.P., Strandberg, J.D., and Zink, C.M. Endothelial cells from diverse tissues exhibit differences in growth and morphology. *Microvasc. Res.* 55: 65-76, 1998.
26. Obeso, J., Weber, J., and Auerbach, R. A hemangioendothelioma-derived cell line: Its use as a model for the study of endothelial cell biology. *Lab. Invest.* 63: 259-269, 1990.
27. Bachem, C.W.B., van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M., and Visser, R.G.F. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant J.* 9: 745-753, 1996.
28. Eberwine, J. Amplification of mRNA populations using aRNA generated from immobilized oligo-dT-T7 primed cDNA. *Biotechniques*, 20: 584-586, 1996.
29. Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettle, M., and Coleman, P. Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. U. S. A.* 89: 3010-3014, 1992.
30. Eberwine, J., Crino, P., and Dichter, M. Single cell mRNA amplification: Basic science and clinical implications. *Neuroscientist*, 1: 200-211, 1995.
31. O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*, 79: 315-328, 1994.
32. Cao, Y., O'Reilly, M.S., Marshall, B., Flynn, E., Ji, R.-W., and Folkman, J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. *J. Clin. Invest.* 101: 1055-1063, 1998.
33. Boehm, T., Folkman, J., Browder, T., and O'Reilly, M.S. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, 390: 404-407, 1997.
34. Arap, W., Pasqualini, R., and Ruoslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science*, 377-380, 1998.

McLeskey, Sandra W.
DAMD17-94-J4173

APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS

aRNA	amplified ribonucleic acid
AFLP	amplified fragment-length polymorphism
cDNA	copy DNA
DNA	deoxyribonucleic acid
MVD	microvessel density
PCR	polymerase chain reaction
PECAM-1	platelet-endothelial cell adhesion molecule 1
RNA	ribonucleic acid
RT-PCR	reverse transcription followed by the polymerase chain reaction
T7	bacteriophage T7

McLeskey, Sandra W.
DAMD17-94-J4173

Meeting abstracts since the last report: None for this project.

Publications since the last report: None in connection with this project.

Manuscripts in preparation: None to date.

Personnel receiving pay from this negotiated effort during this period:

Sandra W. McLeskey, PhD
Susette Mueller, PhD
Arleen Emanuels, PhD