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Award Number: DAMD17-03-1-0473

TITLE: Stromal Gene Expression and Function in Primary Tumors that Metastasize to Bone Cancer

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REPORT DATE: July 2005

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-05	Annual Summary	07/01/04-06/30/05

4. TITLE AND SUBTITLE
Stromal Gene Expression and Function in Primary Tumors that Metastasize to Bone Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0473

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Melbourne University
Melbourne, 3010, Australia

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Abstract can be found on next page.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	31	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

ABSTRACT

Tumor progression and metastasis is mediated not only by tumor cells but by the surrounding stroma as well, including the vascular endothelium. Knowledge of the molecular and cellular interactions that promote metastasis is required to determine prognostic markers and therapeutic targets for metastatic breast cancer. A clinically relevant syngeneic model of breast cancer metastasis has been used to determine gene expression alterations that occur in both tumor epithelial cells and the associated vascular endothelium throughout metastatic progression. Expression profiles of immunopurified cell populations derived from primary tumors of varying metastatic potential have identified aberrant gene expression in endothelial (Cathepsin D, SNAIL and FoxP1) and tumor/epithelial (Stefin A1 and Breast Cancer Metastasis Suppressor gene 1, BRMS1) cells. Analysis of matched primary tumors and spine metastases revealed the additional up-regulation of the cathepsin inhibitor Stefin A1 at sites of distant metastasis, including lung and bone. Further, we have preliminary evidence of prognostic significance of Stefin A expression in primary human breast tumors, with a significant increase in Stefin A positivity in tumors derived from patients that developed soft tissue and bone metastases (N=24). In a small study, human breast cancer bone metastases were positive for Stefin A, indicating the clinical validity of the murine model and the potential significance of Stefin A in bone metastasis. In co-cultures, Stefin A1 expression is induced *in vitro* in highly metastatic cells when co-cultured with stroma. The role of cathepsins and the inhibitor Stefin A1 in breast cancer metastasis are under investigation by examining the effect of stefin A over-expression on metastasis and the associated role of the cathepsins.

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INTRODUCTION

Breast cancer, although relatively treatable at an early stage, is a clinical problem once it has progressed to metastatic disease in tissues such as lung and bone. It is therefore crucial to determine the molecular mechanism by which primary tumor cells migrate and develop metastases at secondary organs. It is becoming increasingly evident that tumor progression and metastasis is mediated not only by tumor cells but by the surrounding stroma as well (1-4), including the vascular endothelium. Knowledge of the molecular and cellular interactions that promote metastasis is required to determine prognostic markers and therapeutic targets for metastatic breast cancer.

The importance of tumor-stromal interactions to tumorigenesis and metastatic progression has illustrated the absolute requirement for use of *in vivo* models that allow for such an interaction. For this reason conventional cell culture studies *in vitro* have become inadequate, with researchers developing models for 3D cultures (5, 6) that aim to mimic the cellular microenvironment *in vivo*. The ideal model, however, is an *in vivo* model that encompasses the entire process of breast cancer metastasis, including primary tumor formation and spontaneous metastasis to distant sites applicable to the human disease. Additionally, unlike the commonly used human xenograft and intra-cardiac models, the ideal animal model should be syngeneic to ensure tumor and host stroma compatibility and to allow the use of immunocompetent animals. Recent studies on mammary gland development have proven that matched host stroma is critical for mammary gland formation (unpublished results, Era of Hope Meeting) and the importance of the immune system in tumorigenesis has also been documented. The contribution of stroma also raises another important issue. 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 is important to identify gene candidates in stromal cells that could have been masked using whole tumor analysis.

Our laboratory has previously developed a spontaneous metastasis model that mimics the clinical disease with primary tumor formation, invasion of cells through the stroma and into the circulation and colonization at distant organs (7, 8). To our knowledge, this is the only syngeneic model of the entire process and has great potential both for gene discovery and as a tool for analysing the functional significance of gene candidates in breast cancer metastasis. Using this model (Figure 1) and immunopurification of specific cell populations and microarray profiling, we have identified gene expression alterations that occur in both tumor epithelial cells and the associated host vascular endothelium throughout metastatic progression. We have identified a number of genes aberrantly expressed in these cell populations, including those previously implicated in tumorigenesis and/or metastasis and also novel candidates. The clinical relevance of this study to the human disease has been verified by further analysis of one such gene, the cathepsin inhibitor Stefin A. This gene was found in the model upregulated in tumor cells derived from highly metastatic primary tumors and was expressed at even higher levels in matched metastases in lung and bone. Expression of Stefin A1 in metastatic tumor cells is only induced *in vivo* and is not seen in cells in culture unless co-cultured with appropriate stromal cell populations (ie. mammary fat pad) indicating a role for the microenvironment in its induction. The influence of the microenvironment again emphasizes the need for model systems that mimic the *in vivo* microenvironment. Our studies in human breast cancer have supported the murine studies, with an increase in Stefin A expression in primary tumors derived from patients that developed soft tissue and bone metastases and was also detected in metastatic bone lesions.

The main objectives are to study the molecular events involved in metastasis, specifically we aim to 1) verify gene candidates expressed in both tumor epithelial and tumor-derived endothelial cells that were identified as associated with metastasis, 2) determine whether these candidates are also expressed in human breast cancer, 3) study the function of these genes *in vitro,* 4) explore the role of candidates in distant metastasis to lung and 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 was completed in the 1st year of the award. Refer to progress report July 01, 2003- June 30, 2004.

Summary of findings-

We used a clinically relevant syngeneic model of breast cancer metastasis to determine gene expression alterations that occur both between primary breast cancers with varying metastatic potential and between matched primary and bone metastases. For this study, we immunopurified epithelial and endothelial cell populations and profiled them separately to identify a number of tumor and stroma associated genes, some of which have not been associated previously 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. These included enhanced expression of Cathepsin D, macrophage migration inhibitory factor (MIF), LASP-1 and a reduction in expression of FoxP1, LKB-1 and LATS-2 (Table 1). Functional groups from genes aberrantly expressed in tumor epithelium included developmental genes, metastasis suppressors and genes involved in cytoskeletal organization, cell cycle progression, apoptosis and transformation. Stefin A1and DACH-1 were increased while BMP-4 and BRMS-1 were decreased (Table 2). Analysis of epithelium from matched spine metastases revealed some genes that were up-regulated further at the metastatic site. Stefin A1, already up-regulated in highly metastatic primary epithelium, was increased a further 9-fold in matched bone metastases.

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).

The realtime RT-PCR verification of selected candidate genes from Task 1 has been completed and was described in the July 01, 2003- June 30, 2004 annual progress report.

In summary-

The differences in expression for several gene candidates has now been confirmed by real time quantitative RT-PCR. From the summary list of epithelial genes altered in highly metastatic primary breast cancer (Table 2), we have compared the expression of BMP4, Dach1 and Stefin A1 and also 2 ESTs (NM028729 and BC042445). Of interest was the decreasing expression of BMP4 with increasing metastatic capacity and the reverse response for Dach1. BMP4 is a member of the TGFβ family, has a role in development, induces senescence and is a negative regulator of Dach1, which stimulates proliferation and inhibits TGFβ induced apoptosis. Functional analysis of this interaction will be further studied *in vitro*. Stefin A1 (a cathepsin inhibitor) was expressed at much higher levels in the highly metastatic 4T1.2 and 4T1.13 primary tumor epithelium compared to a lack of expression in tumor cells derived from non- or weakly-metastatic primary tumors. 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 of cDNA samples from immunopurified epithelial populations derived from both the primary tumor and matched spine metastases (Figure 2). 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.

We have also verified the microarray data derived from primary tumor vascular endothelium (Table 1), before proceeding to a study of the expression of the genes *in situ* in tumors. Aberrant expression of FoxP1, LKB1, MIF, LATS2 and Snail in vascular endothelium of highly metastatic primary tumors has now been verified by real time quantitative RT-PCR. 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 (9).

New data (mouse and human verification studies)
In the last annual report it was stated that we were attempting to optimise an *in situ* hybridisation technique to verify cell specific candidate gene expression in tissues derived from the model. This is due to the lack of antibodies recognising the candidate proteins, with some anti-human antibodies available but a lack of those that recognise murine antigens. Since then an *in situ* hybridisation method has been optimized. The method involved generation of a cocktail of riboprobes spanning gene transcripts (designed across intron/exon junctions). Riboprobes were generated by end-labelling 5' and 3' ends of PCR primers with T7 and SP6 promoter sequences and PCR amplification of DNA sequences of interest followed by *in vitro* transcription using T7 and SP6 polymerase, generating sense and anti-sense probes respectively. The *in vitro* transcription includes labelling of transcripts with FITC. Paraffin embedded sections of 67NR, 66cl4, 4T1.2 and 4T1.13 primary mammary tumors and their corresponding metastases (see Figure 1) were used for *in situ* hybridisation (ISH). Protocols were modified from those previously described(9, 10), including deparaffinization and fixation of tissues, pre-treatment for access to target nucleic acid sequence and riboprobe hybridization overnight, with the use of FITC riboprobe labelling replacing DIG as previously used. Riboprobe/FITC signal was detected and amplified using the GenPoint™ Fluorescein Tyramide Signal Amplification System (DakoCytomation). The signal was visualized using DAB staining followed by nuclear counter staining with hematoxylin.

Using *in situ* hybridisation, the increased expression of Stefin A1 throughout metastatic progression was verified. As a positive control, mouse embryos were stained to reveal positive cells within the liver at day 15.5. By staining sections from primary mammary tumors, expression (positive staining) was observed in only the highly metastatic 4T1.2 and 4T1.13 tumors, and such expression was limited to only specific subsets of tumor cells (Figure 2C). In contrast, sections from matched lung metastases revealed staining of metastatic lesions that appeared in a large proportion of tumor cells (Figure 2C). This supports the hypothesis that the higher levels of expression observed in metastatic lesions compared to primary tumors detected by quantitative RT-PCR was due to only a subset of cells in the primary tumor expressing the gene and either a selection of these cells that metastasise and grow in distant sites, or induction of stefin A1 in tumor cells once they reached the microenvironment of the lung or bone.
With the availability of an anti-human Stefin A antibody, we performed immunohistochemistry (IHC) on human primary breast tumors and found that some tumors expressed Stefin A. This showed a relevance to human cancer and therefore this study was extended to a small cohort of 24 primary breast tumors derived from patients with known metastatic outcome. The IHC study revealed that metastatic primary breast tumors expressed Stefin A at the protein level (Figure 3). Interestingly, there was a significant increase in

Stefin A positivity in metastatic breast tumors compared to those that did not metastasize (Figure 3B). Further, Stefin A was detected in human bone metastases (Figure 4) revealing that expression is maintained, and enhanced, once tumor cells lodge and grow in tissues at distant sites. This indicates the potential of Stefin A as a prognostic factor and also as a target for treatment. We are now going to investigate a large cohort of primary breast cancer tissues (178) that were derived from patients with known metastatic outcome. This will reveal the prognostic significance of Stefin A expression in human breast cancer.

We have also performed verification studies on two genes found upregulated in highly metastatic primary tumor endothelial cells, Snail and PRL-3.

Snail and PRL-3 were originally identified in recent work on gene expression changes in vascular endothelium associated with human invasive breast cancer. As these two genes were not included on our mouse 15k array, I used quantitative RT-PCR to investigate whether these genes are expressed in primary tumors in our model and, if so, whether the expression was altered in highly metastatic tumors. Studies using RNA derived from immunopurified epithelial and endothelial cells isolated from non-metastatic (67NR), weakly metastatic (66cl4) and highly metastatic (4T1.2, 4T1.13) primary tumors showed a similar expression pattern as seen in human cancer, yet revealed additional information regarding their regulation during metastasis. PRL-3 was expressed solely in the vascular endothelium of invasive breast cancer in humans, with no expression observed in the tumor epithelium (Figure 5A). This was also seen in the murine model, with expression only obverved in the purified tumor endothelium (Figure 5B). Interestingly, the expression of PRL-3 increased in the endothelial cells derived from highly metastatic tumors indicating a possible role in metastasis. Snail was expressed in both the tumor epithelial cells and associated endothelium in invasive breast cancer (Figure 5A). Again, this was seen in the murine model, with both epithelial and endothelial cell populations expressing Snail. As with PRL-3 there was an enhanced expression of Snail with metastasis and this was specific to the endothelial cells, resulting in much greater endothelial expression of Snail in the highly metastatic primary tumors (Figure 5C) such that it could be detected by *in situ* hybridisation (Figure 5D). We are now looking at the functional effects of altering the expression of the genes in primary endothelial cells to determine the effect on endothelial growth and interactions with tumor cells in culture.

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).

Due to the impact of the microenvironment on Stefin A expression, we decided to investigate the induction of Stefin A in co-cultures *in vitro*. As summarised in Task 2, we have found that expression of Stefin A1 is only observed in highly metastatic tumor cells *in vivo*. We wanted to determine whether we could stimulate the expression of Stefin A in these cells when co-cultured with stromal cells *in vitro* to investigate the effect of the surrounding microenvironment. Stromal cells were isolated from mammary fat pads and cultured either alone, or in contact with tumor cells. When co-cultured with the non-metastatic 67NR cells or weakly metastatic 66cl4 cells there was no induction of stefin A1 expression. Interestingly, when the highly metastatic 4T1.2 cells were co-cultured with the fatpad stroma, a significant induction in expression was observed by quantitative RT-PCR (Figure 6A). This effect was not observed when cells were cultured together in transwell inserts (and therefore not in contact) indicting a requirement of cell-cell contact for induction of Stefin A1 expression (Figure 6B). Even though it can be hypothesised that cathepsins may be involved in Stefin A1 induction, there was no significant difference in expression of cathepsin B, S, K and C with co-cultures (Figure 7). However, there were some

differences, including the enhanced expression of cathepsin L only in co-cultures with 4T1.2 cells and a decrease in cathepsin D expression except when co-cultured with 4T1.2 cells, where there was maintenance of cathepsin D expression. Cathepsin C was also only expressed in 4T1.2 cells, with a lack of expression in 67NR and 66cl4 cells cultured alone.It remains to be investigated whether there are changes in the activity of specific cathepsins, that may subsequently lead to Stefin A1 expression as a mechanism of inhibition. We are now investigating the possible mode of Stefin A1 induction, including the role of cathepsins (since Stefin A1 is an intracellular inhibitor of cathepsins) and whether stromal cells isolated from other organs (tissues that have metastatic growth in the 4T1.2 model and those that do not develop any tumor burden after primary tumor formation) can also induce Stefin A1 expression upon contact co-culture. This work (including an extension of the human studies) will be continuedd and funded through a Concept Award from the Department of Defense for the fiscal year July 2005-June 2006.

As stated in task 2 we are currently in the process of cloning Snail and PRL-3 for over-expression studies in endothelial cells to determine the effect on endothelial growth, trans-endothelial migration of tumor cells and other metastatic properties. This will then be extended to functional studies.

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 if interest to measure the effect on bone metastasis (months 18-36).

As stated above in task 3, we were particularly interested in Stefin A1 for initial functional studies. Although this gene was induced in the tumor cells themselves and not surrounding stroma, its expression was induced by the microenvironment, with a lack of expression in metastatic cells cultured *in vitro* and enhanced expression in primary tumors *in vivo* and in co-culture experiments with appropriate stromal cells. It was of interest to determine whether enhancing the expression of Stefin A1 in 4T1.2neo1 cells *in vitro* would alter the metastatic burden when cells were injected into the mammary fat pad. Stefin A1 was cloned into the pBabe vector (containing a puromycin resistance gene) and transfected into the phoenix viral packaging line. The 4T1.2 cells were then infected with virus either containing the pBabe base vector (BV, as a control) or the pBabe-Stefin A1 plasmid and stably selected by growth in puromycin. Expression was confirmed by quantitative RT-PCR (Figure 8A) and high expressers were pooled for *in vivo* studies.

When Stefin A1 and BV clones were injected into the mammary glands of Balb/c mice there was no significant difference in primary tumor growth (Figure 8C,D), and even though Stefin A1 was induced in the 4T1.2-BV primary tumors (as expected), the enhanced expression was maintained in 4T1.2-StfA1 tumors (Figure 8B). Interestingly, both plasma calcium levels (Figure 9A) and lung and bone metastases (Figure 9B,D) decreased in the over-expression lines. This indicated that over-expression of Stefin A1 decreases metastasis, in fact it almost totally inhibits bone metastasis with most spines having no detection of any tumor burden (by QPCR of the neomycin tag) and the return of plasma calcium concentrations to that of "normal" mice that do not have tumor burden. The expression of Stefin A1 in primary tumors and the spine metastatic burden was compared to reveal whether there is a correlation between Stefin A1 expression and metastasis. In 4T1.2-BV cells, moderate Stefin A1 levels correlated with increased metastasis (as seen by previous verification studies) whereas in the 4T1.2-StfA1 clones, primary tumors with much higher levels of stefin A1 do not metastasise at all, which is also the case when there is very low levels the gene expressed in the primary tumor. This needs to be investigated further, but it seems that there is a threshold of Stefin A1 expression that correlates with metastasis and expression levels considerably above or below this level do not have spine metastatic involvement. There are a number of hypotheses as to why increased Stefin A1 may decrease metastasis. Firstly, it was seen from task 2 that

Stefin A1 is only seen in specific cell populations within a primary tumor. Expression in all cells prior to injection may inhibit cathepsins that are important in metastasis (eg cathepsin B, D, K). Alternatively, the cellular localization of stefin A1 may be altered, again resulting in the inhibition of cathepsins that are pro-metastatic. In base vector cells, the localised expression of Stefin A1 may be serving to inhibit the lysosomal activity of pro-apoptotic cathepsins (eg cathepsin L) and those involved in immune recognition/antigen presentation (eg cathepsin S). Another hypothesis is that Stefin A1 induction in 4T1.2 cells *in vivo* may just be a marker of an increased cathepsin activity. We are currently investigating these hypotheses, which form the basis of the recently funded DOD-concept award.

Another gene that we have recently cloned for functional analysis is Breast Cancer Metastasis Suppressor gene 1 (BRMS1). As reported in Task 1, this gene is suppressed in the tumor cells of highly metastatic tumors. We are currently generating 4T1.2 clones expressing BRMS1 to determine whether re-expression inhibits metastasis to bone, a study that hasn't been possible before due to the lack of models of spontaneous breast to bone metastasis.

As summarised in Task 3, we will also be looking at the functional significance of the endothelial associated genes, Snail and PRL-3 on metastasis. This will include transgenic Balb/c mice expressing the endothelial gene of interest under control of an endothelial specific promoter (such as Tie 2) to determine the effect on metastasis. This will be extended to studies aimed to inhibit the pro-metastatic function of such genes (small molecule antagonists etc.). It is realistic that this study will extend beyond the time remaining in the Postdoctoral Award, and work of the next year will be used as preliminary data in future grant applications.

Considering work that has been completed, we are well within the timeline limits and the next year will involve further investigations on the functional significance of genes we have verified as potential regulators of metastatic progression in our "clinically relevant" model of breast cancer metastasis.

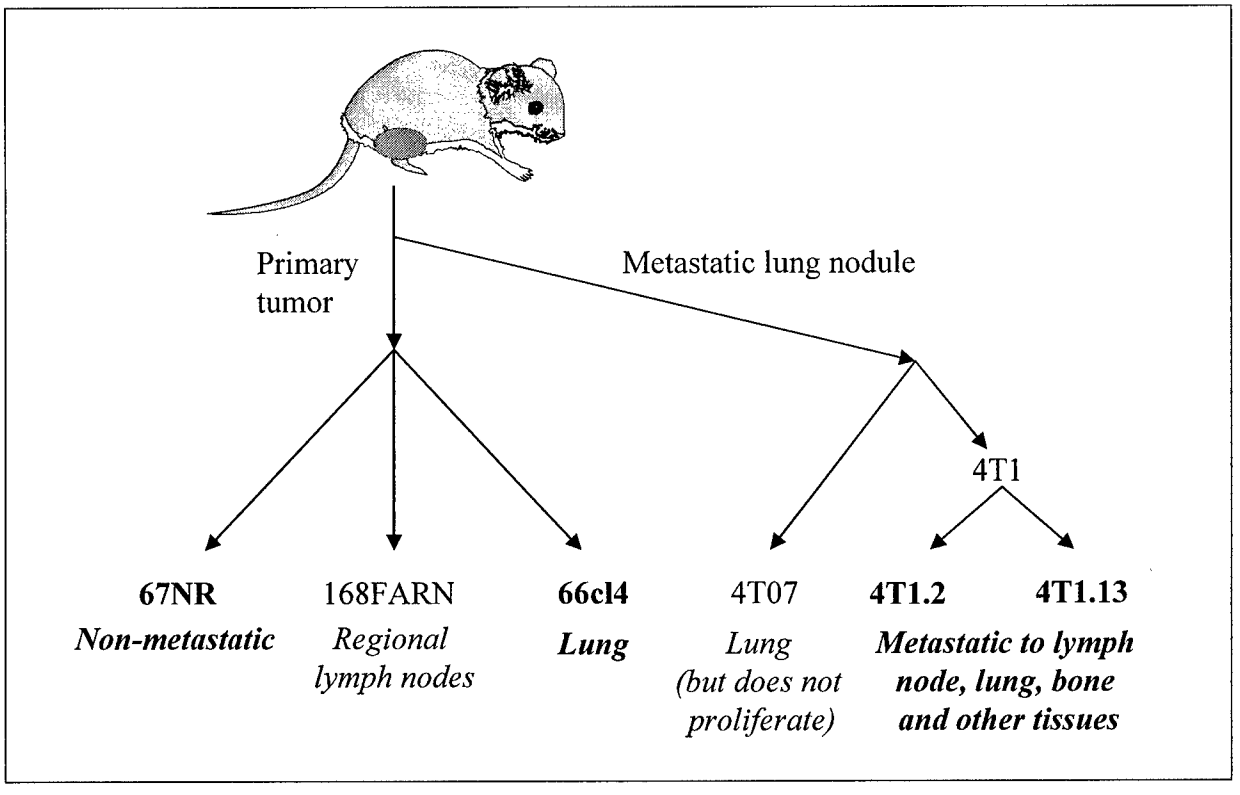


Figure 1. Orthotopic model of breast cancer metastasis to bone. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, while 168FARN, 66cl4 and 4T07 are weakly metastatic and have a tissue 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

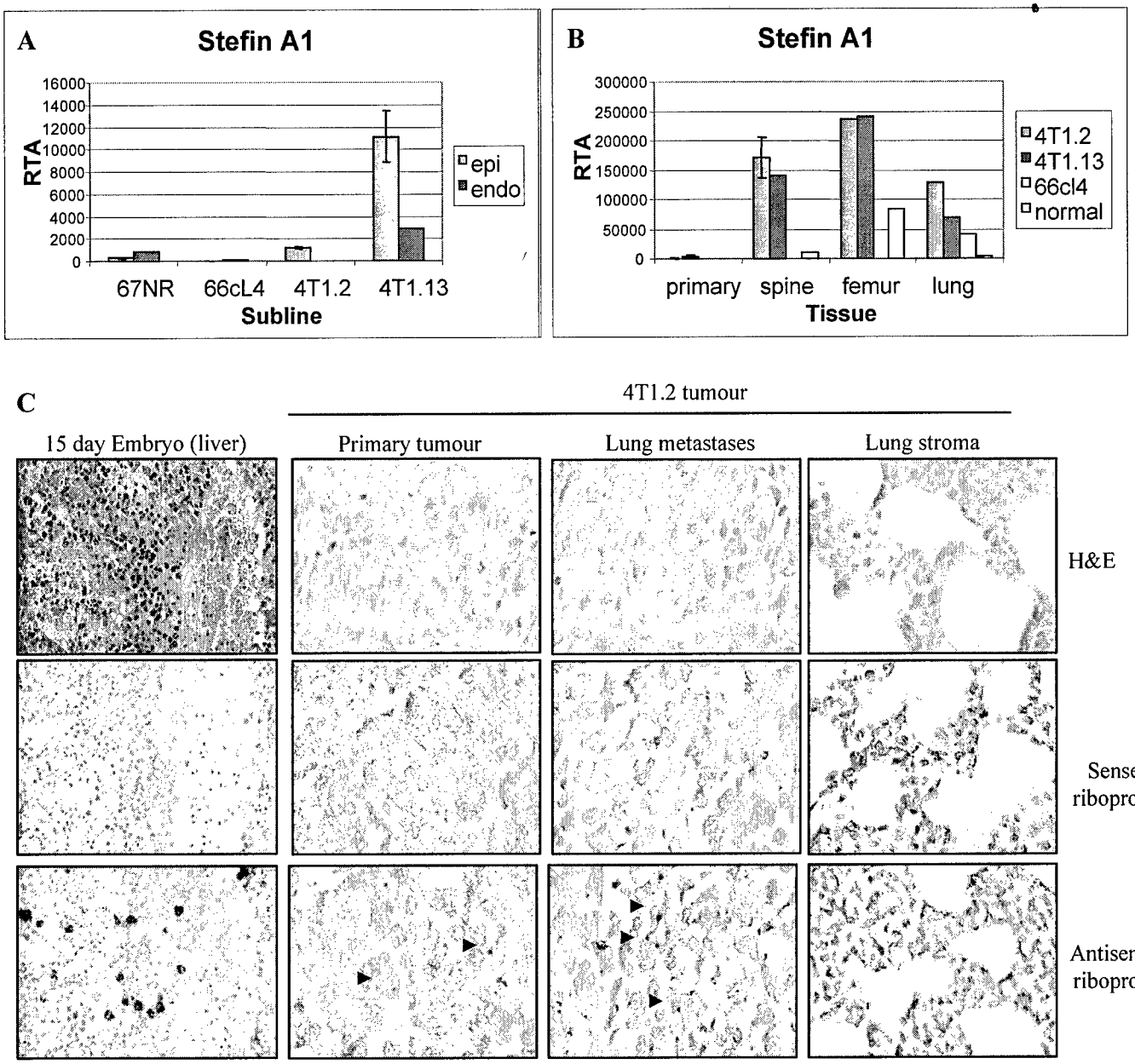
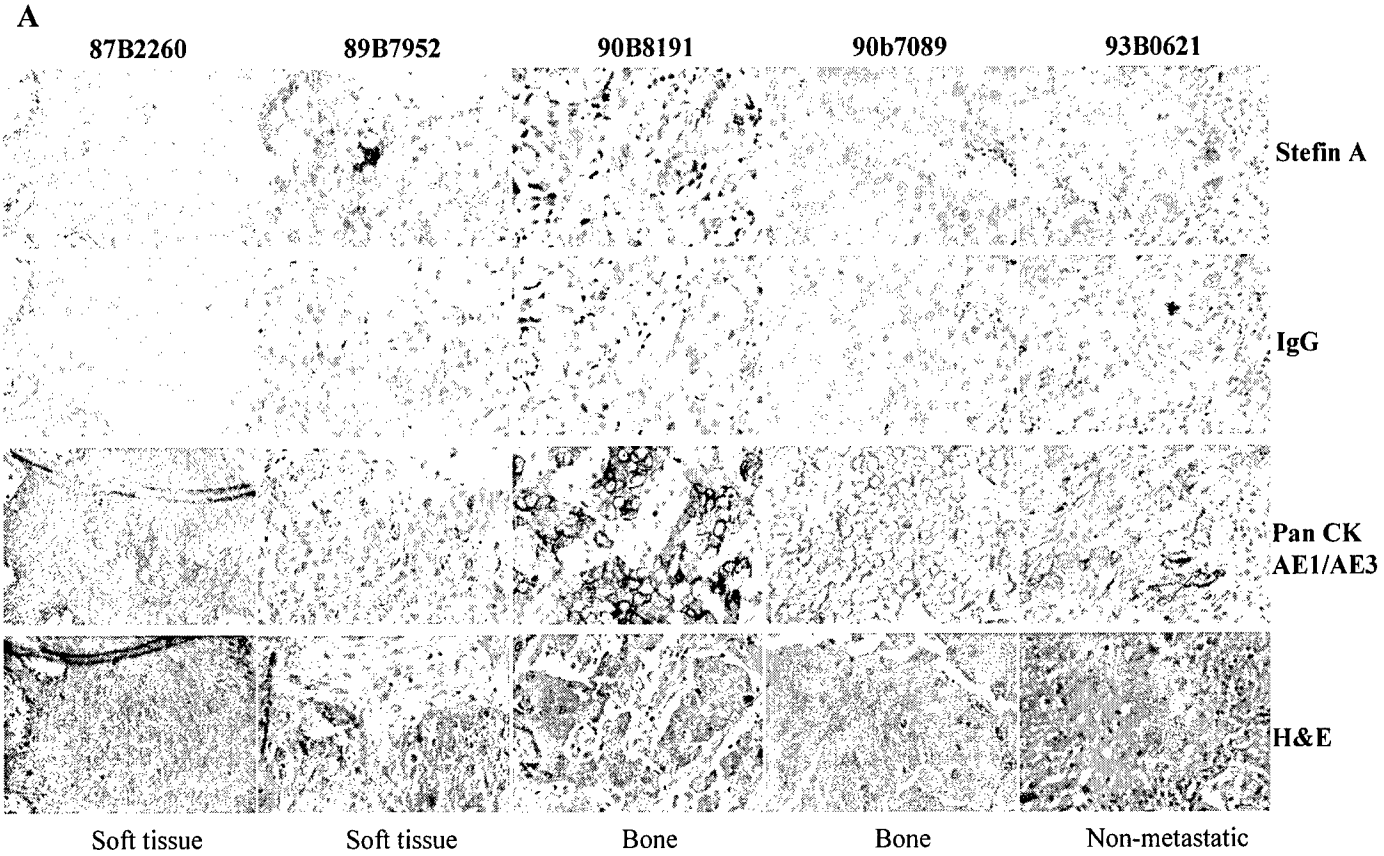


Figure 2 Expression of Stefin A1 (Stfa1) in primary and metastatic tumors.
Stefin A1 was found over-expressed in highly metastatic primary tumor epithelium and at even higher levels in epithelium isolated from the spine of mice containing bone metastases. This was verified by using quantitative RT-PCR, revealing an increase in transcript level in highly metastatic sublines at the primary site (A), and a further increase in metastatic lesions (B). Expression was validated by *in situ* hybridisation (C). 4T1.2 primary tumors and lung metastases were stained with stefin A1 antisense riboprobes and sense probes as controls. Riboprobes were FITC labelled and detected using anti-FITC HRP followed by DAB staining and counter staining with hematoxylin. Staining was detected in specific cells within the primary tumor and there was an increase in expression in the matched lung metastases (as indicated by arrows -brown staining). This yet again validates the array and RT-PCR data. Mouse embryo (15.5 days) was stained as a positive control and staining was detected in the liver.



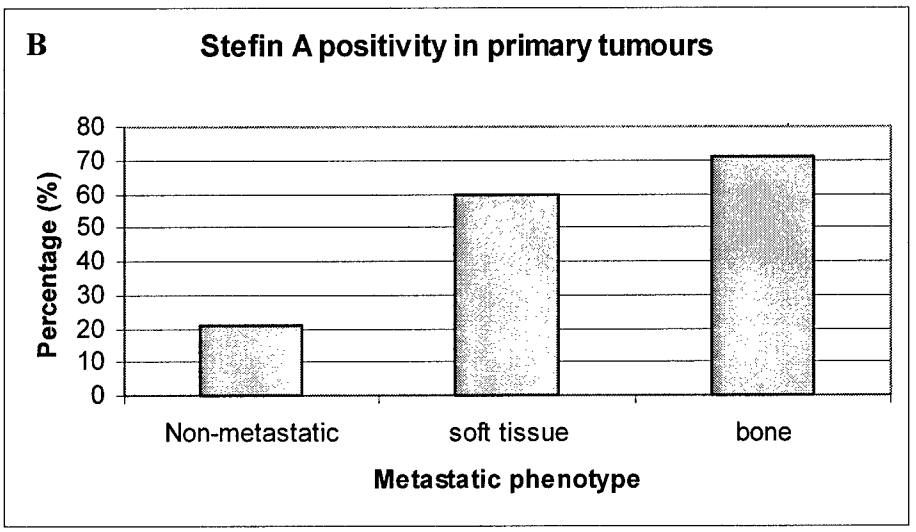


Figure 3 Immunohistochemistry of Stefin A expression in human breast cancer

A) Primary breast tumor tissues derived from patients with known metastatic outcome (as indicated below each panel) were stained with mouse anti-human Stefin A antibody (Serotec) or control mouse IgG. Serial sections were stained with the epithelial specific AE1/AE3 pan cytokeratin antibody to confirm epithelial expression. Subsets of tumor cells expressed Stefin A at the protein level. B) Results of Stefin A expression in the panel of 24 human primary breast tumours with known metastatic outcome. Stefin A expression was detected in numerous primary tumours, with the majority of positive tissues being those that metastasised to lung and bone (60 and 71% respectively, compared to 21% of non-metastatic tumors).

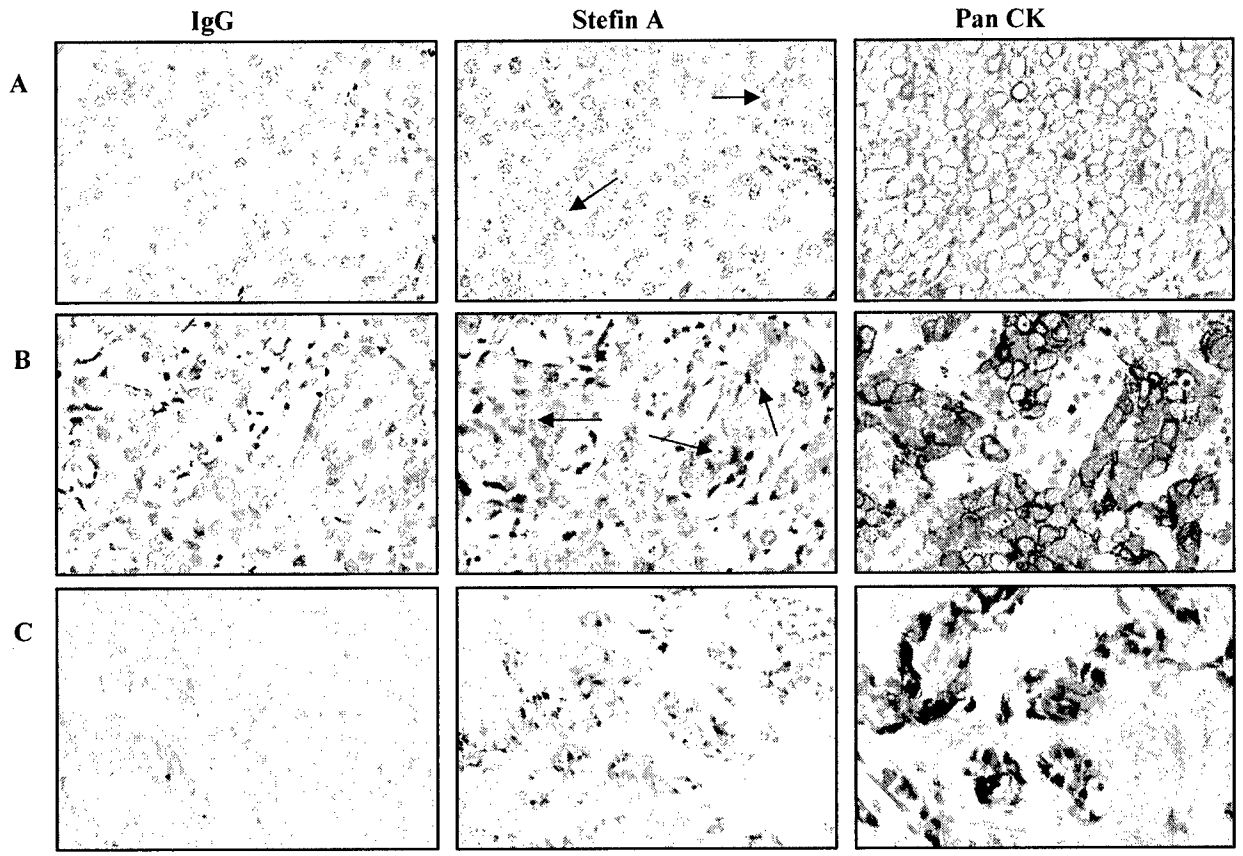


Figure 4 Stefin A expression in human bone metastases derived from breast cancer
To validate expression of Stefin A in metastatic lesions, as hypothesised by the RT-PCR and ISH in the murine model, a bone metastases derived from breast cancer was stained with anti-human stefin A. Tumours cells stained positively for stefin A, as verified by identical staining patterns to the epithelial specific antibody, AE1/AE3 pan cytokeratin. A-C represent different areas from the same sample. It should be noted that all tumour cells stained positive for stefin A, that is not the case for primary tumors where only a subset of cells are positive.

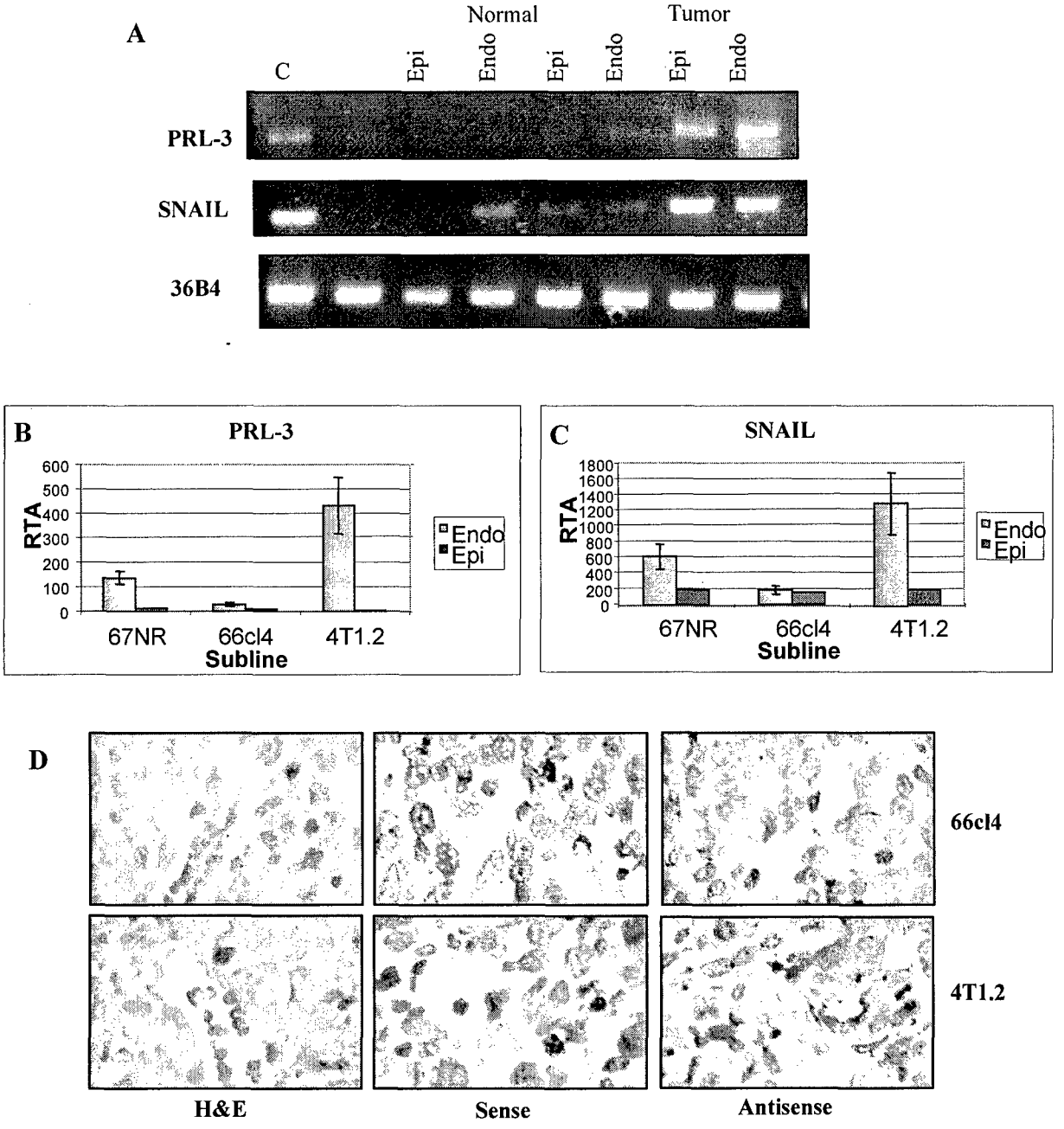
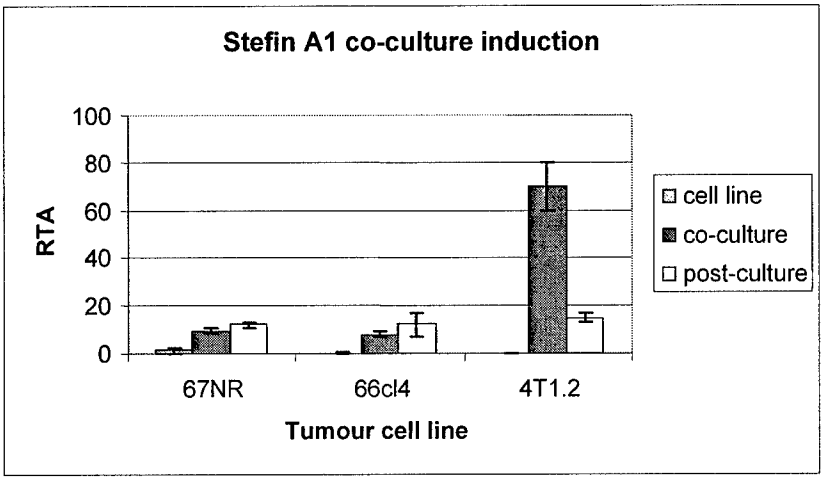


Figure 5 Comparison of endothelial expression of PRL-3 and Snail in human and murine breast cancer
A) Human RNA expression (RT-PCR) of PRL-3 and Snail in epithelial and endothelial cell populations immunopurified from normal mammoplasty tissue or from 2 freshly resected invasive breast cancer samples. Similar expression patterns for PRL-3 and Snail were detected by quantitative RT-PCR in epithelial and endothelial cells immunopurified from primary tumors from the murine model (B,C). The use of primary tumors with varying metastatic potential also revealed the increase in expression of both genes in endothelium derived from highly metastatic 4T1.2 tumors. The increase in endothelial expression of Snail was detected by ISH, with binding of the anti-sense cocktail of riboprobes (as indicated by brown staining) and absence of staining in sense controls (D).

A



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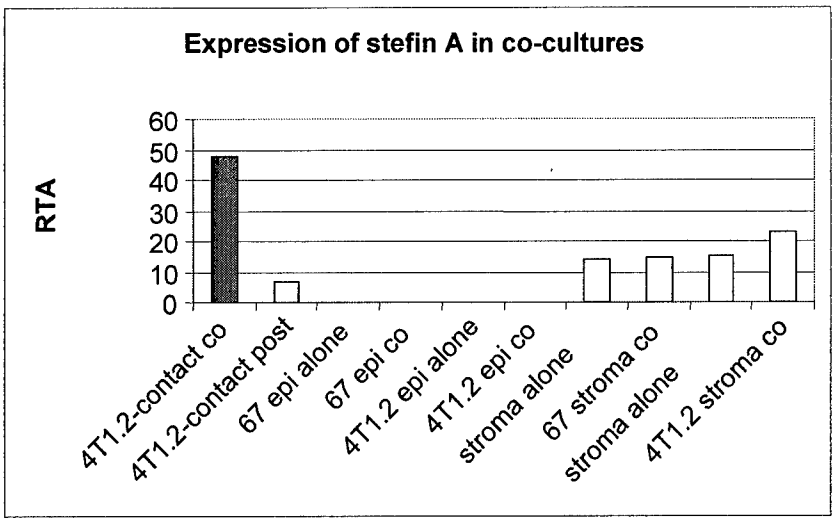


Figure 6 Co-culture induction of Stefin A1

Mammary fat pads were resected from Balb/c mice and collagenase A digested into single cell suspensions. Filtered cells were then plated and allowed to attach overnight. For contact co-cultures (A), tumor cells (67NR, 66cl4 or 4T1.2) were then added to stromal populations (co) or cultured separately (cell line) overnight in serum-free media. Post-culture represents cells that were incubated separately and then mixed in lysis buffer to determine the additive expression of Stefin A1 in tumor and stromal cells when not cultured together. Tumor cells alone did not express Stefin A1, and a significant induction was seen only in contact co-cultures of the highly metastatic 4T1.2 epithelium with fat pad stroma. B) In contrast, cells co-cultured in transwell inserts (and therefore not in contact) did not induce Stefin A1 expression revealing the need for cell contact for induction, rather than stimulation by secreted factors. As can be seen in the stromal populations in panel B, the stromal cells express a low level of Stefin A and this expression is not altered in co-cultures. Expression was detected using quantitative RT-PCR and a comparison to GAPDH.

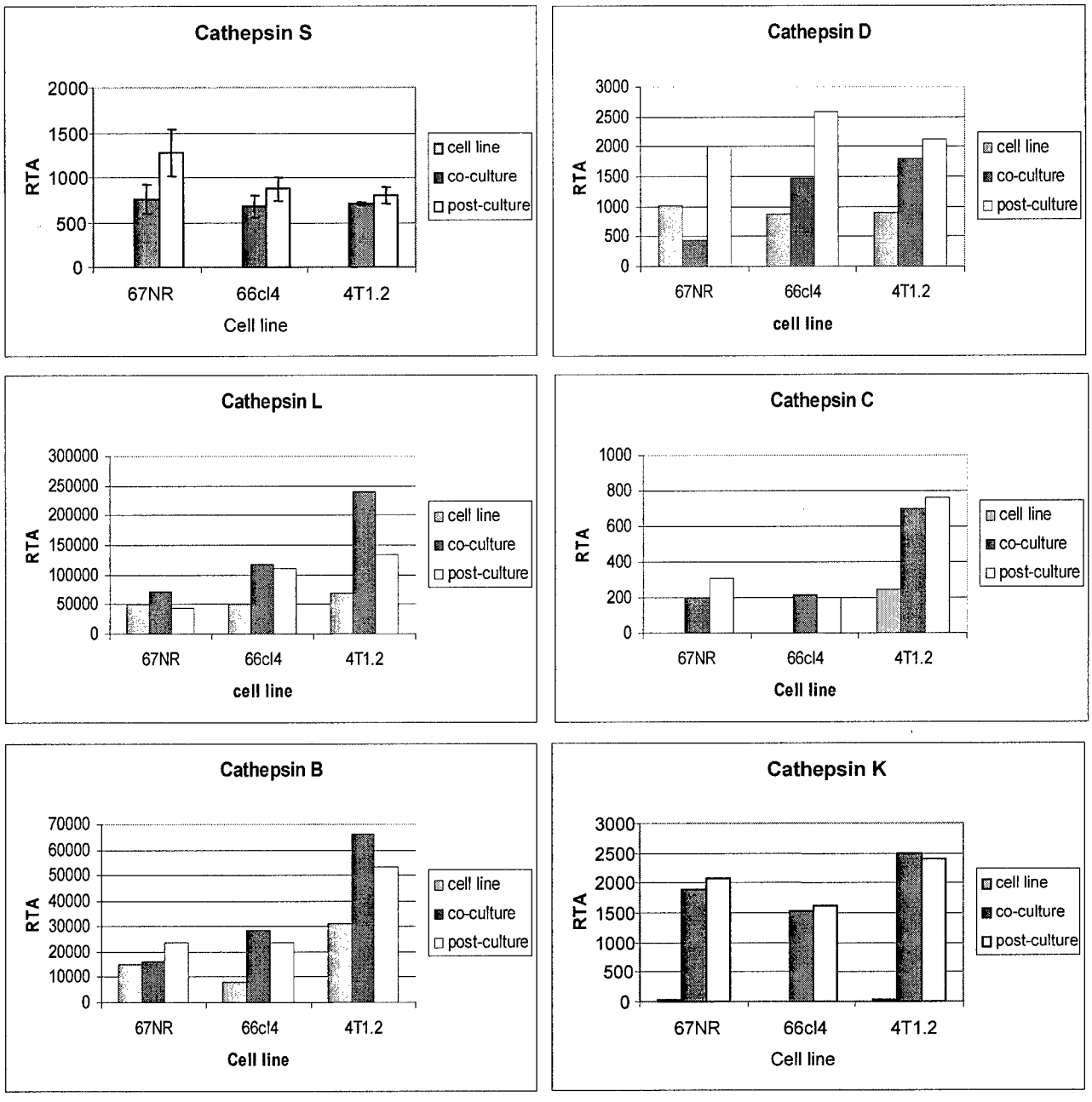
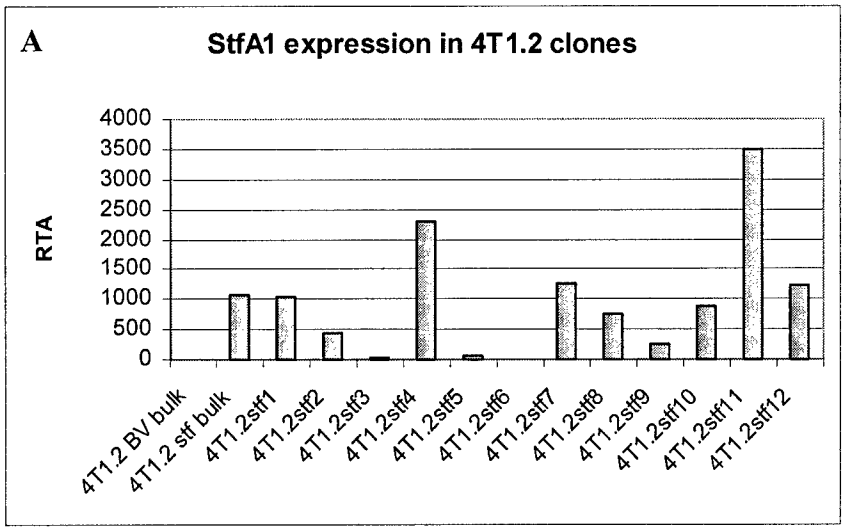
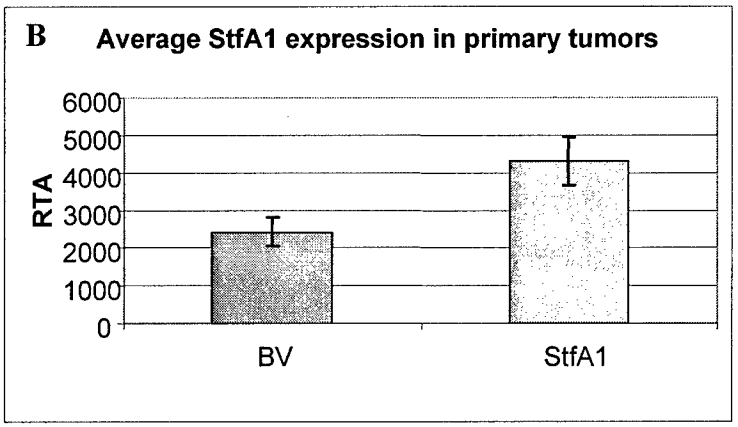
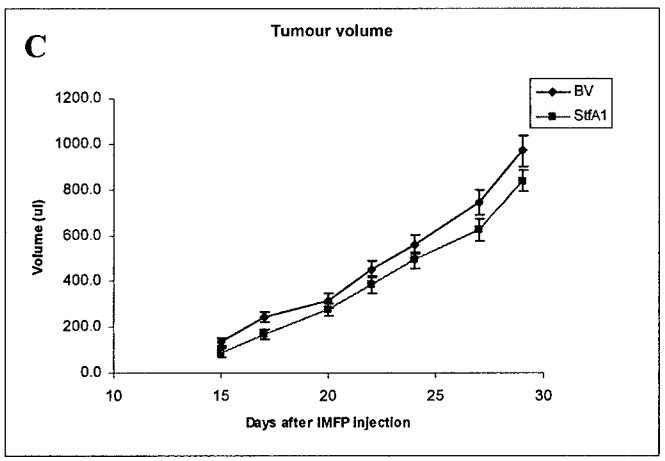


Figure 7 Cathepsin expression in contact co-culture experiments

67NR, 66cl4 and 4T1.2 cells were cultured alone (cell line) or in contact with mammary with mammary fat pad stroma (as in figure 6A). The expression of cathepsin S, D, L, C, B and K was measured by quantitative RT-PCR. In most cases, there was no significant difference between co-culture and post-culture induction for all lines indicating that co-cultures do not enhance cathepsins at the expression level. There were some differences, including the enhanced expression of cathepsin L only in co-cultures with 4T1.2 cells and a decrease in cathepsin D expression except when co-cultured with 4T1.2 cells, where there was maintenance of cathepsin D expression. Cathepsin C was also only expressed in 4T1.2 cells, with a lack of expression in 67NR and 66cl4 cells cultured alone.







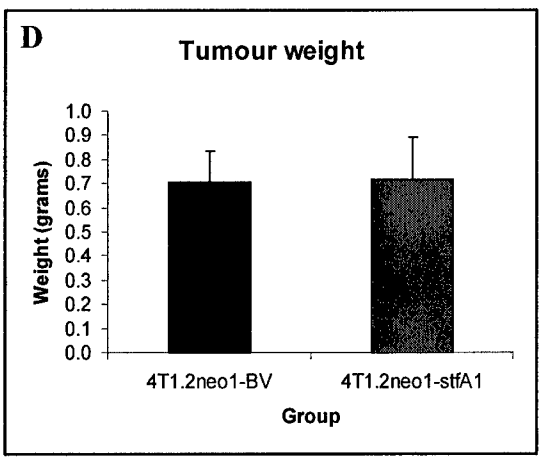


Figure 8 Stefin A1 over-expression in 4T1.2 cells does not effect primary tumor growth in vivo
Bulk 4T1.2 neo1-StfA1 cells were single cell cloned and quantitative RT-PCR was used to detect Stefin A1expression (A). High expressers were pooled (clones 1, 4, 7, 11, 12) and used for *in vivo* experiments along with the 4T1.2neo1-base vector (BV) bulk cell population as a control. Primary tumors derived from injection of 4T1.2neo1-BV cells gained expression of Stefin A1 (as expected by previous *in vivo* studies) yet the 4T1.2-StfA1 clones maintained an average 2-fold higher expression (B). Primary tumor growth and weight at time of harvest was not significantly different between the 2 groups (C, D), indicating that Stefin A1 expression does not alter the growth of the primary tumor. Base vector

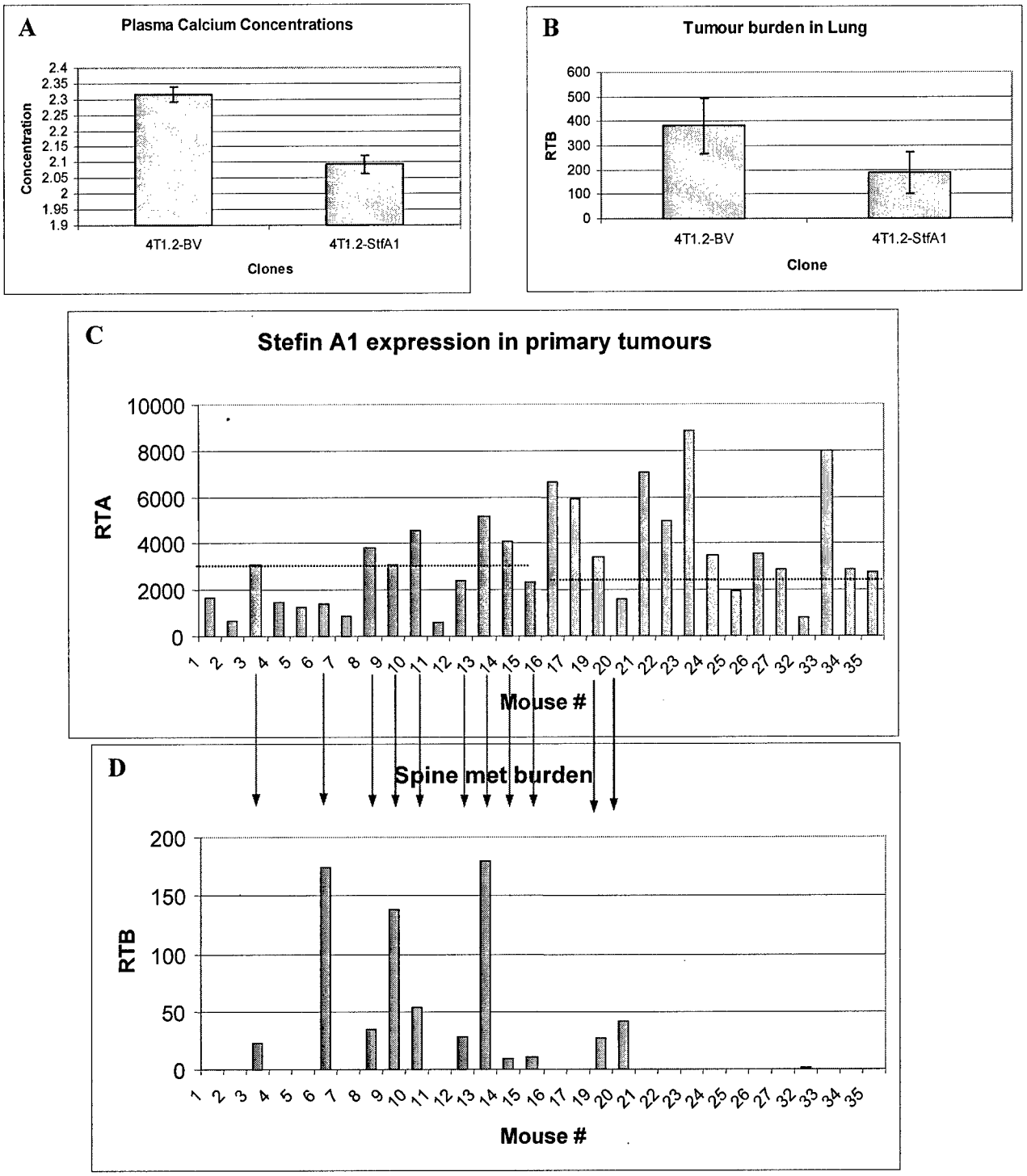


Figure 9 Stefin A1 over-expression in vitro leads to reduced lung and bone metastasis in vivo

A) The concentration of plasma calcium in all 30 mice was measured as an indication of distant metastatic involvement. As can be seen, mice injected with 4T1.2-StfA1 clones had reduced plasma calcium, in fact the concentration was equivalent to levels seen in mice that do not have tumor burden (~2.1). This indicates a decrease in metastatic burden and this was confirmed by realtime QPCR detection. In the lungs (B) there was a marked decrease in tumor burden (neomycin tagged tumor cells compared to vimentin signal). This effect was even greater in spine metastases, with an almost complete inhibition of spine metastases in the 4T1.2 cells over-expressing StfA1 (mice 16-35, blue)(D). When spine metastatic burden is compared to Stefin A1 expression in the primary tumor of each mouse (C), the highest stefin A1 expression correlated with a lack of metastasis.

Description	Common	Function
endothelial-derived gene	Eg1	angiogenesis
AXL receptor tyrosine kinase	Axl	angiogenesis, cell adhesion and proliferation
cathepsin D	Ctsd	angiogenesis, ECM degradation
macrophage migration inhibitory factor	Mif	angiogenesis, endo growth and migration
ornithine decarboxylase, structural	odc	tumor invasion/angiogenesis/endo proliferation (supression of endostatin)
transducer of ERBB2, 2	Tob2	antiproliferative/inhibits cell cycle progression
H1 histone family, member 0	H1f0	DNA replication progression through cell cycle
serine/threonine kinase 11	Stk11	tumor suppressor/cell cycle blockage/p53 apoptotic regulation
a disintegrin and metalloprotease domain 8	ADAM8	cell adhesion/growth/osteoclast maturation
RAB20, member RAS oncogene family	Rab20	cell adhesion/migration
integrin beta 1 (fibronectin receptor beta)	Itgb1	cell adhesion/spreading
cell adhesion molecule-related/down-regulated by oncogenes	Cdon	cell adhesion
RAP2B, member of RAS oncogene family	Rap2b	chemotaxis/cell adhesion, morphology, motility/migration
villin 2	Vil2	cytokeleton architecture
LIM and SH3 protein 1	Lasp1	cytoskeleton architecture/cell motility/downstream target of hedgehog
S100 calcium binding protein A9 (calgranulin B)	S100a9	transendothelial migration/adhesion to ECM
phosphatase and tensin homolog	PTEN	tumor suppressor/proliferation/migration
forkhead box P1	Foxp1	growth suppression/transcriptional repressor
suppressor of cytokine signaling 3	Socs3	inhibition of antiproliferative proteins/inhib T cell recognition/osteoclast diff.
BTB and CNC homology 2	Bach2	oxidative stress/reduced proliferation/spontaneous cell death
SWI/SNF related, subfamily a, member 5	Smarca5	decreased expression = cell specific differentiation
Snail homolog 1	Snail	Role in EMT, repression of E-cadherin, over-expressed in cancer
protein tyrosine phosphatase type IVA, member 3	PRL3	Tyrosine phosphatase, expressed in metastatic colorectal cancer

Table 1- Expression profiling of isolated endothelial cells.
Microarray profiling revealed genes that had increased (purple) or decreased (pink) expression in endothelial cells derived from primary tumors that are metastatic (compared to non-metastatic tumors).

Description	Common	Function
stefin A1	stfa1	cystatin, cathepsin inhibitor
breast cancer metastasis-suppressor 1	Brms1	Supression of metastasis
ets variant gene 6 (TEL oncogene)	ETV6	cellular aggregation, transformation
complement component 3	C3	up in cancer patients, including highly metastatic human melanoma cells
histidine decarboxylase	Hdc	synthesis of histamine, tumor cell proliferation
SMT3 (supressor of mif two, 3) homolog 1	Smt3h1	protection against TNF etc cell death, cell cycle progression
histidine decarboxylase	Hdc	synthesis of histamine, tumor cell proliferation
dachshund 1 (Drosophila)	DACH1	cell proliferation, inhibits apoptosis (TGFB), developmental gene
bone morphogenetic protein 4	BMP4	member of TGF-beta superfamily, induce cell senescence
topoisomerase (DNA) I	Top1	realxation of supercolied DNA through breakage, cell cycle checkpoint
mitogen activated protein kinase 1	MAPK1	activation of ERK1/ERK2, roles in differentiation
MAP kinase-interacting serine/threonine kinase 2	Mknk2	limit/inhibit translation
developmental pluripotency associated 5	dppa5	development
maternal effect gene	Mater	early development
eomesodermin homolog (Xenopus laevis)	Eomes	early developmental gene
envoplakin	Evpl	cytolinker protein
Rho GTPase activating protein 8	Arhgap8	cytoskeletal organization, cell cycle regulation, ras-mediated transformation
Rho guanine nucleotide exchange factor (GEF) 3	Arhgef3	cytoskeletal rearrangement
ARP3 actin-related protein 3 homolog (yeast)	ARP3	actin nucleation, organization of the cytoskeleton, controls polarised cell growth
heat shock protein 8	HSP8/73	chaperone
autophagy 7-like (S. cerevisiae)	Apg7l	enzyme essential for autophagy
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	stress induced, induced upon anti-metastatic activity (lung adenocarcinoma cells)
zinc finger protein X-linked	Zfx	inhibits angiogenesis, involved in embryonic growth (sex differentiation)

Table 2 Genes altered in highly metastatic epithelial cells.
Microarray profiling revealed genes that had increased (purple) or decreased (pink) expression in immunopurifed epithelial cells from primary tumors that metastasize to lung and bone.

KEY RESEARCH ACCOMPLISHMENTS

Refer to progress report July 2004-June 2005 for accomplishments for that year.

Additional progress is as follows-

- Optimization of an *in situ* hybridisation method for verification studies
- ISH verification of Stefin A1 expression in primary and metastatic tumors
- ISH verification of endothelial genes (Snail and PRL-3)
- Verification of Stefin A expression in human breast cancer and preliminary evidence of prognostic significance
- Co-culture induction of Stefin A with mammary fat pad stroma
- Stefin A1 over-expression in highly metastatic cells (4T1.2) *in vitro* inhibits bone metastasis and reduces lung metastasis *in vivo*

REPORTABLE OUTCOMES

Awards

2005 Awarded US Army Department of Defense (DOD) Breast Cancer Research Program (BCRP) Concept Award

2005 Finalist- Cure Cancer Australia Young Researcher of the Year

Publications relating to project

Eckhardt, B.L., Parker, B.S., van Laar, R.K., Restall, C.M., Natoli, A.L., Tavaria, M.D., Stanley, K.L., Sloan, E.K., Moseley, J.M., and Robin L. Anderson. (2005). Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. *Molecular Cancer Research*, 3, 1-13.

Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S and Madden SL (2004). Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Research,* 64, 7857-7866.

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.

Conference presentations

Genetic aleteration in tumor epithelium and host endothelium associated with metastatic progression in a murine model of breast cancer metastasis. Parker, B.S and Anderson, R.L. 4th Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June 2005. Philadelphia, Pennsylvania. Poster **and** platform presentation.

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

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 cDNA microarrays.

A number of candidates have been identified as over-expressed or suppressed in tumor endothelium and in the tumor cells themselves during metastatic progression. Some of these have been verified by quantitative RT-PCR and by *in situ* hybridisation and are being analysed further for their functional role in metastasis, and for their role in human breast cancer.

One such gene, Stefin A1, has enhanced 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 preliminary results revealing a prognostic significance to expression of Stefin A in a cohort of primary breast tumors with known metastatic outcome. Stefin A has also been detected in human bone metastases and this study will be extended to a cohort of 178 patients to further study the potential of stefin A expression as a prognostic marker.

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 possible in other studies, and may be responsible for the lack of molecular markers as prognostic indicators and targets for treatment. This study has combined the use of a syngeneic model of spontaneous breast cancer metastasis with immunopurification of tumor and stromal cell populations. This allows for interactions of tumor cells with compatible stroma and the use of an immunocompetent model, two factors that have been found to be extremely important in cancer progression. Genes that have previously been associated with human cancer progression have been identified in this study and the fact that we have verified expression of one of our candidates in human breast cancer, with potential prognostic significance, reveals the clinical relevance of this model in investigating breast cancer metastasis.

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APPENDICES

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TERTIARY QUALIFICATIONS

1998-2001	**PhD**, Department 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

2005	Awarded US Army Department of Defense (DOD) Breast Cancer Research Program (BCRP) Concept Award

2005 Finalist- Cure Cancer Australia Young Researcher of the Year

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

2003 Awarded- Department of Defense Breast Cancer Research Program (BCRP) Postdoctoral Fellowship.
(July 2003-July 2006)
- Susan Komen Foundation 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 La Trobe University Postgraduate scholarship

SCIENTIFIC PUBLICATIONS

Eckhardt, B.L., Parker, B.S., van Laar, R.K., Restall, C.M., Natoli, A.L., Tavaria, M.D., Stanley, K.L., Sloan, E.K., Moseley, J.M., and Robin L. Anderson. (2005). Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. *Molecular Cancer Research*, 3, 1-13.

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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*, 14, 279-290.

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ONGOING RESEARCH SUPPORT

DOD/BCRP – Postdoctoral Fellowship (DAMD17-03-1-0473)
Stromal gene expression in primary breast tumors that metastasize to bone. July 2003-July 2006
Role: PI

DOD/BCRP- Concept Award (W81XWH-05-1-0444)
The role of Stefin A in breast cancer metastasis
July 2005-July 2006
Role: PI

CONFERENCE PRESENTATIONS

Genetic aleteration in tumor epithelium and host endothelium associated with metastatic progression in a murine model of breast cancer metastasis. Parker, B.S and Anderson, R.L. 4th Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June 2005. Philadelphia, Pennsylvania. Poster **and** platform presentation.

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

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.

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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
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