

AD _____

Award Number: DAMD17-03-1-0473

TITLE: Stromal gene expression and function in primary breast tumors that metastasize to bone cancer

PRINCIPAL INVESTIGATOR: Belinda Parker
Robin L. Anderson

CONTRACTING ORGANIZATION: University of Melbourne
Victoria 3010 Australia

REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

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.

20050113 037

PII Redacted

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

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 this 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 (Leave blank)		2. REPORT DATE July 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 03-30 Jun 04)	
4. TITLE AND SUBTITLE Stromal gene expression and function in primary breast tumors that metastasize to bone cancer			5. FUNDING NUMBERS DAMD17-03-1-0473	
6. AUTHOR(S) Belinda Parker Robin L. Anderson				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Melbourne Victoria 3010 Australia E-Mail: Belinda.parker@petermac.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command 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 (Maximum 200 Words): A clinically relevant syngeneic model of breast cancer metastasis has been used to determine gene expression alterations that occur both between primary breast cancers with varying metastatic potential and between matched primary and bone metastases. We have immunopurified epithelial and endothelial cell populations and profiled them separately to identify differentially expressed genes, some of which have not previously been associated with breast cancer metastasis. Expression profiles of vascular endothelium derived from primary tumors of varying metastatic potential identified aberrant expression of genes involved in angiogenesis, cell cycle progression, cytoskeletal structure and tumor suppression. Those altered in the primary tumor epithelium included developmental genes, metastasis suppressors and genes involved in cytoskeletal organization, cell cycle progression, apoptosis and transformation. The microarray data was confirmed by quantitative RT-QPCR. Further analysis of epithelium from matched spine metastases revealed some genes that were up-regulated further at the metastatic site. These included stefin A1 (inhibitor of cathepsin S) that was up-regulated in highly metastatic primary epithelium and increased a further 9-fold in matched bone metastases. The expression in spine metastases was verified by <i>in situ</i> hybridisation whilst the expression of stefin A1 in subsets of tumor cells in invasive human breast cancer was confirmed by immunohistochemistry.				
14. SUBJECT TERMS No subject terms provided.			15. NUMBER OF PAGES 26	
			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

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	19
Reportable Outcomes.....	20
Conclusions.....	21
References.....	22
Appendices.....	23

INTRODUCTION

Breast cancer is a high frequency disease that can often be treated successfully if detected early, yet once metastasis occurs there is a reduced chance of patient survival. A large proportion (~70%) of patients who die of their disease have skeletal involvement (1), indicating that bone metastasis is a major cause of morbidity in patients with breast cancer. Due to the high frequency and severity of breast cancer metastasis to bone, and the lack of markers that allow for early detection and therapy, it is necessary to determine the cellular and molecular mechanisms by which the specific spread of breast cancer cells to the bone occurs. There are a number of steps in the metastatic process, including local invasion of breast cancer cells out of the stroma and the ability to overcome cell-cell interactions to enter the circulation and the subsequent interaction between the cancer cell and the secondary host environment to stimulate metastatic tumor growth. The steps involved in tumor progression is influenced by both tumor and stromal genes, with recent evidence revealing that stimulation of host stromal genes is important in breast cancer progression (2-4). Identification of the genes critical to allowing specific growth in bone and other metastatic sites is important for the development of therapeutic strategies.

Although many genes have been found to be associated with breast cancer metastasis, a single accurate predictor to identify patients who will develop bone disease is still lacking. This is largely due to the lack of clinically relevant models of breast carcinoma metastasis to bone to evaluate the function of genes already implicated in the metastatic process and to find new gene candidates. Models used to find these genes have usually relied on cell culture or xenograft models of bone metastases in the absence of primary breast tumor formation (eg. MDA-MB-231 injected into left ventricle of nude mice). This makes it difficult to study the steps of metastatic progression from the initial invasion of cells into the circulation to the growth in the bone microenvironment. Also, whole tumor gene expression analysis ignores the contribution of tumor-associated stromal cells to growth and invasion of tumor cells. The use of cell specific profiling may therefore identify gene candidates in stromal cells that could have been masked using whole tumor analysis. A recent study reported major alterations in gene expression of breast cancer associated myoepithelium (5), changes that have not been identified in past studies.

A recent spontaneous metastasis model has been developed in our Laboratory. This model mimics the clinical disease in that a primary breast tumor develops, the cells invade through the stroma into the circulation and colonize at a distant organs (Figure 1) (6). This is the first reported model of spontaneous metastasis of breast tumor cells from the primary site in the mammary gland to different organs, including bone. The model is of enormous importance for a comparison of the expression profiles of primary tumors with different metastatic capacities. This proposal aims to determine cell-specific expression changes during breast cancer metastatic progression. The model can then be used for functional analysis of genes identified to determine whether they are in fact involved in the metastatic process and whether they have therapeutic potential.

The main objectives are to study the molecular events involved in metastasis, specifically we aim to 1) identify genes expressed in tumor-derived endothelial cells that may be associated with metastasis; 2) determine whether these genes are also expressed in human breast cancer, 3) study the function of these genes using *in vitro* metastasis and angiogenesis assays; 4) explore the role of these genes in metastasis to bone *in vivo*.

BODY

TASK 1: Isolate tumor and stromal cells from primary tumors of differing metastatic capacity and subject the RNA isolated from these cells to microarray analysis (months 1-12)

- a. Purify specific cell populations (epithelial, endothelial and fibroblast cells) using immunopurification from fresh tumors and by laser capture microdissection (LCM) from frozen tumors (months 1-9).
- b. Perform microarray analysis of specific cell types, comparing RNA from cells derived from a non-metastatic tumor to that from a metastatic tumor (months 6-12).

Task 1 has now been completed. We have separated epithelial cells, endothelial cells and remaining stromal cells from the primary tumors of the tumor lines shown in Figure 1. The separation was done individually for six tumors of each line following 28 days growth in the mammary gland of Balb/c mice. Fresh resected tissue (normal fat pad, primary tumor tissue or the metastatic sites spine, femur and lung) was obtained and cell separation completed using an immunobead protocol adapted from Vogelstein and colleagues (7). Purity of the epithelial and endothelial cell populations was confirmed by immunostaining for carcinoembryonic antigen (CEA) and von Willibrand factor (vWF), respectively and also by RT-PCR of epithelial (CK18) and endothelial (vWF, P1H12) associated genes (Figure 2). During the cell isolation procedure, epithelial cells, endothelial cells, B and T cells and red blood cells are removed. The composition of the "remaining stromal cells" is likely to be primarily fibroblasts, with a small number of other cells including macrophages and adipocytes likely to be present.

Amplified RNA isolated from epithelial and endothelial cells was used for cDNA microarray analysis using the NIA mouse 15K custom array (Microarray Facility, Peter MacCallum Cancer Centre) with newborn mouse RNA as a reference. Microarray results were analysed using Genepix and Genespring v.6 software and gene expression profiles of epithelial and endothelial samples were compared to determine those that were significantly different ($p < 0.05$) in high metastatic samples compared to low metastatic and normal samples, by at least 1.8 fold. It should be noted that amplification of RNA did not produce any bias as confirmed by the lack of significant difference between expression profiles of the same sample that was or was not amplified (data not shown).

Gene expression profiles have been obtained from the tumor epithelial and endothelial cells within the primary tumors. For each cell type, a set of genes have been identified whose expression is different between the highly aggressive bone metastasizing lines (4T1.2 and 4T1.13) and the poorly metastatic lines (66cl4 and 67NR); and in endothelial comparisons also different from that in the normal mammary gland. We have also isolated RNA from the remaining stromal cell component of each of the six primary tumors of each tumor line in the metastasis model in preparation for gene expression profiling of this cell population. Further, we have isolated RNA from the three different cell populations within metastases, where they occur, in preparation for cDNA microarray analysis of lung and bone metastases.

The genes identified aberrantly expressed in highly metastatic tumor-associated endothelium are listed in Table 1 and include, not surprisingly, genes involved in angiogenesis, proliferation, adhesion and motility. Also of interest are some genes whose expression is suppressed in the

endothelial cells of the highly metastatic tumors, including two well known tumor suppressor genes, PTEN and LKB1. There are also genes involved in, or regulated by the Hedgehog signalling pathway: Lasp1, CREBBP/EP300 inhibitory protein 1 and FoxP1. Of interest as well are a number of differentially regulated ESTs, shown in Table 2. Interestingly, one EST down-regulated in highly metastatic tumor endothelium has recently been identified as a homolog of large tumor suppressor 2 (LATS2), again revealing endothelial-cell specific decrease in expression of tumor suppressor genes.

Some of the genes in Table 1 have been linked previously to cancer, but many have not yet either been associated with cancer or even reported before in endothelial cells. It is intended that the function of some of these genes will be examined in tumor associated endothelial cells using our metastasis model. Those of specific interest include serine/threonine kinase 11 (Stk11 or LKB1), Forkhead box P1 (FoxP1) and macrophage migration inhibitory factor (MIF).

Genes found altered in highly metastatic primary tumor epithelium are summarised in Table 3. Upregulated genes include those involved in cellular proliferation and apoptotic inhibition (DACH1), cytoskeletal organization and development (Dppa5, Mater) whereas those found to be decreased in expression in highly metastatic tumor cells include differentiation and apoptosis genes (BMP4) and metastasis suppressor genes (BRMS1). A number of ESTs were also identified as aberrantly expressed and a selection validated in Task 2.

The genes of interest for further study are listed in Table 4, along with their chromosomal localization, previous loss of heterozygosity (LOH) or gene amplification documented for that region and associated functions that may indicate a role in metastasis. It should be noted that these genes are yet to be studied for chromosomal and epigenetic aberrations and it will be of future interest to do so in our future studies (ie DNA amplification, promoter methylation for up-regulated and down-regulated genes respectively).

TASK 2 Verify expression of differentially expressed genes found in the mouse model in the relevant cells of human breast tumors, using immunohistochemistry or in situ hybridization (months 9-18).

- a. Use realtime RT-PCR and immunohistochemistry in cell culture and in tissue sections of the mouse model to confirm the microarray data (months 9-15).
- b. Confirm that these genes are also relevant to breast cancer metastasis to bone in humans by using human tissue arrays to measure expression of the identified genes in the relevant human cell type (endothelial, fibroblast or epithelial) (months 12-18).

As reported under Task 1, we have generated gene expression profiles of isolated epithelial cells from tumors that are highly metastatic compared to those that are weakly metastatic or non-metastatic. We have used our metastasis model in which spontaneous metastasis to various sites, including bone, occurs following growth of a tumor in the mammary gland. In Table 1, 2 and 3 we reported some of the genes we found to be aberrantly expressed in tumor epithelial cells and in associated host endothelium. The differences in expression for several of these genes have now be confirmed by real time quantitative RT-PCR. From the lists of epithelial genes altered in highly metastatic primary breast cancer (table 3), we have compared the expression of BMP4, Dach1 and two ESTs – NM028729 and BC042445 as shown in Figure 3. Of interest is the decreasing expression of BMP4 with increasing metastatic capacity and the reverse response for Dach1. BMP4 is a member of the TGF β family, has a role in development, induces senescence

and is a negative regulator of Dach1, which stimulates proliferation and inhibits TGF β induced apoptosis. Functional analysis of this interaction will be further studied *in vitro*.

Stefin A1 was also found to be expressed at much higher levels in the highly metastatic 4T1.2 and 4T1.13 primary tumor epithelium (Table 3). Stefin A1 has been reported to be an inhibitor of cathepsin S and a marker of malignancy in some tumor systems. When comparing expression profiles of epithelium isolated from primary tumors and from matched spine metastases (from 4T1.2 and 4T1.13 sublines), stefin A1 had even higher levels of expression in the bone metastases compared to the primary tumor, suggesting an important role in metastasis to bone and the possibility that only a subset of cells in the primary tumor express the gene and these cells are selected for in the bone metastases. The expression patterns were validated by RT-PCR (Figure 4) of cDNA samples from immunopurified epithelial populations derived from both the primary tumor and matched spine metastases. These results suggest that Stefin A1 has potential not only as a prognostic marker at the primary site, but also as a target for treatment of metastatic cancer since its expression is maintained (and enhanced) in bone metastases.

To determine whether Stefin A1 expression was relevant in human cancer (and hence that these studies are clinically relevant) immunohistochemistry was carried out on human primary tumors using an anti-human stefin A antibody. Interestingly, the hypothesis that only a subset of cells expressed Stefin A (and hence the relatively low expression in primary tumors compared to spine and femur metastases) held true, with only 1-2 of 6 primary samples analyzed being positive for stefin A and of those tumors only a subset of tumor cells expressed stefin A at the protein level (Figure 5). It should be noted that this antibody was not reactive to murine stefin A and therefore could not be used for verification in the mouse tumor tissues. *In situ* hybridization will be used to detect the expression of stefin A1 in primary and metastatic tumors of the mouse model.

We have also verified the microarray data derived from primary tumor vascular endothelium, before proceeding to a study of the expression of the genes *in situ* in tumors. Expression of FoxP1, LKB1, MIF, LATS2 and Snail by real time quantitative RT-PCR is shown in Figure 6. With increasing metastatic capacity, endothelial expression of FoxP1, LKB-1 and LATS2 decreased. On the other hand, there was a trend toward increased expression of MIF and SNAIL in the endothelium of highly metastatic 4T1.2 tumors. SNAIL has been shown in a previous study to be up-regulated in human breast tumor endothelium compared to normal endothelium (8).

Since a group of genes have now been verified from the microarray studies by RT-PCR on immunopurified samples, *in situ* hybridisation is now being optimised to verify histologically the cell specific expression of these genes of interest and their expression both in the primary tumors and also in metastatic lesions. Due to the lack of antibodies available, riboprobes are being made by cloning 2-3 ~400 bp cDNA fragments of each gene into pGEM Teasy and utilizing the T7 and Sp6 bidirectional promoters for generating anti-sense and sense RNA probes. The method is being optimised using the vascular endothelial specific gene KDR (VEGFR2). Constructs have already been made for KDR, FoxP1, SNAIL and LATS2. Therefore studies in the near future will be concentrating on the endothelial genes of interest, in order to determine the best candidates to take on to functional studies.

TASK 3 *In vitro* functional analysis of the selected candidates (months 18-36).

- a. Perform *in vitro* invasion and migration assays using tumor cells co-cultured with endothelial cells or fibroblasts isolated from primary tumors with known metastatic potential (months 18-24).
- b. Generate endothelial cells or fibroblasts transiently infected with a retrovirus expressing a cDNA construct for one of the genes of interest. Use these cells in the invasion and migration assays described above with tumor cells of varying invasive potential (months 18-36).

TASK 4: *Explore the function of the selected genes in metastasis in vivo* (months 18-36).

- a. If available, obtain mice null for the stromal gene of interest. Backcross onto a Balb/c background (months 18-30).
- b. Measure the metastatic capacity of the bone metastasizing clone in mice lacking the relevant stromal gene (months 30-36).
- c. In normal Balb/c mice, use neutralizing antibodies, an antagonist or a small molecule inhibitor of the gene of interest to measure the effect on bone metastasis (months 18-36).

As expected by the timeline, functional studies (Task 3 and 4) have not yet been carried out for the genes of interest in Task 2. Stefin A1 is currently being cloned in preparation for retroviral infection into 67NR, 66cl4 and 4T1.2 lines. This gene will be studied for a role in metastasis *in vitro* and in the metastasis model *in vivo*. Upon verification by *in situ* hybridisation, a number of genes derived from vascular endothelial studies will be cloned and studied functionally as per Task 3 and of the proposal.

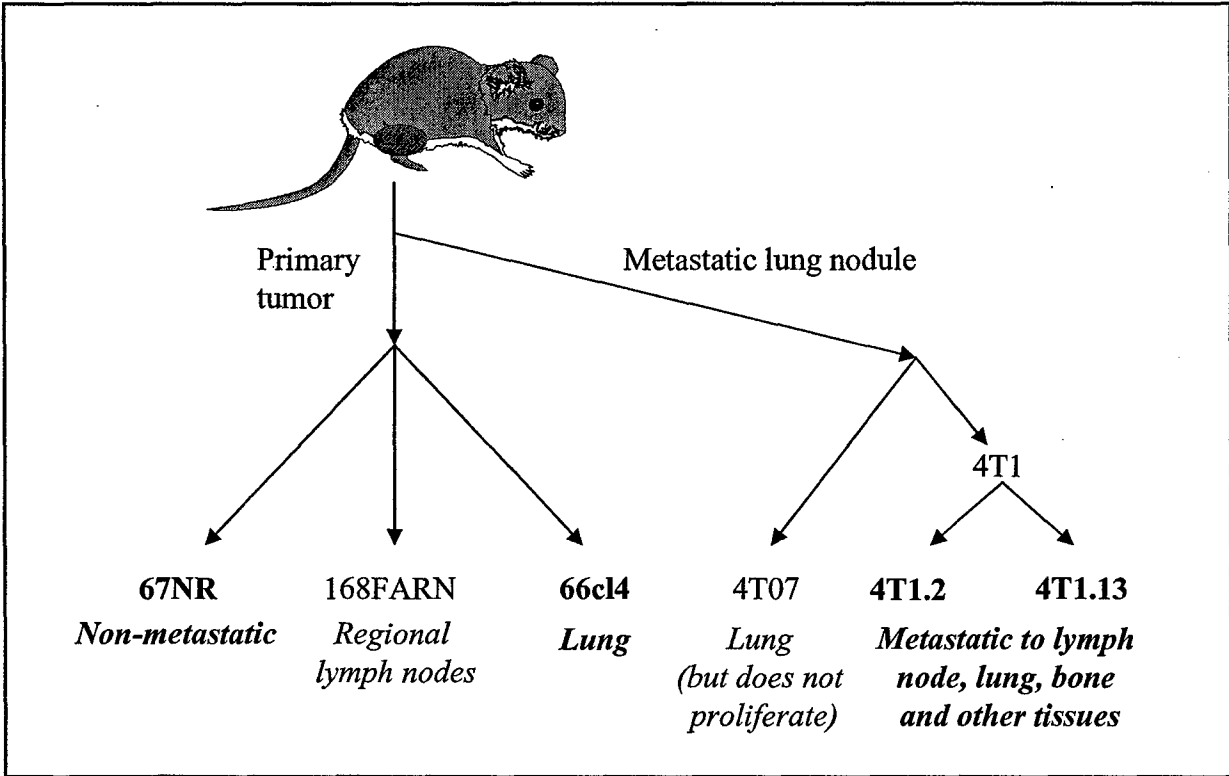


Figure 1. Orthotopic model of breast cancer metastasis to bone. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, while 168FARN, 66c14 and 4T07 are weakly metastatic and have a tissue restricted metastatic distribution. 4T1.2 and 4T1.13 are two bone metastasizing tumor clones derived from the lung metastasizing 4T1 subline. Sublines in **bold** are those that were included in the analysis

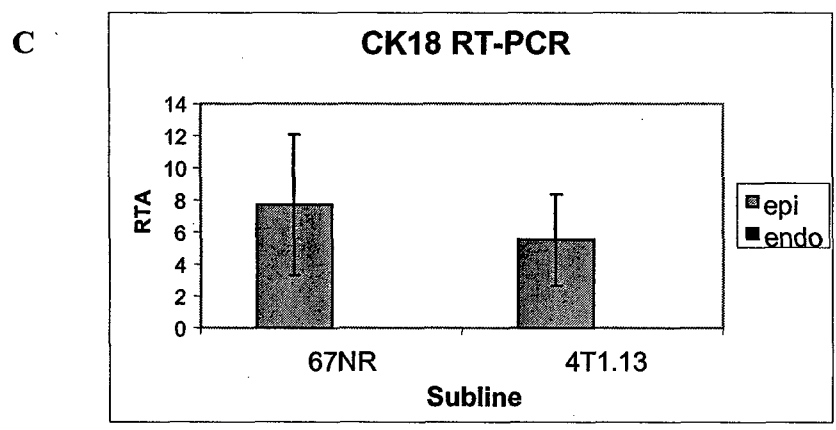
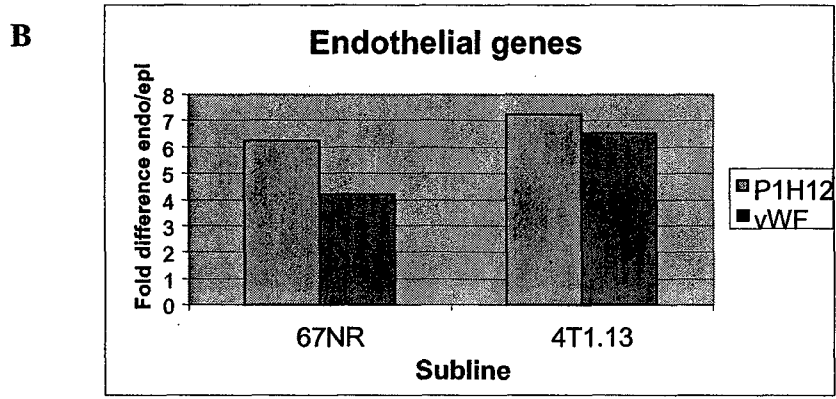
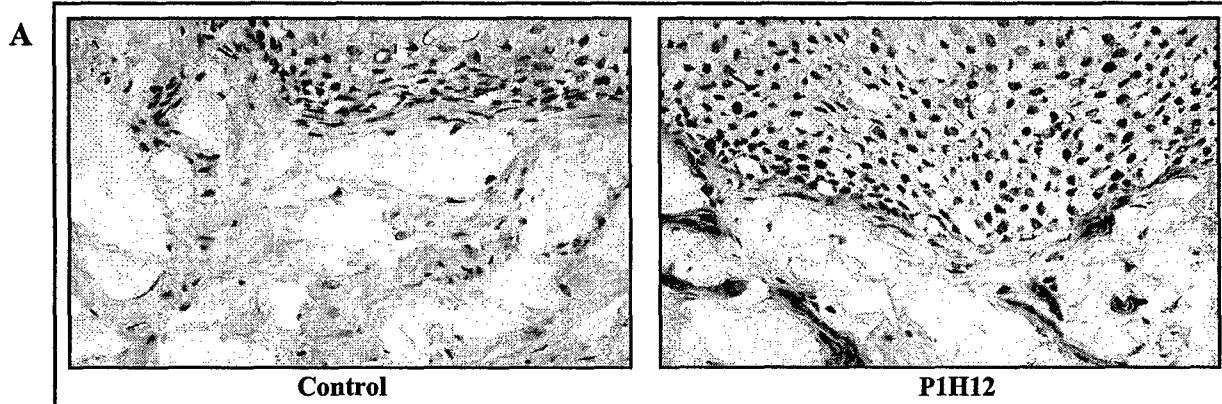
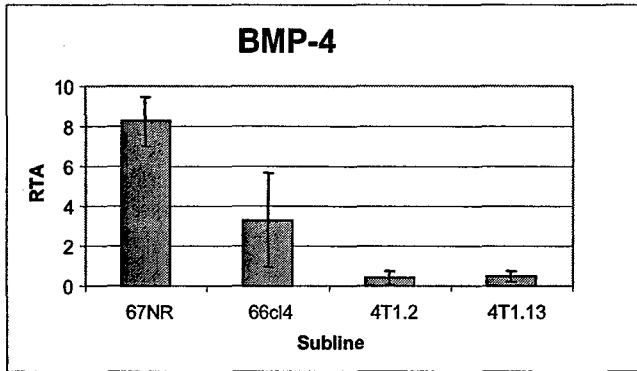
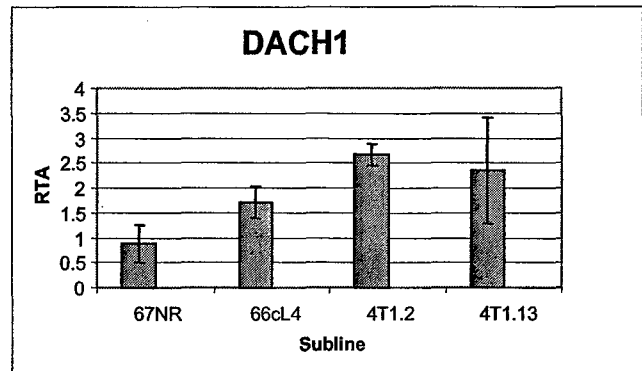


Figure 2 Verification of endothelial cell identity

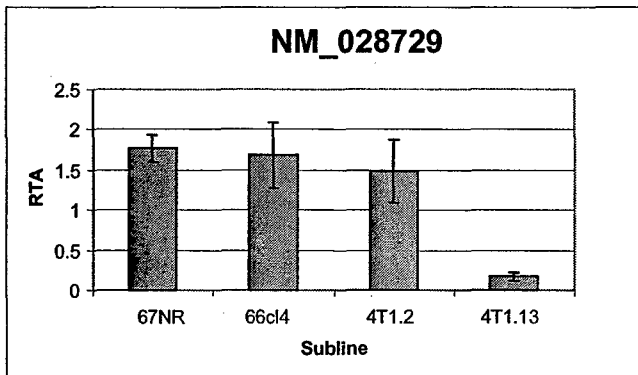
To verify the specific binding of P1H12 to vascular endothelium a number of methods were used. These included immunohistochemistry on human foreskin (A) and mouse hind skin (data not shown) and RT-PCR of immunopurified populations of epithelial and endothelial populations with genes specific for endothelium (vWF, P1H12) and epithelium (CK18), shown in panels B and C respectively.



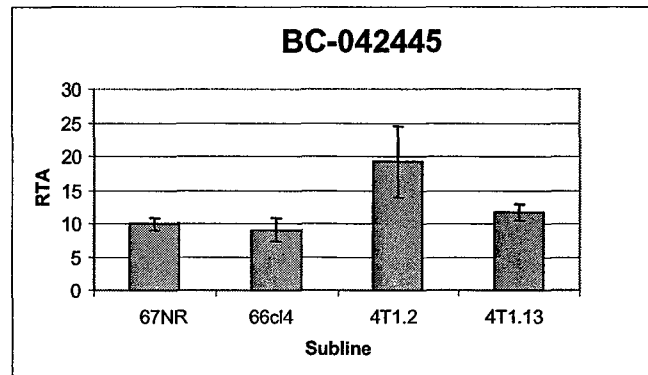
Microarray= Decreased 2.5 fold 4T1.2, 4T1.13 v's 67NR, 66cl4 in epithelium



Microarray= Increased 2.4 fold high met epithelium



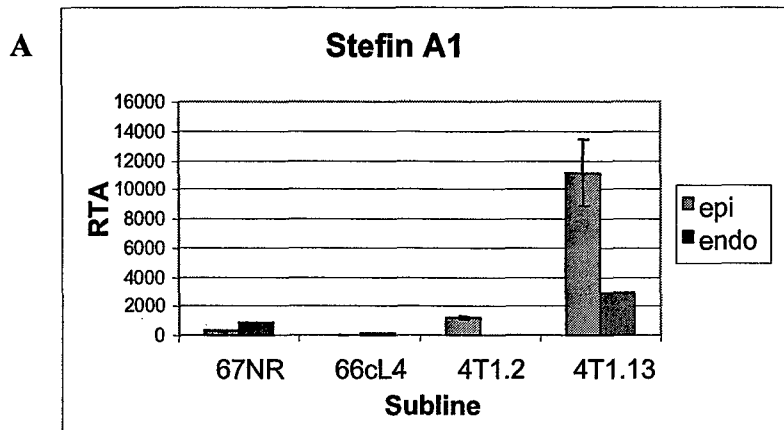
Microarray = 2.2-fold decrease epi specific



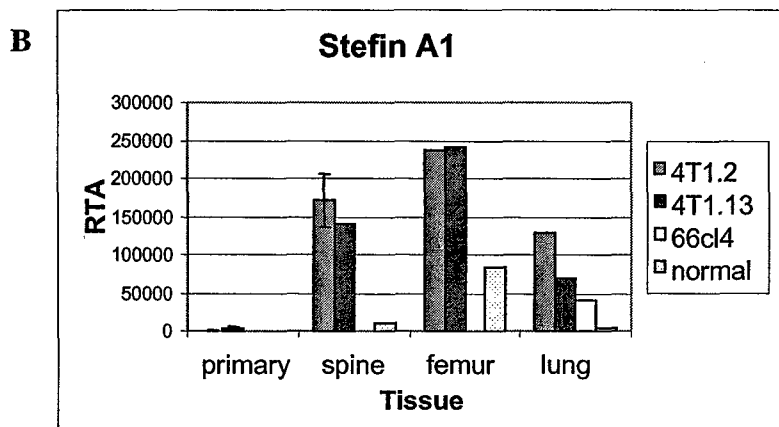
Microarray = 1.9-fold increase epi specific

Figure 3 Genes with altered expression in highly metastatic primary epithelium

Genes (BMP-4, DACH1) and ESTs (NM_028729, BC-042445) found differentially expressed by microarray in epithelial cells immunopurified from highly metastatic primary tumors were verified by quantitative RT-PCR. RNA samples from 5 duplicate immunopurified samples from primary tumors of each subline were reverse transcribed using Qiagen Sensiscript RTase. Quantitative RT-PCR was performed using SYBR green and gene specific primers, and GAPDH as a control for normalization. RTA represents the relative transcript abundance when CT values for each gene were normalised to GAPDH. Fold-difference between epithelium derived from high metastatic and low metastatic primary tumors as determined by microarray is indicated below each graph.



Microarray= Increased 7.6 fold in epithelium



Microarray= increased 9-fold in spine epithelium compared To primary epithelium

Figure 4 Expression of Stefin A1 (*Stfa1*) in primary and metastatic tumors.

Stefin A1 was found over-expressed in highly metastatic primary tumor epithelium and at even higher levels in epithelium isolated from the spine of mice containing bone metastases. This was verified by using quantitative RT-PCR, revealing an increase in transcript level in highly metastatic sublines at the primary site (A), and a further increase in metastatic lesions (B). 4T1.2 spine epithelium was derived from 6 duplicates, as were all primary epithelial samples. Comparisons to microarray results are shown below each graph.

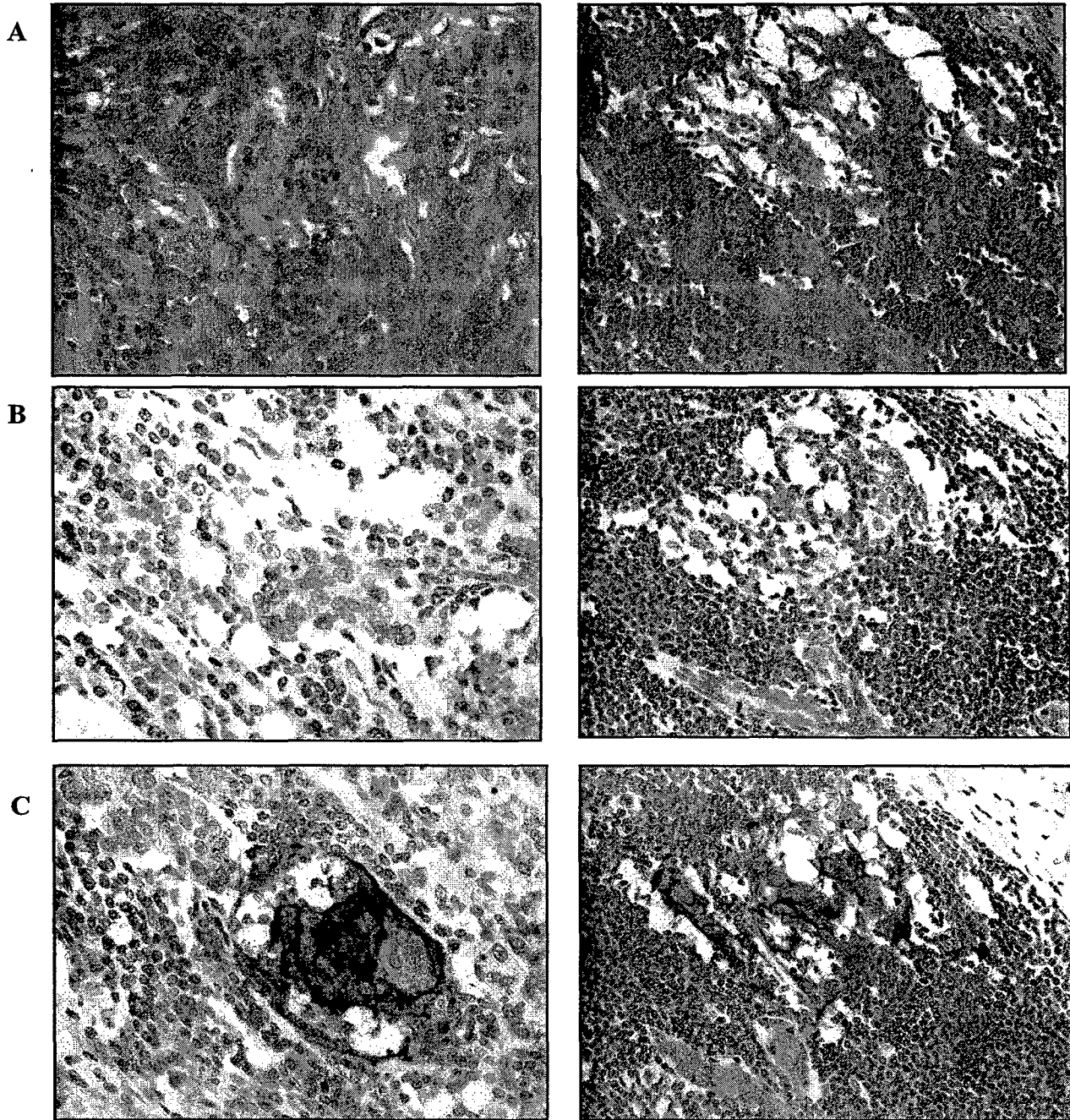
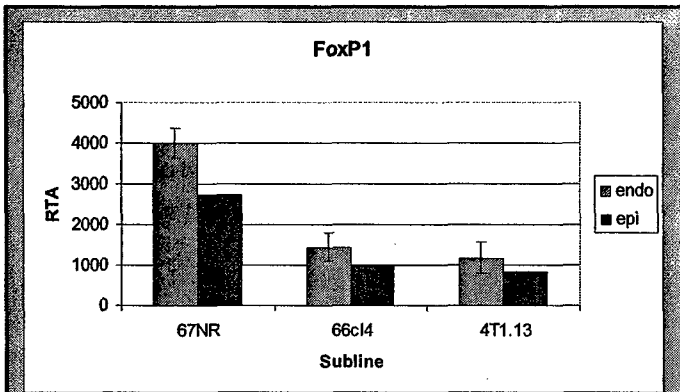
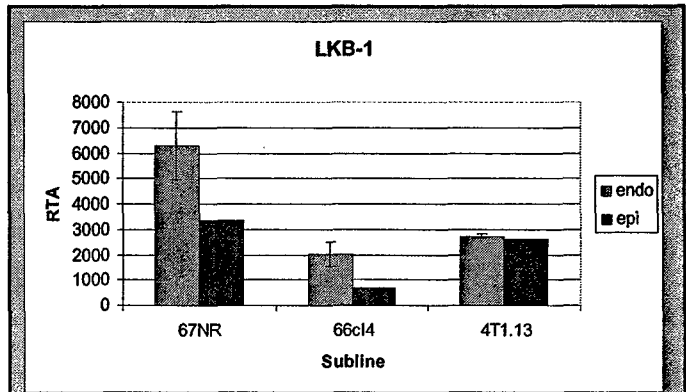


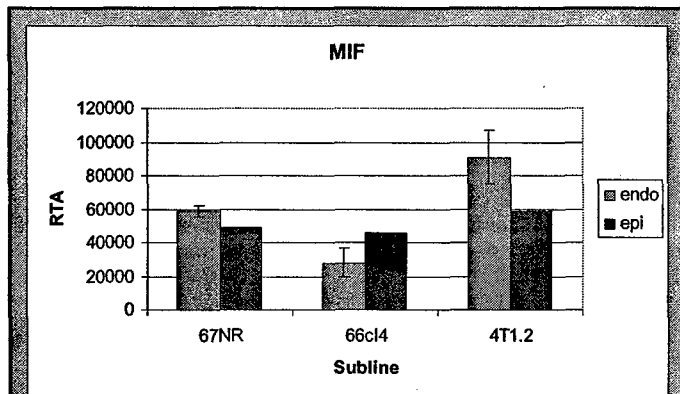
Figure 5 Immunohistochemistry of Stefin A in human breast cancer
2 regions of a primary breast tumor and shown stained with hemotoxylin and eosin as a reference (A), IgG negative control (B) and the anti-human steffin A antibody.



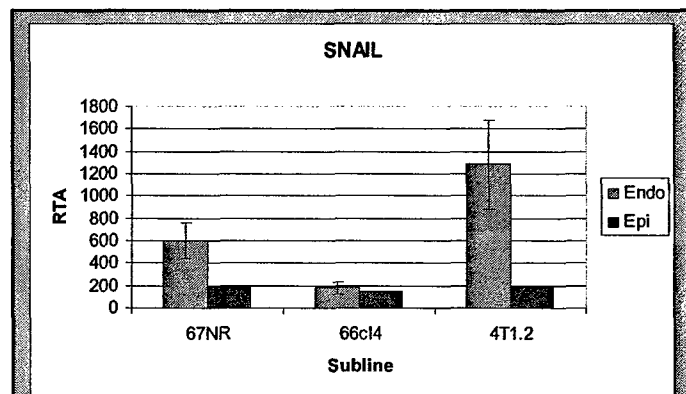
Microarray = ~3-fold decrease in high met tumor endothelium - also decreased in epithelium



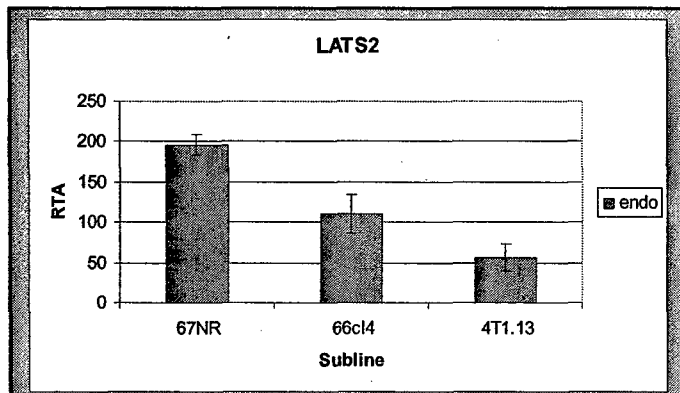
Microarray = 4-5-fold decrease in high met primary endo - no great difference in epithelium



Microarray = 3.5-fold high met/low met endothelium



Not on microarray slide but up in human tumor endo 10-fold compared to normal mammary endothelium.



Microarray = Decreased 3-fold in high met endothelium compared to low met primary tumor endothelium

Figure 6. Genes with altered expression in highly metastatic primary endothelium

FoxP1, LKB1, MIF, LATS2 and SNAIL found to be differentially expressed by microarray in endothelial cells immunopurified from highly metastatic primary tumors, were verified by quantitative RT-PCR. RNA samples from 5 duplicate immunopurified samples from primary tumors of each subline were reverse transcribed using Qiagen Sensiscript RTase. Fold-difference between endothelium derived from high metastatic and low metastatic primary tumors as determined by microarray is indicated below each graph.

Accession ID	Description	Common	P-value	High met/ normal	High met/ low met	normal/ high met	Low met/ high met	Function
BG077636	endothelial-derived gene	Eg1	2.60E-04	3.01	1.83			angiogenesis
BG075854	AXL receptor tyrosine kinase	Axl	0.008018	9.01	2.16			angiogenesis, cell adhesion and proliferation
BG087383	cathepsin D	Ctsd	0.014202	7.08	1.98			angiogenesis, ECM degradation
BG077749	macrophage migration inhibitory factor	Mif	5.49E-04	4.64	3.48			angiogenesis, endo growth and migration
BG069647	ornithine decarboxylase, structural	odc	0.014948	3.12	1.96		3.29	tumor invasion/angiogenesis/endo proliferation (suppression of endostatin)
BG074926	transducer of ERBB2, 2	Tob2	0.005109			5.49		antiproliferative/inhibits cell cycle progression
BG072463	H1 histone family, member 0	H1f0	0.006185	3.06	2.5		5.2	DNA replication/progression through cell cycle
C87546	serine/threonine kinase 11	Stk11	0.004594			4.39		tumor suppressor/cell cycle blockage/P53 apoptotic regulation
BG069799	α -disintegrin and metalloprotease domain 8	ADAM8	9.82E-05	53.47	1.92			cell adhesion/growth/osteoclast maturation
BG066967	RAB20, member RAS oncogene family	Rab20	0.002162	8.93	0.85			cell adhesion/migration
AW544628	Integrin beta 1 (fibronectin receptor beta)	Itgb1	0.001659	2.92	2.09			cell adhesion/spreading
BG088280	cell adhesion molecule-related down-regulated by oncogenesis	Cdon	0.00251	3.36	1.86			cell adhesion
BG082041	RAP2B, member of RAS oncogene family	Rap2b	0.004098	4.94	1.34			chemotaxis/cell adhesion, morphology, motility/migration
AW542365	Villin 2	Vil2	0.010279			3.14	2.47	cytoskeleton architecture
BG077548	LIM and SH3 protein 1	Lasp1	1.54E-04	3.56	1.8			cytoskeleton architecture/cell motility/downstream target of hedgehog
BG072801	S100 calcium binding protein A9 (calgranulin B)	S100a9	0.004227	14.78	1.43			transendothelial migration/adhesion to ECM
BG071424	integral membrane protein 2C	Lim2c	0.003416	3.91	1.3			integral transmembrane protein/adhesion????
BG063236	phosphatase and tensin homolog	PTEN	0.013317			3.984	2.68	tumor suppressor/proliferation/migration
BG076288	forkhead box P1	Foxp1	0.004227			3.69	2.98	growth suppression/transcriptional repressor
BG076991	suppressor of cytokine signaling 3	Socs3	0.007995	4.17	1.47			inhibition of anti-proliferative proteins/inhib T cell recognition
BG085276	peroxiredoxin 5	Prdx5	1.62E-04	6.77	2.86			antioxidant/protective and proliferative role
BG088394	selenoprotein P, plasma	Sepp1	8.17E-05	9.67	1.3			oxidative stress response
AW553304	BTB and CNC homology 2	Bach2	0.017182			11.63	2.17	oxidative stress/reduced proliferation/spontaneous cell death
BG077758	histidine triad nucleotide binding protein	Hint	0.011782					protection of cells from damage by LDL
BG085960	apolipoprotein C-II	ApoC2	1.62E-04	2.88	1.45			cholesterol homeostasis (like apoB2)
BG069532	Niemann Pick type C2	Npc2	8.55E-06	5.25	1.47			glycolytic enzyme/induced by hypoxia
BG064823	phosphoglycerate mutase 1	pgam1	0.002216	4	1.93			tubulin production/microtubule maintenance
BG084568	tubulin cofactor a	tbc a	0.005996	2.44	1.18			acetylase/transcriptional activator
BG073029	GREBBP/EP300 inhibitory protein 1	Ctfl1	5.51E-04	2.7	1.21			signalling intermediate
C87464	tumor protein D82	tpd52	4.34E-06	3.64	0.96		1.99	decreased expression = cell specific differentiation
BG068028	SWI/SNF related, matrix associated, actin dependent regulator	Smarca5	0.005919			3.73		
BG078674	cathepsin B	Ctsb	0.001984	6.54	0.69			invasion- but not met specific- up in all primary tumor endothelium

Table 1 Genes differentially expressed in endothelium of highly metastatic primary tumors compared to normal mammary gland endothelium or endothelium from tumors with low metastatic capacity

Accession ID	Description	P-value	High met/ normal	High met/ Low met	Low met/ high met	normal/ high met	Human homolog
AW544006	Mus musculus cDNA clone CO176G03.3, mRNA sequence	0.0065	5.56	3.92	1.42	3.14	Homo sapiens BAC clone RP11-372K14
BG065803	RIKEN cDNA: E230006M18, gene	0.004469	2.93	2.17	1.44	2.96	Homo sapiens 3' BAC RP11-630D12*
BG066117	Hypothetical protein: 5230400M03	1.90E-04	8.23	1.85	1.68	3.03	Human DNA sequences from clone RP11-14E8*
BG071783	DNA segment: Chr 9, Wayne State University 18, expressed	0.006698	2.53	1.44	2.23	12.01	Homo sapiens acid cluster protein 33, mRNA
BG088489	Hypothetical protein: 4732466J24	4.14E-04	3.1	1.15	2.37	3.77	Homo sapiens endonuclease G-like 1 (ENDOGL1), mRNA
BG072866	Mus musculus, clone MGC 28609 IMAGE4218591, mRNA, complete cds	1.19E-05	7.15	0.8	2.46	15.32	Homo sapiens 3q BAC RP11-396A13*
BG072106	RIKEN cDNA: 9590027K23, gene	1.60E-05			2.51	3.97	Homo sapiens hypothetical protein FLJ21918, mRNA
BG086714	RIKEN cDNA: 1200013B22, gene	0.005894			2.8	3.02	Homo sapiens likely ortholog of rat SNF1/AMP-activated protein kinase (SNARK)
BG071241	RIKEN cDNA: 9430028L20, gene	0.013949			3.11	4.15	Homo sapiens Kruppel-like factor 13 (KLF13), mRNA
AU022584	DNA segment: Chr 8, ERATO Doi 812, expressed	0.013024			4.62	5.53	Homo sapiens hypothetical protein FLJ20244, mRNA
BG082241	RIKEN cDNA: 2810489L22, gene	0.007995			4.88	14.66	Homo sapiens structural maintenance of chromosomes 6-like 1 (SMC6L1), mRNA
BG082333	RIKEN cDNA: 2810489L22, gene	0.010697					Homo sapiens structural maintenance of chromosomes 6-like 1 (SMC6L1), mRNA
AU022611	RIKEN cDNA: 2810489L22, gene	0.0028					Homo sapiens structural maintenance of chromosomes 6-like 1 (SMC6L1), mRNA
BG088755	ESTs, Moderately similar to T14792 hypothetical protein DKFZ556G0322.1 (H. sapiens)	0.016369					Homo sapiens structural maintenance of chromosomes 6-like 1 (SMC6L1), mRNA
BG075419	Hypothetical protein LOC219024	0.005797					Homo sapiens protein associated with Myc (PAM), mRNA
BG072804	Mus musculus mRNA, up-regulated by FUS-ERG, 3' region, cDNA fragment: C14G220, *	0.002249					Homo sapiens APEX nuclease (multifunctional DNA repair enzyme) (APEX) gene
BG084366	RIKEN cDNA: 2810452K22, gene	0.002216					Homo sapiens large tumor suppressor, homolog 2 (Drosophila) (LATS2), mRNA
BG078034	Expressed sequence tag: A1320141						Homo sapiens cyclin-dependent kinase 2-interacting protein, mRNA

*short homologous region ~50 bp

Table 2 Analysis of genes differentially expressed in endothelium of highly metastatic primary tumors compared to normal mammary gland endothelium or endothelium from tumors with low metastatic capacity. The corresponding human homologs of these ESTs are shown.

Accession ID	Description	Common	P value	high met/ low met	low met/ high met	Function
BG074171	stfai1	stfai1	0.007	7.6		cystatin, inhibitor of Cathepsin S, decrease antigen processing and presentation
BG085518	ets variant gene 6 (TEL oncogene)	ETV6	0.004	7.2		cellular aggregation, transformation
BG072171	histidine decarboxylase	Hdc	0.003	5.8		protection against histamine, tumor cell proliferation
BG064722	SMT3 (suppressor of mit two, 3) homolog 1 (S. cerevisiae)	Smt3h1	0.001	3.9		protection against TNF etc cell death, cell cycle progression
BG087429	SMT3 (suppressor of mit two, 3) homolog 1 (S. cerevisiae)	Smt3h1	3.00E-04	3.9		protection against TNF etc cell death, cell cycle progression
BG072394	complement component 3	C3	0.004	3.8		up in cancer patients, including highly metastatic human melanoma cell line
BG071895	histidine decarboxylase	Hdc	0.003	3.5		synthesis of histamine, tumor cell proliferation
BG082584	developmental pluripotency associated 5	dppa5	7.00E-04	3		development
BG079103	envelopin	Evp1	0.008	2.6		cytolinker, protein
BG075820	histone cell cycle regulation defective homolog A (S. cerevisiae)	Hira	0.007	2.5		Histone chaperone, nucleosome assembly, cell cycle control
BG077636	dachsinski 1 (Drosophila)	DACH1	0.004	2.4		cell proliferation, inhibits apoptosis (TGFB), developmental gene
BG066360	endothelial-derived gene	Eg1	0.006	2.4		angiogenesis (originally identified in tumor endothelium-also found in tumor epithelium)
BG071729	Rho GTPase-activating protein 8	Rafgap8	0.004	2.3		cytoskeletal organization, cell cycle regulation, ras-mediated oncogenic transformation
BG065738	maternal effect gene	Mater	0.004	2.2		early development
BG065879	Rho guanine nucleotide exchange factor (GEF) 3	Argef3	0.008	1.8		cytoskeletal rearrangement
BG071892	sonesoderm homolog (Xenopus laevis)	Eomes	0.006	1.8		early developmental gene
BG088006	RAD21 homolog (S. pombe)	Rad21	0.008	1.8		up in prostate cancer, chromatin cohesion protein
BG077363	signal transducer and activator of transcription interacting protein 1	statp1	0.01	1.8		cytokine signalling, stat3 interacting protein, stat 3 has role in oncogenesis
BG064270	topoisomerase (DNA I)	Top1	0.001	2.1		realization of supercoiled DNA through breakage, cell cycle checkpoint
BG064129	breast cancer metastasis-suppressor 1	Brms1	0.002	2.2		Suppression of metastasis, function not clear, may be involved in transcription regulation
BG078804	mitogen-activated protein kinase 1	MAPK1	0.005	2.4		activation of ERK1/ERK2, roles in differentiation
BG064070	golgi SNAP receptor complex member 1	Gosl1	0.003	2.5		trafficking membrane protein b/n ER and golgi
BG085457	bone morphogenetic protein 4	BMP4	6.00E-05	2.5		member of TGF-beta superfamily, induce cell senescence
BG077621	ARP3 actin-related protein 3 homolog (yeast)	ARP3	0.006	2.6		actin nucleation, organization of the cytoskeleton, controls polarised cell growth
BG087426	MAP-kinase-interacting serine/threonine kinase 2	Mknk2	0.003	2.6		limit/inhibit translation
BG063869	lamina	Lmna	0.006	2.7		exp. correlated with non-prolif. cells and tissues, nuclear intermediate filament protein
BG082615	heat shock protein a1	HSP873	0.005	2.8		chaperone
BG077186	autophagy 7-like (S. cerevisiae)	Apg7l	0.002	3		enzyme essential for autophagy
BG064223	DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	0.003	3		stress induced, induced upon anti-metastatic activity (in lung adenocarcinoma cells)
BG065503	heat shock protein 8	HSP873	0.01	3		chaperone
BG088147	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21 (RNA helicase)	Dox21	4.00E-04	3.1		rRNA biogenesis
BG081794	integrin beta 5	Iigb5	0.008	3.1		cell-ECM interaction
BG068990	zinc finger protein X-linked	Zfx	0.004	3.1		inhibits angiogenesis, involved in embryonic growth (sex differentiation)
BG080311	similar to Mifc-1 related chloride channel 1	Mclc	0.007	3.2		regulate transcription through maintenance of chromatin structure
BG085841	suppressor of Ty 6 homolog (S. cerevisiae)	supt6h	2.00E-04	3.7		ABC protein, membrane transporter
	KIP-binding cassette, sub-family B (MDR/TAP), member 1B	Abcb1b	0.006	4		transcription factor, differentiation
	nuclear factor, erythroid derived 2-like 2	Nfe2l2	0.002	4.6		

Table 3 Genes differentially expressed in highly metastatic primary tumor epithelium compared to tumors with low metastatic capacity

Gene	Up/down	Human chrom. location	LOH/gene amplification	Function/Cancer association
Stefin A1	Up	3q21	LOH	-Cystatin, inhibitor of Cathepsin S (antigen presentation) -Marker of malignancy in some experimental tumors
Smt3h1/ SUMO	Up	21q22.3	gene amp	-protection against TNF cell death - interacts with MDM2 -cell cycle progression
Dach 1	Up	13q22	LOH & gene amp	-Cell proliferation, inhibits TGF β -induced apoptosis -Developmental gene -Role in skeletal development (stimulated by FGF and inhibited by BMP4)
EST (BC-042445)	Up	15q21.3	Allelic imbalance	-no known function - homology to human KIAA1584 protein (AB046804)
Lamin A	Down	1q21.2-21.3	LOH	-nuclear intermediate filament protein -expression correlated with non-prolif. cells and tissues - apoptotic protein
BMP4	Down	14q22-23	LOH	-member of TGF-beta superfamily - developmental role, induces cell senescence - may have a role in hedgehog signalling
EST (NM_028729)	Down	11q13.5	LOH & gene amp	- no known function - homology to human hypothetical protein FLJ25416 (BC039268)

Table 4: List of candidate genes aberrantly expressed in highly metastatic primary tumor epithelium. Genes that are increased (up) or decreased (down) in the highly metastatic tumors (4T1.2, 4T1.13) compared to those that do not metastasize to bone are indicated, along with the human chromosomal localization and known gene functions and previous documentation in cancer. Also included is past evidence of LOH or gene amplification in that chromosome region in breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Purification of epithelial, endothelial and “stromal” cells from primary tumors derived from an *in vivo* model of breast cancer metastasis
- Confirmation of endothelial cell purity using the immunopurification method
- Microarray gene expression profiling of cell specific alterations with increasing metastatic propensity of primary tumors
- Generation of tables of genes statistically different in endothelial and epithelial cells from highly metastatic primary tumors
- Quantitative RT-PCR verification of genes altered in the endothelium (FoxP1, LKB-1, MIF, SNAIL, LATS2) and epithelium (Stefin A1, BMP-4, DACH1, and 2 ESTs)
- Verification of high Stefin A1 expression in epithelial cells purified from bone metastases (higher than that in matched primary tumors)
- Detection of Stefin A expression also in human breast cancer by immunohistochemistry

REPORTABLE OUTCOMES

Awards

2003 AACR Special Conference Scholar-in-Training Award (provided by the Avon Foundation).

Publications relating to project

Parker, B.S., Eckhardt, B.L. and Anderson, R.L. (2004). Models of breast cancer metastasis to bone: characterization of a clinically relevant model. In Bone Metastasis, Eds. G. Singh and F.W. Orr, Kluwer Press, The Netherlands.

Parker, B.S., Argani, P, Cook, B.P., Liang Fen, H., Chartrand, S.D., Zhang, M., Saha, S., Bardelli, A., Yiang, Y., St. Martin, T.B., Nacht, M., Teicher, B.A., Klinger, K.W., Sukumar, S. and Madden, S.L. (2004). Alterations in vascular gene expression in invasive breast cancer. *Cancer Research*, Accepted.

Conference presentations

Cell specific gene expression profiling in a murine model of breast cancer metastasis. Parker, B.S. and Anderson, R.L. Metastasis Research Society, 10th Int'l Congress, Genoa Italy September 17-20, 2004

Aberrant gene expression in breast cancer endothelium. Parker, B.S., Madden, S.L., Sukumar, S.S. and Anderson, R.L. Advances in Breast Cancer Research: Genetics, Biology, and Clinical Implications (AACR Special Conference), 2003. Huntington Beach, CA, USA.

Aberrant gene expression in breast cancer endothelium . Parker, B.S., Madden, S.L., Sukumar, S.S. and Anderson, R.L. 5th Peter MacCallum Cancer Centre Symposium, November, 2003. Melbourne, Australia.

CONCLUSIONS

This study has investigated cell specific gene expression alterations during metastatic progression of breast cancer in a clinically relevant *in vivo* model. Endothelial and epithelial cells have been successfully purified from primary breast tumors and (subsequent to RNA amplification and labelling) have been expression profiled using microarray.

A number of candidates have been identified over-expressed or suppressed in tumor endothelium and in the tumor cells themselves during metastatic progression. These candidates have been verified and will be analysed further for their functional role in metastasis, and for their role in human breast cancer.

One such gene, stefin A1, has an enhanced gene expression in tumor cells with greater metastatic propensity and this expression is elevated in matched spine and femur metastases. Importantly, this gene also has relevance in human breast cancer, with detection of protein expression in a subset of primary tumors. This work is going to be continued to determine the prognostic significance of stefin A expression in human breast cancer.

This work has many implications to breast cancer research. The use of a clinically relevant model of breast cancer metastasis was not only useful for finding gene candidates but is also of enormous importance in determining the functional role of such genes in the metastasis process. This has not been available in other studies, and may be responsible for the lack of molecular markers as prognostic indicators and targets for treatment. Additionally, many genes that have previously been associated with human cancer progression have been identified in this study and that fact that we have already verified expression of one of our candidates of interest in human breast cancer reveals the clinical relevance of this model in researching breast cancer metastasis.

REFERENCES

- 1) Rubens, R.D., *Bone metastases - the clinical problem*. European Journal of Cancer, 1998. 34: p. 210-213.
- 2) Bissell, M.J. and D. Radisky, *Putting tumors in context*. Nature Reviews (Cancer), 2001. 1: p. 1-19.
- 3) Cunha, G. R. and Matrisian, L. M. It's not my fault, blame it on my microenvironment. *Differentiation*, 70: 469-472, 2002.
- 4) Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res*, 59: 5002-5011, 1999.
- 5) Allinen, M., Beroukhi, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A., Schnitt, S., Sellers, W.R., and Polyak, K. *Cancer Cell*, 6: 17-32, 2004.
- 6) Lelekakis, M., Moseley, J. M., Martin, T. J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F. R., Slavin, J., and Anderson, R. L. A novel orthotopic model of breast cancer metastasis to bone. *Clinical and Experimental Metastasis*, 17: 163-170, 1999.
- 7) St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. Genes expressed in human tumor endothelium. *Science*, 289: 1197-1202, 2000.
- 8) Parker, B.S., Argani, P, Cook, B.P., Liang Fen, H., Chartrand, S.D., Zhang, M., Saha, S., Bardelli, A., Yiang, Y., St. Martin, T.B., Nacht, M., Teicher, B.A., Klinger, K.W., Sukumar, S. and Madden, S.L. (2004). Alterations in vascular gene expression in invasive breast cancer. *Cancer Research*, Accepted.

APPENDIX

CURRICULUM VITAE

Name: Belinda Sheree Parker
[Redacted]
Work Address: Peter MacCallum Cancer Centre
Department of Research
St Andrews Place, East Melbourne, 3002
[Redacted]
Telephone: (BH) 03 96561306 (AH) 0402 849305
Fax: 03 96561411
Email: belinda.parker@petermac.org
[Redacted]

PII Redacted

TERTIARY QUALIFICATIONS

1998-2001 **PhD** (submitted 23/08/001), Dept. of Biochemistry, La Trobe University
Supervisors : Dr D.R.Phillips and Dr S. M. Cutts

1997 **Honours year** Biochemistry, La Trobe University -Result H1

1994-1996 **Bachelor of Biological Sciences** La Trobe University
Majors: Biochemistry and Human Genetics

POSTDOCTORAL EXPERIENCE

March 2003-present Postdoctoral Fellow, Department of Research, Peter MacCallum Cancer Centre.
Field of Study- Breast Cancer
PI: Dr. Robin Anderson

Nov 2001-March 2003 Postdoctoral Fellow, Department of oncology, Johns Hopkins University, MD, USA.
Field of study- Breast Cancer
PI: Professor Saraswati Sukumar

AWARDS AND FELLOWSHIPS

2003 AACR Special Conference Scholar-in-Training Award (provided by the Avon Foundation).

- 2003 Awarded- Department of Defence Breast Cancer Research Program
 (BCRP) Postdoctoral Fellowship.
 (July 2003-July 2006)
 - Susan Komen Postdoctoral Fellowship (declined)
 - Peter Doherty Postdoctoral Fellowship (March 2003-July 2003
 then declined)
- 2000 Australian Society of Biochemistry and Molecular Biology (ASBMB)
 travel Fellowship.
- 1998 LaTrobe University Postgraduate scholarship

SCIENTIFIC PUBLICATIONS

Parker, B.S., Eckhardt, B.L. and Anderson, R.L. (2004). Models of breast cancer metastasis to bone: characterization of a clinically relevant model. In *Bone Metastasis*, Eds. G. Singh and F.W. Orr, Kluwer Press, The Netherlands.

Parker, B.S., Rephaeli, A., Nudelman, A., Phillips, D.R. and Cutts, S.M. (2004). Formation of mitoxantrone adducts in human tumor cells: Potentiation by AN-9 and DNA methylation. *Oncology Research*. In press.

Parker, B.S., Buley, T., Evison, B.J., Cutts, S.M., Neumann, G.M., Iskander, M.N. and Phillips, D.R. (2004). A molecular understanding of mitoxantrone-DNA adduct formation: Effect of cytosine methylation and flanking sequences. *J. Biol. Chem.* In Press (Epub).

Parker, B.S., Argani, P., Cook, B.P., Saha, S., Fen, H.L., Bardelli, A., Madden, S.L., Sukumar, S. (2004). Aberrant expression of genes in human breast tumor vascular endothelium: Genetic profiling and validation. Submitted.

Waly, M., Olteanu, H., Banerjee, R., Choi, S.-W., Mason, J.B., Parker, B.S., Sukumar, S., Shim, S., Sharma, A., Benzecry, J.M., Power-Charnitsky, V.-A. and Deth, R.C. (2004). Activation of methionine synthase by insulin-like growth factor-1 and dopamine: A target for neurodevelopmental toxins and thimerosal. *Molecular Psychiatry*. In Press (Epub).

Parker, B.S., Cutts, S.M., Nudelman, A., Rephaeli, A., Phillips, D.R. and Sukumar, S. (2003). Mitoxantrone mediates Demethylation and re-expression of cyclin D2, estrogen receptor and 14-3-3 σ in breast cancer cells. *Cancer Biol. & Ther.* 2(3), 259-263.

Parker, B.S. and Sukumar, S. (2003). Distant metastasis in breast cancer: Molecular mechanisms and a search for therapeutic targets. *Cancer Biol. & Ther.* 2(1), 14-21.

Parker, B.S., Cutts, S.M. and Phillips, D.R. (2001). Cytosine methylation enhances mitoxantrone-DNA adduct formation at CpG dinucleotides. *J. Biol. Chem.* 276(19), 15953-15960.

Cutts, S.M., Parker, B. S., Swift, L. P., Kimura, K. and Phillips, D.R. (2000). Structural requirements for the formation of anthracycline-DNA adducts. *Anticancer Drug Design.* 15(5), 373-386.

Parker, B.S., Cutts, S.M., Cullinane, C. and Phillips, D.R. (2000). Formaldehyde activation of mitoxantrone yields CpG and CpG specific DNA adducts. *Nucleic Acids Research* 28, 982-990.

Parker, B.S., Cullinane, C. and Phillips, D.R. (1999). Formation of DNA adducts by formaldehyde-activated mitoxantrone. *Nucleic Acids Research* 27, 2918-2923.

ONGOING RESEARCH SUPPORT

DOD/BCRP - DAMD17-03-1-0473

Stromal gene expression in primary breast tumors that metastasize to bone. July 2003-July 2006

Role: PI

CONFERENCE PRESENTATIONS

Aberrant gene expression in breast cancer endothelium . Parker, B.S., Madden, S.L., Sukumar, S.S. and Anderson, R.L. 5th Peter MacCallum Cancer Centre Symposium, November, 2003. Melbourne, Australia.

Aberrant gene expression in breast cancer endothelium. Parker, B.S., Madden, S.L., Sukumar, S.S. and Anderson, R.L. Advances in Breast Cancer Research: Genetics, Biology, and Clinical Implications (AACR Special Conference), 2003. Huntington Beach, CA, USA.

Enhancement of drug-DNA binding at methylated cytosine residues by the CpG specific anticancer drug mitoxantrone. Parker, B. S., Cutts, S. M. and Phillips, D.R. Annual Conference of the American Association of Cancer Research, March 2001. New Orleans, LS, USA.

The role of CpG methylation on mitoxantrone-DNA adduct formation. Parker, B.S., Swift, L. P., Cutts, S. M. and Phillips, D.R. Australian Society of Biochemistry and Molecular Biology Annual Conference, December 2000. Wellington, New Zealand.

Formaldehyde mediated DNA alkylation by mitoxantrone. Parker, B.S., Cullinane, C. and Phillips, D.R. Molecular Determinants of Sensitivity to Antitumour Agents, American Association of Cancer Research, March, 1999. Whistler, Canada

Molecular and cellular studies of the activation of mitoxantrone by formaldehyde. Parker, B.S., Cullinane, C. and Phillips, D.R. 11th Lorne Cancer Conference, Feb, 1999. Lorne, Australia

RELEVANT EXPERIENCE

Aug 2001-Nov 2001 Casual senior research assistant, Biochemistry Department, La Trobe University

1998-2001 Demonstrating 2nd and 3rd year biochemistry practical classes

REFEREES

Dr. Robin Anderson
Head, Cancer Biology
Department of Research
Peter MacCallum Cancer Center
St Andrews Place
East Melbourne, 3002
Phone: 61 (0)3 96561286
Email: robin.anderson@petermac.org

Professor Don R. Phillips
Reader in Biochemistry
La Trobe University
Bundoora, 3086
Australia
Phone: 61-3-9479 2182
Email: D.Phillips@latrobe.edu.au

scutts@bioserve.latrobe.edu.au

Prof. Sara Sukumar
Professor of Oncology
Sidney Kimmel Comprehensive
Cancer Center at Johns Hopkins
1650 Orleans St
Baltimore, MD, 21231, USA
Phone: 11 410 6142479
Email: sukumsa@jhmi.edu

Dr. Suzanne M. Cutts
Research Associate
School of Biochemistry
La Trobe University
Bundoora, 3086
Australia
Phone: 61-3-9479 1182
Email: