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13. ABSTRACT (Maximum 200 Words) We are investigating interactions between breast cancer and bone stromal cells and their role in regulating metastasis of breast tumor cells to bone and osteoclast development using an orthotopic mouse model. Results from <i>in vivo</i> experiments indicate that PTHrP is an important factor regulating tumor growth but is not required for specific metastasis of breast tumors to bone. <i>In vitro</i> co-culture of bone stromal and tumor cells leads to up-regulation of RANKL and down-regulation of OPG, a response likely to contribute to the establishment of breast metastases in bone and to increases in bone resorption. The expression of RANKL and OPG <i>in vivo</i> is being investigated using our orthotopic model and immunohistochemistry. The expression of MMP9 significantly increases in these co-cultures and its expression <i>in vivo</i> following tumor cell injection into the mammary gland appears to be most prominent at sites of bone resorption and the bone stroma-breast metastasis interface. Thus, both tumor and stromal-derived MMP9 are likely to contribute to metastatic development and accompanying osteolysis. This is being investigated further using MMP9 null mice and RNAi technology. Work investigating osteoclast requirement in tumor-mediated osteolysis using <i>in vivo</i> administration of OPG, β 3 integrin peptidomimetics, and/or β 3 integrin and M-CSF null mice is ongoing.				
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INTRODUCTION

Breast cancer is the leading cause of cancer mortality among western women. The high incidence of metastatic spread of breast tumors to bone in these women is, for the vast majority, associated with osteolytic lesions leading to significant clinical complications including osteoporosis, hypercalcemia, intractable pain, spinal cord compression and fracture of the long bones that invariably impair the quality of life of those affected (reviewed in (1)). Whilst the mechanisms leading to the preferential metastasis of breast cancer cells to bone remain poorly understood, recent findings from in vitro experiments using normal breast epithelial cells or tumour cells lines (2, 3) and the development of transgenic or xenograft animal models of breast cancer metastasis (reviewed in (4)) strongly support the critical role of the bone stromal microenvironment in the metastatic process and point to considerable cross-talk/interactions between bone cells and tumor cells leading to the establishment of secondary tumors in this organ. In particular, the use of an intracardiac injection model (5, 6) has been particularly useful in unravelling the mechanisms and factors affecting bone remodelling and late stage progression of breast tumor metastasis to bone. However, whilst informative, current models are limited by the low incidence of spontaneous bone metastases and/or only allow the study of specific stages of the metastatic cascade.

We have developed an orthotopic mouse model of breast cancer metastasis to bone (7). In this model, tumor sublines of varying metastatic potential derived from a spontaneous mammary gland carcinoma from a Balb/cfC3H mouse are injected into an orthotopic site (mammary fat pad) of syngeneic animals. These animals consistently develop primary breast tumors that spontaneously progress to form metastases in bone and/or soft tissues (Figure 1). This model has the advantage over existing models in that it mimics the entire metastatic cascade of the human disease, including the ability to evade immune surveillance. Importantly, the model enables us to study the interactions between stromal and tumor cells at both primary and metastatic sites in the same animal.

The overall objective of this project is to identify factors that regulate the establishment of breast cancer metastases in bone. Specifically, we aim to:

- 1- Determine the factors expressed by metastatic breast tumor cells and/or bone stromal cells in the bone microenvironment that regulate tumor cell growth and/or osteoclast development.
- 2- Investigate the extent of osteoclast requirement in tumor-mediated osteolysis
- 3- Investigate the mechanisms of osteoclast activation by breast cancer cells

BODY

Task #1. To determine the expression of cytokines and osteoclast regulatory factors by breast tumor and bone stromal cells

The expression of osteoclast regulatory factors and cytokines by tumor cells has been implicated in the progression of bone metastases. Our orthotopic mouse model enables us to investigate in detail the expression of genes implicated in the literature to be important in the metastatic tumor environment, and to compare expression of these genes in the primary and secondary tumor sites. The expression patterns of selected molecules are being examined by immunohistochemistry (IHC), immunofluorescence (IF) and/or *in situ* hybridization (ISH).

The first gene taken for analysis this way was parathyroid hormone related protein (PTHrP). PTHrP has been implicated as a regulator of osteoclast activation, by upregulating expression of RANKL in osteoblasts (8). Over-expression of PTHrP in MDA-MB-231 cells enhances osteolytic activity in femurs after intracardiac injection of tumor cells into nude mice (9). However other data, both in animal models and clinical, cast doubt on the importance of PTHrP in regulating breast cancer metastasis to bone (10-12). Both primary tumors and metastatic tumor deposits of the bone metastasizing 4T1.2 tumor line stain strongly for PTHrP, as do some normal bone cells (Figure 2). Interestingly, the 66cl4 tumor line that does not metastasize to bone (see Figure 1) also stains strongly for PTHrP when grown as a primary tumor in the mammary gland. Further, when 66cl4 cells are injected directly into the tibia, they grow and express large amounts of PTHrP. These data suggest that PTHrP expression does not correlate with bone metastasis in our model. Further analysis of the role of PTHrP is described under Task 3.

We have also examined the expression of some other candidate genes, namely IL-1 α , IL-1 β , IL-6, IL-11, IL-17, IL-18, uPA, MMP2, MMP9, RANKL, RANK, OPG by performing reverse transcriptase real time quantitative PCR (RT-RTQ PCR) on RNA extracted from the primary tumors of the bone metastasizing (4T1.2), the lung metastatic (66cl4) and the non-metastatic (67NR) tumors that comprise our model (Figure 3). This study provided an assay of differential gene expression between tumors of varying metastatic capacity and focussed our attention on IL-1 α , IL-6, uPA, MMP2, MMP9, RANKL and OPG. Since the focus of this project is tumor/stromal cell interactions in bone, we have set up co-cultures of mouse osteoblasts and the tumor lines to gain further insight into the potential cross-talk between osteoblasts and metastatic tumor cells. Cultures of bone stromal cell populations enriched for osteoblasts were obtained from collaborative work with Dr Paul Simmons and Brenton Short from the Stem Cell Research Laboratory at Peter MacCallum Cancer Centre. The mRNA expression of RANKL, MMP9, OPG and MT1-MMP has been measured in these co-cultures by RT-RTQ-PCR and compared to the expression patterns in the lines grown separately. In agreement with its role in osteoclast maturation and activation, we found a consistent up-regulation of RANKL mRNA expression in co-cultures of osteoblasts and the bone metastatic 4T1.2 cell line (Figure 4). Conversely, levels of the RANKL decoy receptor, OPG, were down-regulated in these cultures. These results strongly suggest that interactions between tumor and stromal cells may accelerate bone resorption in proximity of tumor deposits by shifting the balance towards pro-osteolytic factors. These interactions are being investigated further *in vivo* by IHC/IF staining. In addition, MMP9 mRNA expression was up-regulated and MT1-MMP was slightly down-regulated in co-cultures compared to mono-culture controls.

In order to validate our in vitro findings and since this RT-RTQ PCR approach does not identify the cell types responsible for the expression and cannot be performed on bone metastases in vivo, IHC and ISH will still be performed as proposed. So far, we have commenced an analysis of MMP9 expression in situ, examining expression both in the tumor and in the bone microenvironment, in normal mice and mice null for MMP9. The results of this analysis are described under Task 3.

In addition to the molecules originally proposed, we have measured expression of a panel of chemokine receptors in the tumors. Chemokines have long been known to be important in the mobilization of immunological cells from thymus, spleen and bone marrow and their ability to home to sites of infection or damage. A recent study (13) highlighted the importance of chemokine Sdf-1 in the homing of breast tumor cells to specific sites, providing a possible mechanism whereby metastatic cells selectively colonize particular secondary organs depending on which chemokine receptor(s) they express. The receptor for Sdf-1 is CXCR4. It was therefore of great interest to determine whether chemokine driven migration may be responsible for the different patterns of metastasis in our model. Expression of a panel of chemokine receptors was measured in the different tumor lines using RT-RTQ PCR (Figure 5). The chemokine receptors were found in varying levels in the tumors, with CCR1, CCR2, CCR5, CX3CR1 and CXCR4 showing the highest levels. However, expression of the ligands for these receptors is not confined to the sites to which the tumor cells metastasize (eg. bone and lung). To further this aspect of the work, we have set up a collaboration with Dr. Shaun McColl from Adelaide University, South Australia. Dr. McColl has a longstanding interest in chemokines and their function. Even though levels of expression of CXCR4 are low in cells of our metastasis model, bone is a major site of expression of its ligand Sdf-1 (13). We have established that the 4T1.2 line has no endogenous expression of Sdf-1. These cells are now being transfected with constructs encoding wild type Sdf-1, a mutant form of Sdf-1 that acts as a CXCR4 antagonist and a mutated non-functional form of Sdf-1. The aim is to determine whether the CXCR4/Sdf-1 axis is required for 4T1.2 metastasis to bone by disrupting the interaction with excess normal Sdf-1 or the receptor antagonist form.

Task #2. To investigate the extent of osteoclast requirement in tumor-mediated osteolysis

Considerable evidence suggests that tumor cells cause bone destruction by recruiting osteoclasts (14). The role of osteoclasts in our mouse model of breast tumor metastasis to bone is being examined using osteoclast inhibitors and mice lacking functional osteoclasts. We are using the osteoclast inhibitors, osteoprotegerin and bisphosphonates, the integrin β 3 antagonist S247 and two strains of knockout mice with impaired osteoclast function – the m-CSF null mouse (op/op) and the integrin β 3 null mouse.

1. Osteoprotegerin

Osteoprotegerin (OPG) is a decoy receptor for the osteoclast differentiation factor, RANKL and therefore competitively inhibits osteoclast activation by osteoblast and/or tumor cells (15). One of the advantages of our model is that we have developed an RTQ-PCR assay to measure metastatic tumor burden in bone and other organs. In the first series of experiments, mice were injected into the mammary gland with the bone metastasizing clone, 4T1.13con2. Once the tumors were palpable, the mice were treated with daily injections of recombinant Fc-OPG (kindly supplied by Amgen) at 1 mg/Kg/day. (Figure 6). In two separate

experiments, the spines of mice treated with Fc-OPG displayed reduced metastatic tumor burden compared to controls. These results border on statistical significance and further experiments are required to validate these results. Nevertheless, the data suggest that metastatic tumor growth in our model is at least partially dependent on osteolysis. Amgen have recently informed us that a new version of OPG, OPG-Fc is more potent and has a longer half-life in mice, so we intend to repeat these experiments using the new OPG fusion protein.

We have also transfected tumor cells with an expression construct for mouse OPG and have generated single cell clones. The aim is to determine whether local expression of OPG in bone is effective in inhibiting osteolysis and therefore bone tumor growth. These clones have been injected into mice either into the mammary gland or directly into bone.

Following inoculation into the mammary gland, primary tumors generated from the control and OPG expressing lines developed at the same rate. However, tumor growth in the spine was reduced substantially (Figure 7).

An *in vivo* assay of metastatic tumor growth, involving injection of tumor cells directly into the tibia, was used to determine the effect of tumor cell OPG expression on local growth in bone. Tumor cells overexpressing OPG showed reduced metastatic tumor burden compared to control (vector transfected) tumor cells 14 days after tumor inoculation (Figure 8). These data provide encouraging indications that osteolysis is an important factor in the regulation of tumor cell growth in bone, probably via the release of stimulatory cytokines. Further experiments will be required to strengthen these results and provide insights into the mechanism of this regulation.

2. Bisphosphonates

Bisphosphonates are used clinically to alleviate bone pain caused by malignant disease. They are able to coat the bone surfaces with a layer resistant to attack by osteoclasts. They have also been shown to induce apoptosis in osteoclasts. We have tested Pamidronate and Zoledronate several times for their ability to protect mice from osteolytic damage caused by the bone metastasising lines 4T1.2 and 4T1.13. We have never seen a reduction in bone tumor burden using these agents, however, we have not yet looked closely to determine if bone loss is reduced.

3. S247

S247 is a peptidomimetic antagonist for the integrin $\beta 3$, being developed by Pharmacia. Experiments are underway to test whether it can block metastasis to bone by disrupting the function of the integrin $\beta 3$.

4. $\beta 3$ integrin

Mice lacking $\beta 3$ integrin display an osteopetrotic phenotype due to the lack of functional osteoclasts (16). These mice are being backcrossed onto a Balb/c background. We have done 8 backcrosses and have verified normal tumor growth in $\beta 3$ integrin wild type offspring. After one more backcross, we will generate wild type and null mice for experiments. 4T1.2 cells will be injected into the mammary gland and growth of the primary tumor and metastasis to lymph nodes, lung and bone will be assessed.

5. M-CSF

M-CSF (op/op) mice also display an osteopetrotic phenotype due to the lack of m-CSF required for osteoclast activation. These mice have been backcrossed onto the Balb/c background as well. They have been backcrossed nine times and normal growth of the 4T1.2 tumor line has been demonstrated in the wild type offspring. We are currently expanding the numbers of wildtype and op/op mice for tumor growth and metastasis experiments.

Task #3. To examine mechanisms of osteoclast activation by tumor cells

The mechanism(s) by which tumor cells induce osteolysis are not well understood and the identification of factors important in this process is a major focus of this project. The role of OPG in osteolysis induced by metastatic tumor cells is being investigated as described in Specific Aim 2. In this section, we are investigating the role of other factors identified in Specific Aim 1 above to promote or inhibit osteolysis. In addition, the role of matrix metalloproteinases in our model is being examined by backcrossing MMP12 and MMP9 knockout mice onto the Balb/c background. The MMP12 knockout mice have been backcrossed nine times and are being expanded to generate wild type and null mice for experiments. This step has already been completed for the MMP9 mice and some experimental data has been obtained. This is described below.

As mentioned above, there is considerable data suggesting that PTHrP is important in breast cancer metastasis to bone. We have investigated the role of PTHrP in our model of metastasis by genetically engineering altered PTHrP expression levels in tumor cell lines. Our data shows that overexpression of PTHrP does not enhance the ability of tumor cells to metastasize to bone. However, inhibition of PTHrP expression using antisense cDNA constructs reduces the growth rate and therefore metastatic tumor burden of 4T1.2 tumor cells (Figure 9). However, when the tumors bearing the PTHrP antisense construct are grown for an extra week so that the primary tumors are the same size as those of the control group, there was no reduction in tumor burden in bone (Figure 10). These data demonstrate that PTHrP is required for tumor cell growth and that antisense PTHrP may block this intracellular function of PTHrP. However, it is not required for metastasis to bone.

Matrix metalloproteinases (MMPs) have been implicated in the metastatic progression of many cancers. However, whilst MMPs have been postulated to contribute to increased migration and invasion of breast tumor cells, their precise role in the metastasis of breast tumor cells to bone is incompletely understood (17). A recent study by Ohshiba et al. (18) reported that co-culture of MDA-MB-231 breast cancer cells with bone calvaria increased bone resorption and this response was associated with increased expression of pro-MMP-2, active MMP-2, pro-MMP9 and MMP-13. These results implicate multiple MMPs in the osteolytic response associated with breast cancer metastasis to bone. We have reported that the bone metastatic cell line 4T1.2 is more invasive in *in vitro* assays and secretes higher levels of MMP9 than the weakly metastatic (66cl4) and non-metastatic (67NR) cell lines (19). The increased expression of MMP9 in co-cultures of 4T1.2 and bone stromal cells discussed above (see Task #1) indicates that expression of tumor-derived MMP9 may be regulated by bone stromal cells. We have extended our *in vitro* findings by performing

immunohistochemical (IHC) analysis of MMP9 in our in vivo orthotopic model of breast cancer metastasis to bone.

Examination of normal femur sections by IHC revealed high levels of MMP9 in the bone marrow and at sites of active remodelling (growth plates) (Figure 11). Further analysis of femoral metastases derived from mammary fat pad injection of 4T1.2 cells demonstrated significant expression of MMP9 in the metastatic lesion, particularly evident at the periphery of the tumor in close contact with the bone stroma and active sites of bone resorption by osteoclasts (Figure 12). A similar pattern of MMP9 reactivity was detected following direct intratibial injection of 4T1.2 cells in wild type mice (Figure 13). Interestingly, when direct intratibial injections were performed in MMP9 deficient animals, intense staining of the entire tumor area was detected whereas, as expected, the unaffected marrow showed no MMP9 reactivity (Figure 14).

Taken together, these results illustrate the interplay between tumor and bone stroma and suggest that both stroma and tumor-derived MMP9 may be critical for the establishment of breast cancer metastasis to bone. However, the possibility that trauma resulting from the intratibial injection procedure may in part contribute to the increased MMP9 expression detected in the tumors of MMP9 null mice cannot be completely ruled out. To address this and to determine if the development of spontaneous metastasis in these animals is also associated with increased MMP9 expression in bone metastases, we have initiated experiments using IMFP injection of 4T1.2 cells in MMP9 deficient and wild type mice and are currently awaiting the development of bone metastasis for histological examination and MMP9 IHC analysis. This will be performed in conjunction with measurements of tumor burden by RTQ-PCR. In addition, we have generated RNAi constructs to block the endogenous production of MMP9 in 4T1.2 cells. The combination of RNAi methodology and MMP9 null mice will clarify whether tumor-derived MMP9, bone stroma-derived MMP9 or both contribute to the establishment of breast cancer metastases in bone and associated bone resorption.

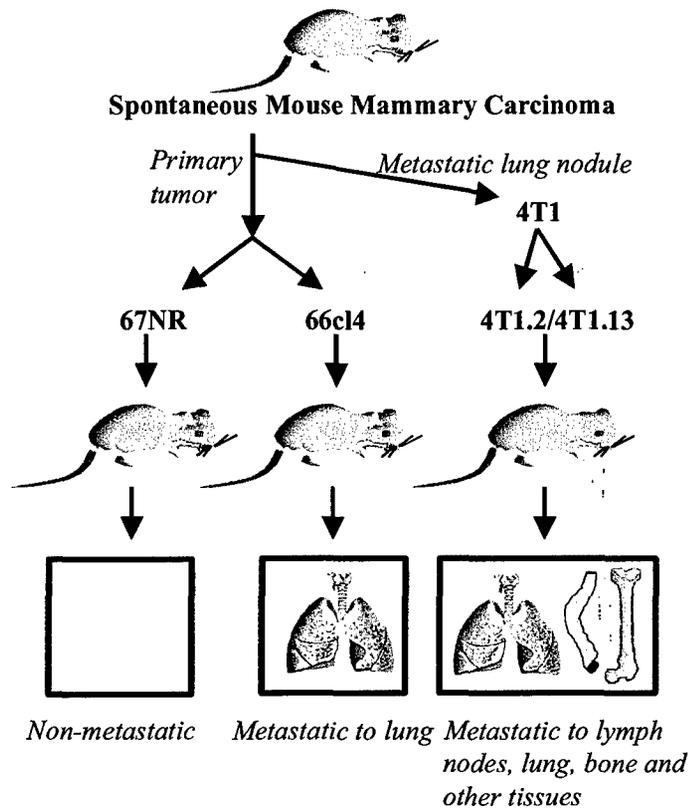


Figure 1. Orthotopic model of breast cancer metastasis. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, whereas 66cl4 is weakly metastatic to lung. 4T1.2 and 4T1.13 are two bone metastasizing tumor clones derived from the lung metastasizing 4T1 tumor subline isolated from a metastatic lung nodule.

Primary Tumor

Spine Metastasis

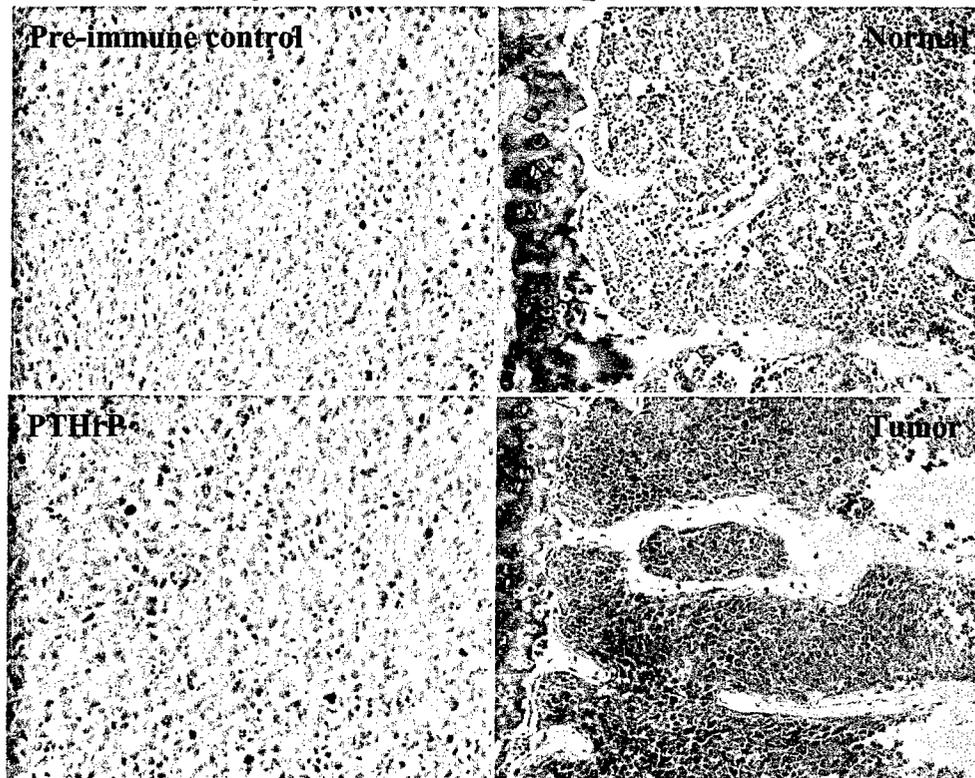


Figure 2. Immunohistochemical Detection of PTHrP in Primary Mammary Tumor and Bone Metastasis. Sections were prepared from primary tumor and spine of mice bearing 4T1.2 tumor, stained with hematoxylin and eosin and immunostained with a peroxidase-conjugated anti-human PTHrP antibody. Top left: section through primary tumor labelled with control pre-immune serum. Bottom left: section of primary tumor labeled with specific antibody directed against PTHrP. Top right: section of normal bone and bone marrow labelled with PTHrP antibody. Bottom right: adjacent section of spine showing large tumor deposit expressing PTHrP.

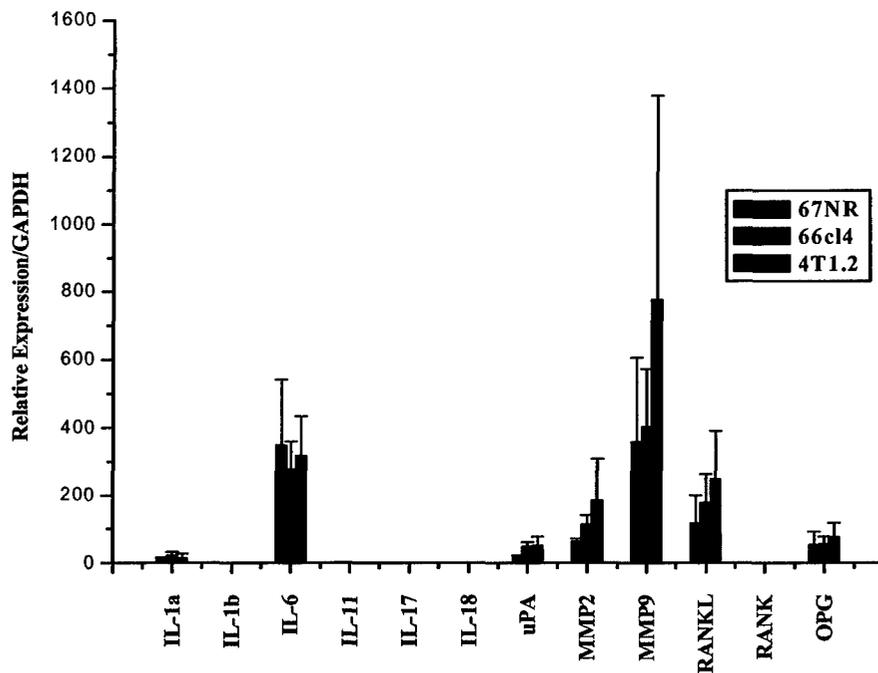


Figure 3. Expression of Candidate Genes in Primary Tumors. RNA was extracted from three primary tumors for each of the tumor lines indicated. cDNA was synthesized and SyBr Green RT-RTQ-PCR performed for each of the genes indicated. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.

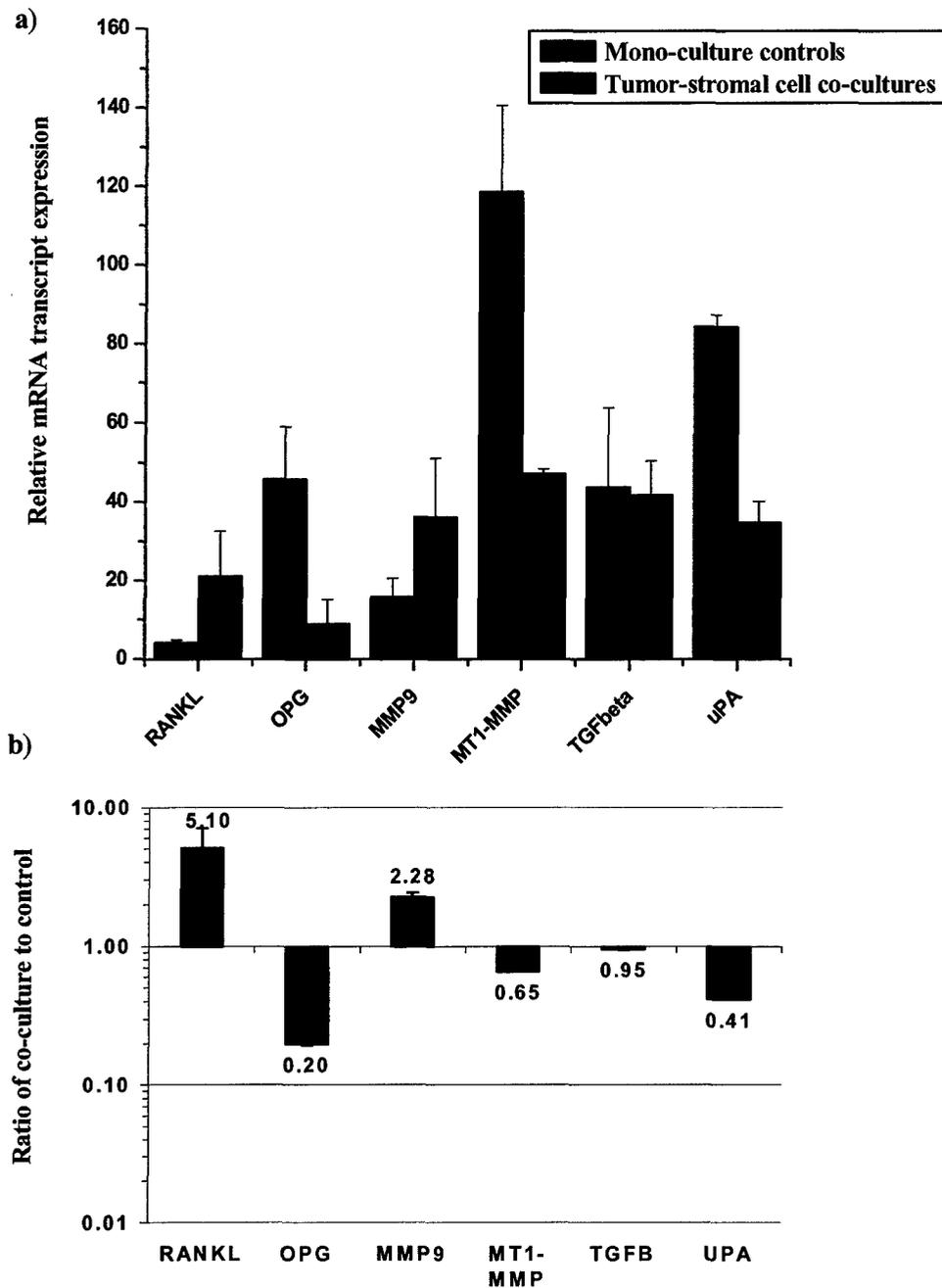


Figure 4. Regulation of gene expression by co-culture of tumor and stromal cells in vitro. RNA was extracted from control mono-cultures or co-cultures of 4T1.2 tumor cells and bone stromal cell population enriched for osteoblasts. CDNAs were synthesized and SyBr Green RT-RTQ-PCR performed for each of the genes indicated. (a) mRNA transcript expression relative to GAPDH was determined and the results represent the means \pm SD of triplicates. (b), the results from panel are expressed as a ration of mRNA expression of co-cultures/control mono-cultures to reflect the changes in transcript abundance for co-cultures of tumor-stromal cells.

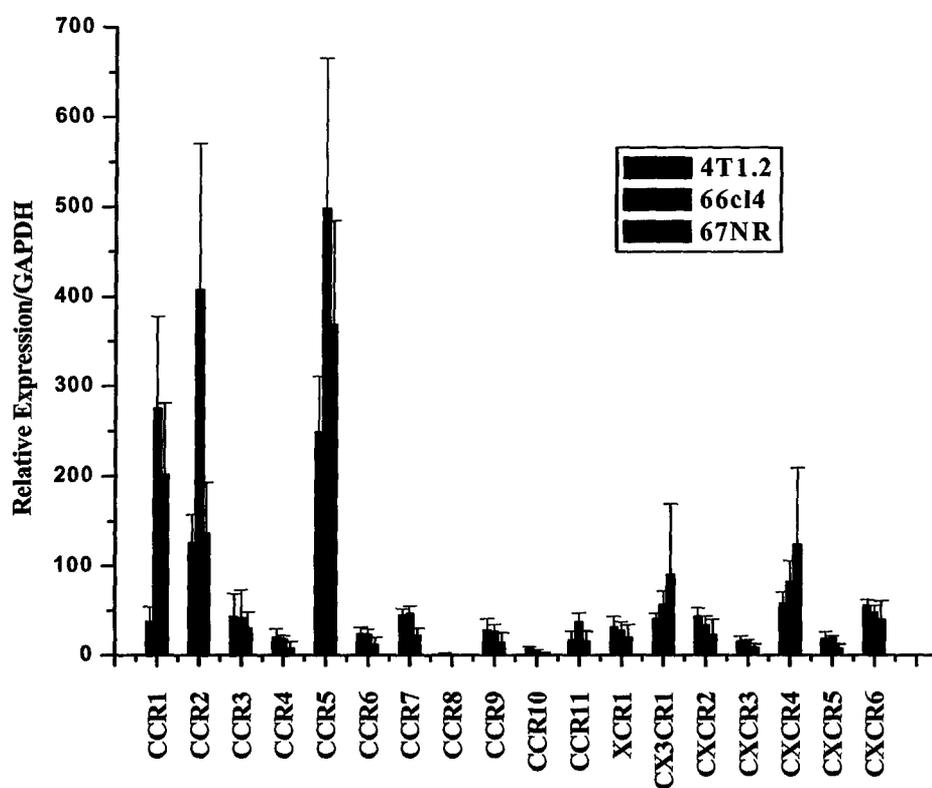


Figure 5. RT-RTQ-PCR Analysis of Chemokine Expression in Tumor Cells. RNA was extracted from three separate primary tumors for each of the tumor lines indicated. cDNA was synthesized and SyBr Green RT-RTQ-PCR performed for each of the chemokine receptors. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.

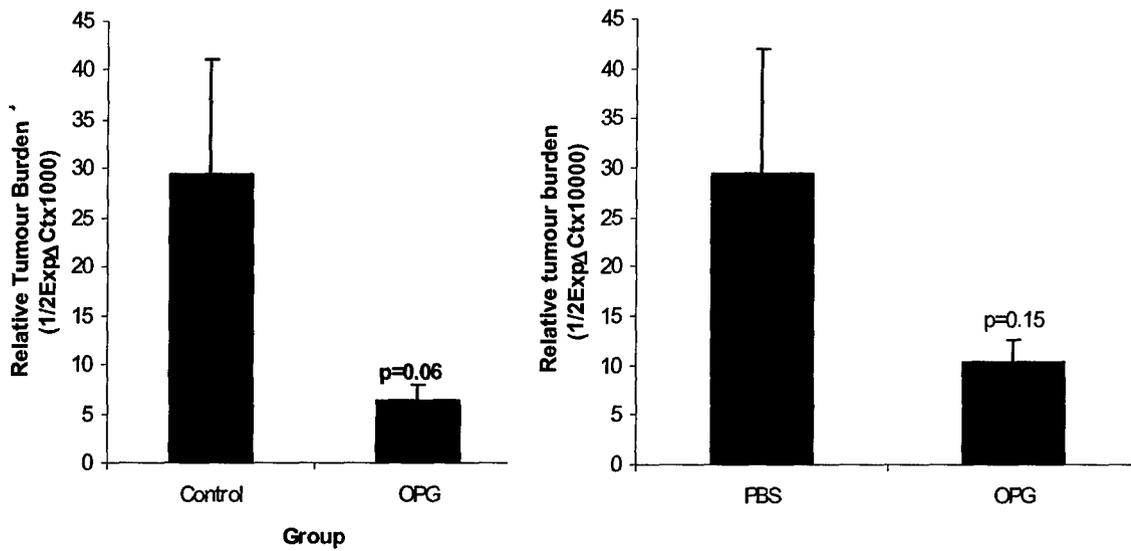


Figure 6. Effect of OPG on Metastatic Tumor Burden in Spine. Mice (n = 15) were injected with 4T1.13Con2 and primary tumors, allowed to develop for either 11 days (panel a) or 14 days (panel b). OPG (1mg/kg/day) was then administered daily by subcutaneous injection for 14 days. Genomic DNA was prepared from spine and RTQ PCR used to determine metastatic tumor burden. Error bars represent standard errors of the mean and p values were determined using the student t test.

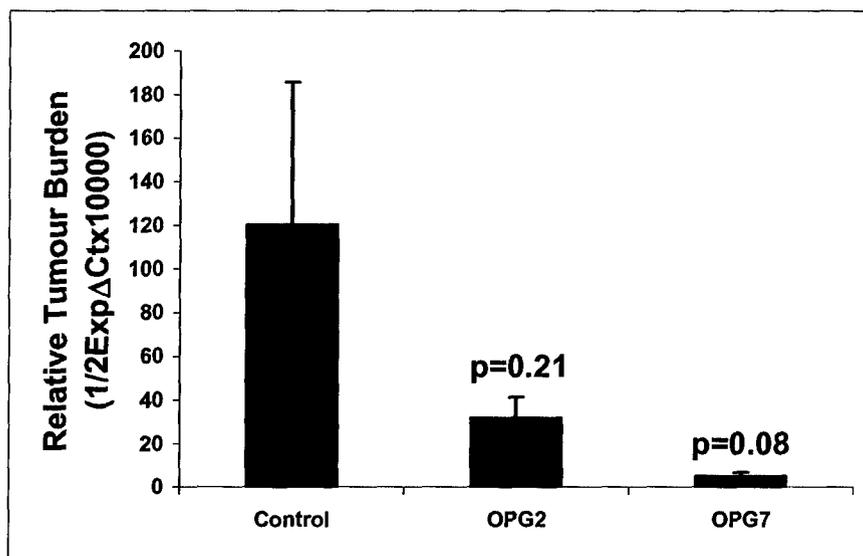


Figure 7. Overexpression of OPG in Breast Tumor Cell Lines Reduces their Bone-Metastatic Potential. Mice (n = 15) were inoculated into the mammary gland with 4T1.13Con2 and primary tumors, allowed to develop for 30 days. Genomic DNA was prepared from spine and RTQ-PCR used to determine metastatic tumor burden. Error bars represent standard errors of the mean and p values were determined using the student t test.

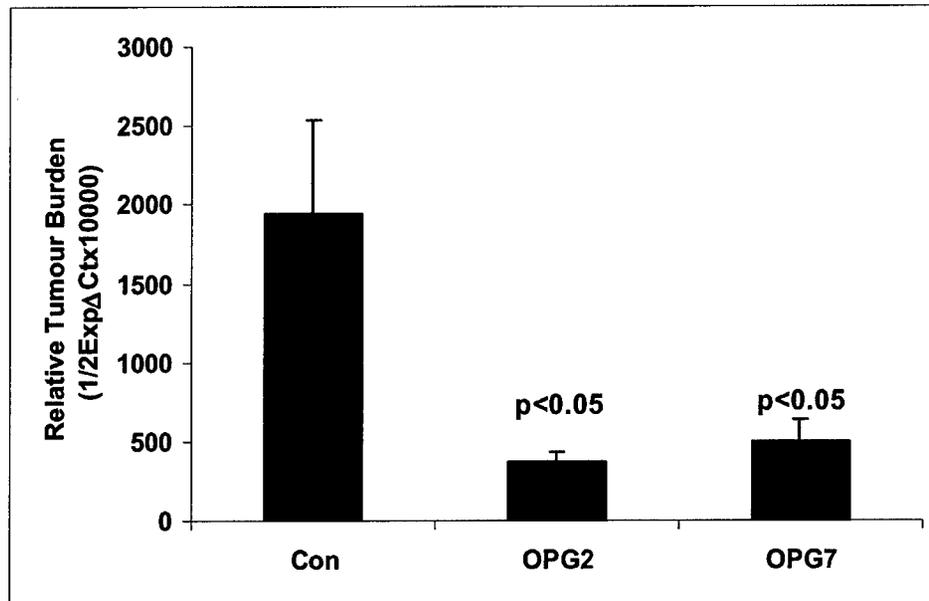


Figure 8. *In Vivo* Growth of Tumor Cells Overexpressing OPG. Two single cell clones overexpressing OPG and one vector transfected clone of 4T1.2 were injected directly into the mouse tibia (n = 8) and tumor cells allowed to grow for 18 days. Tibia were removed and tumor growth measured using RTQ PCR. Error bars represent standard error of the mean and p values were determined using the student t test.

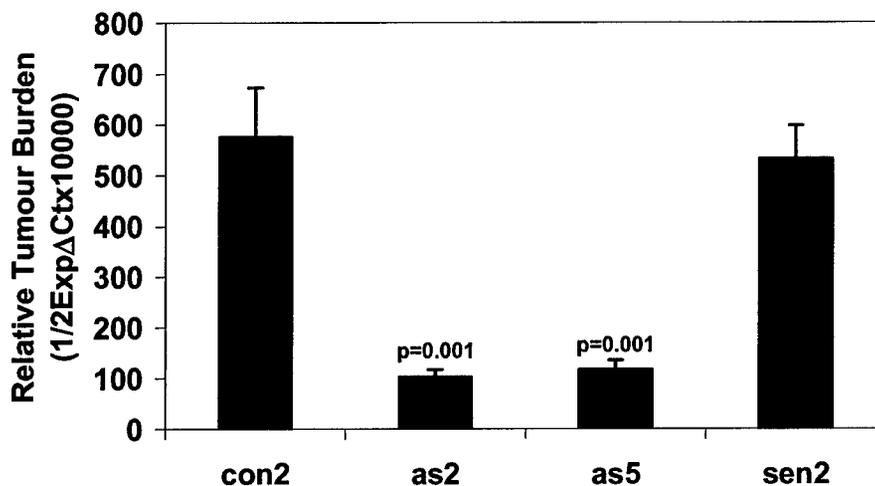


Figure 9. Effect of Antisense PTHrP on Metastatic Tumor Burden. Single cell clones of 4T1.13 tumor cells were transfected with vector (con2), PTHrP sense (sen2) or PTHrP antisense (as2 and as5) and injected into the mouse mammary fat pad (n = 15). Four weeks later, spines were removed and RTQ PCR used to measure metastatic tumor burden. Error bars represent standard error of the mean and p values were calculated using the student t test.

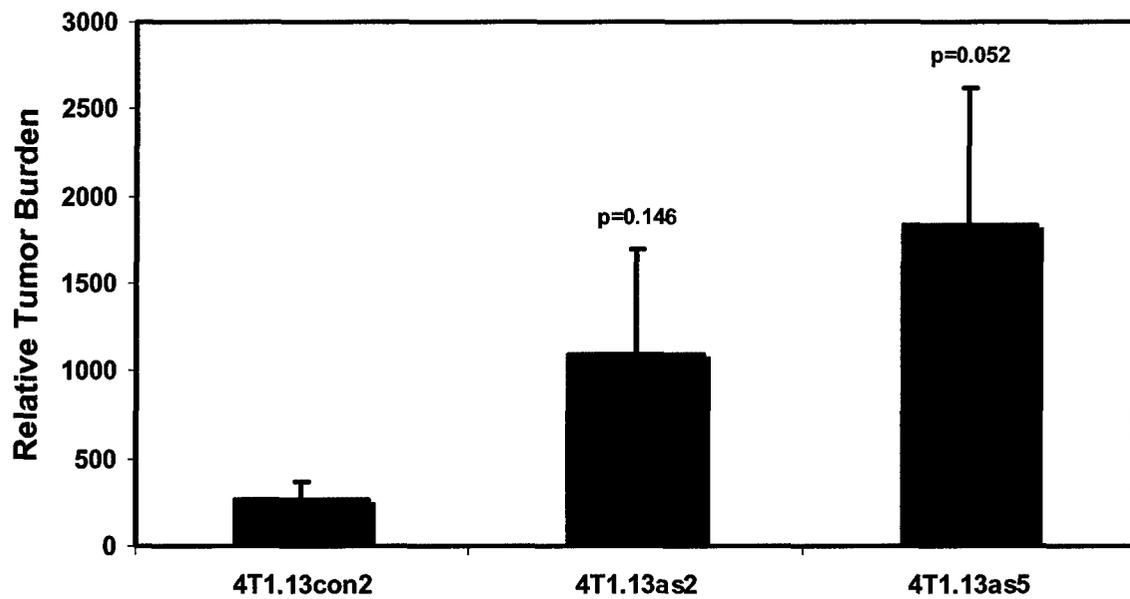


Figure 10. Effect of Antisense PTHrP on Metastatic Tumor Burden. Single cell clones of 4T1.13 tumor cells were transfected with vector (4T1.13con2) or PTHrP antisense (4T1.13as2 and 4T1.13as5) and injected into the mouse mammary fat pad. Four weeks later, spines from mice inoculated with 4T1.13Con2 cells were removed and RTQ PCR used to measure metastatic tumor burden. Spines from mice inoculated with 4T1.13 cells transfected with a PTHrP antisense were harvested and tumor burden measured 7 days later when the primary tumors were the same size as those from control mice. $n = 10$. Error bars represent standard error of the mean and p values were calculated using the student t test.

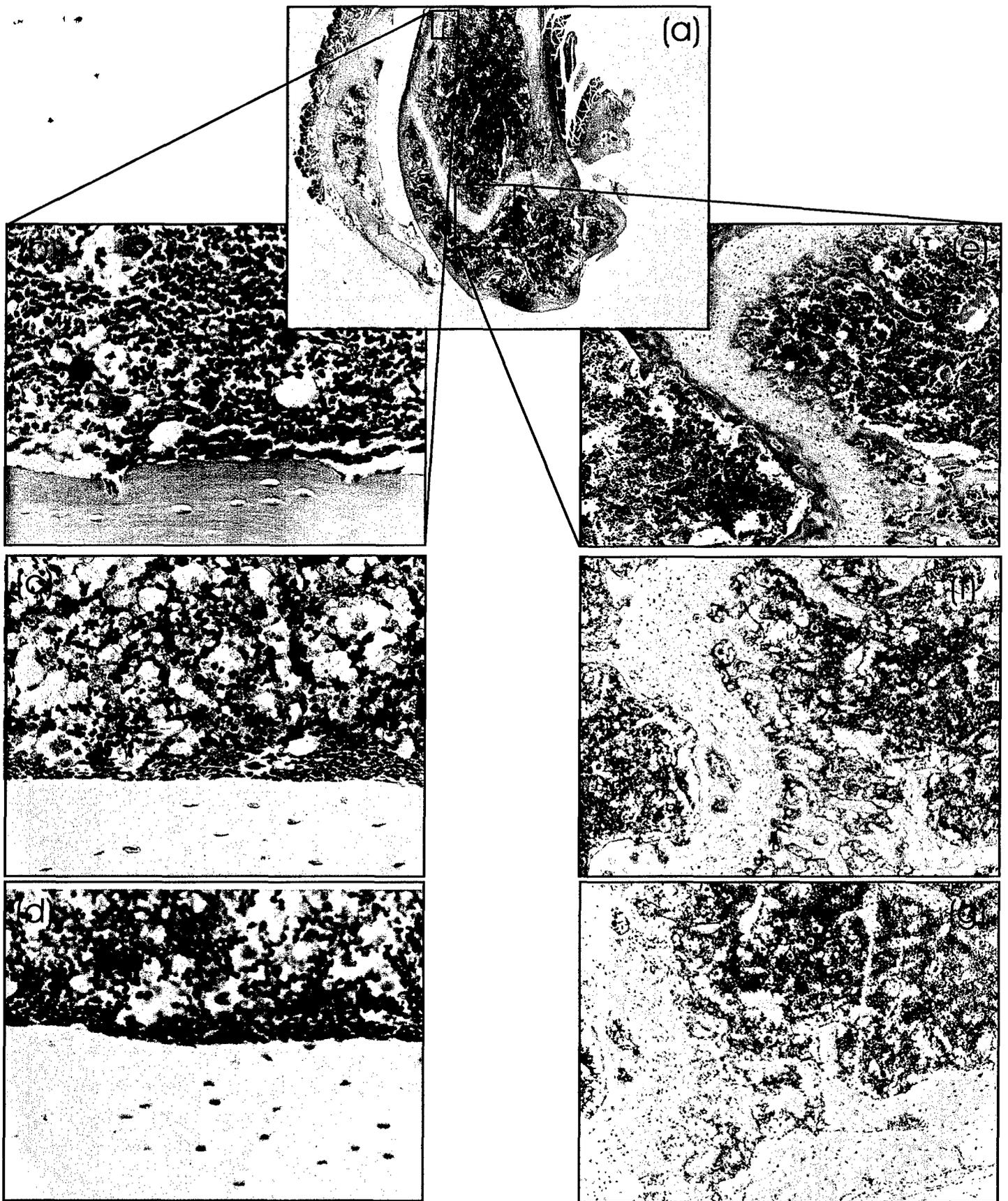


Figure 11. MMP-9 expression in normal femur sections. Femurs from healthy 6-8 week old Balb/c mice were harvested, fixed in formalin and decalcified in EDTA prior to processing for paraffin embedding. Serial sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical detection of MMP-9 using a polyclonal goat antiserum. (a) H&E stained femur epiphysis. (b) H&E staining of marrow-bone interface. (c) Specific MMP-9 reactivity is seen in haematopoietic cells populating the bone marrow. (D) Absence of specific staining in control sections reacted with pre-immune serum. (e) H&E of the growth plate region. (f) MMP-9 stained serial section localising MMP-9 to the zone of ossification (transition zone from cartilage to bone). (g) negative control reacted with pre-immune serum. Magnification: 2.5X (a), 10X (b-g).

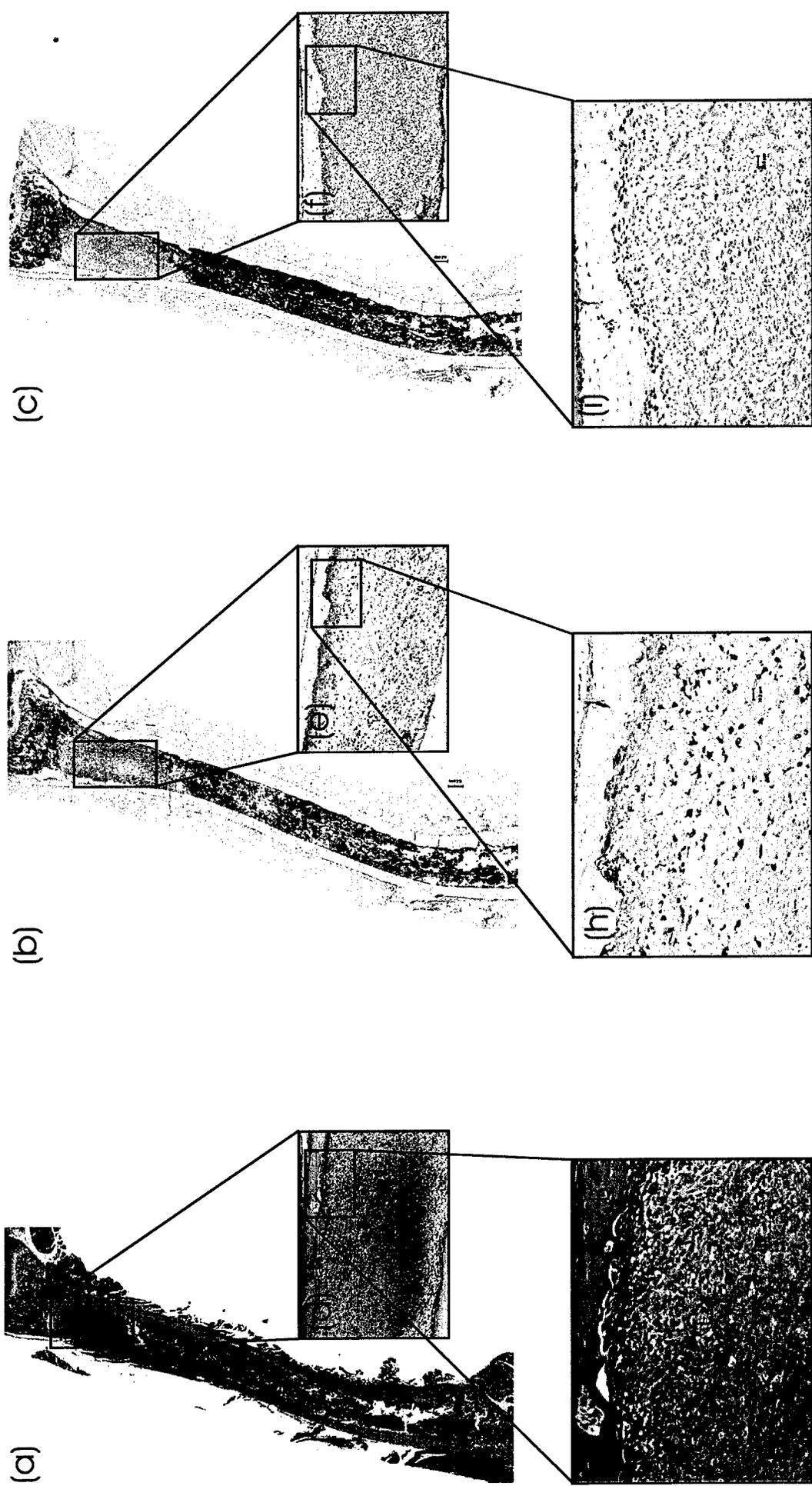


Figure 12. MMP-9 Expression in 4T1.2 femoral metastases. Femurs were harvested 30 days after injection of 4T1.2 tumor cells into the mammary fat pad. Femurs were fixed in formalin, decalcified in EDTA and processed for paraffin embedding. (a) H&E staining shows a large metastatic lesion (t) in the proximal epiphyseal region. (b) serial section reacted with a specific goat polyclonal antiserum directed against MMP-9 following antigen retrieval by trypsin digestion shows MMP-9 immunoreactivity scattered through the marrow and around the metastatic lesion. (c) No specific labeling was detected using a control goat pre-immune serum. (d) H&E stained section. (e) corresponding field stained for MMP-9 demonstrates MMP-9 localization primarily at the periphery of the metastatic lesion. (f) absence of specific staining using pre-immune serum. (g) at higher magnification, H&E of the metastasis-bone interface shows the presence of osteoclasts within resorption lacunae (T = tumor, B = cortical bone, arrows denote osteoclasts and associated resorption lacunae) (h) 20x view of the corresponding stained section, demonstrating MMP-9 immunoreactivity within resorption lacunae and in scattered cells within the tumor mass. (i) 20X magnification of the corresponding negative control section. Magnification: 2.5X (a-c), 10X (d-f), 20X (g-i).



Figure 13. MMP-9 expression in 4T1.2 tumor following intratibial injection into wild type Balb/c mice. Tibias were harvested 11 days after direct intratibial injection of 4T1.2 tumor cells, fixed in formalin, decalcified and processed for paraffin embedding. Serial sections were stained for MMP-9 expression following antigen retrieval by trypsin digestion. (a) H&E stained section showing a large 4T1.2 tumor deposit (T) adjacent to bone / cartilage (B/C). (b) MMP-9 stained serial section revealed strong immunoreactivity at the periphery of the tumor adjacent to the bone surface. MMP-9 immunoreactivity is also clearly visible within the cartilage / bone matrix of the growth plate. (c) Absence of specific staining using a control goat pre-immune serum. Magnification: 10X (a-c).

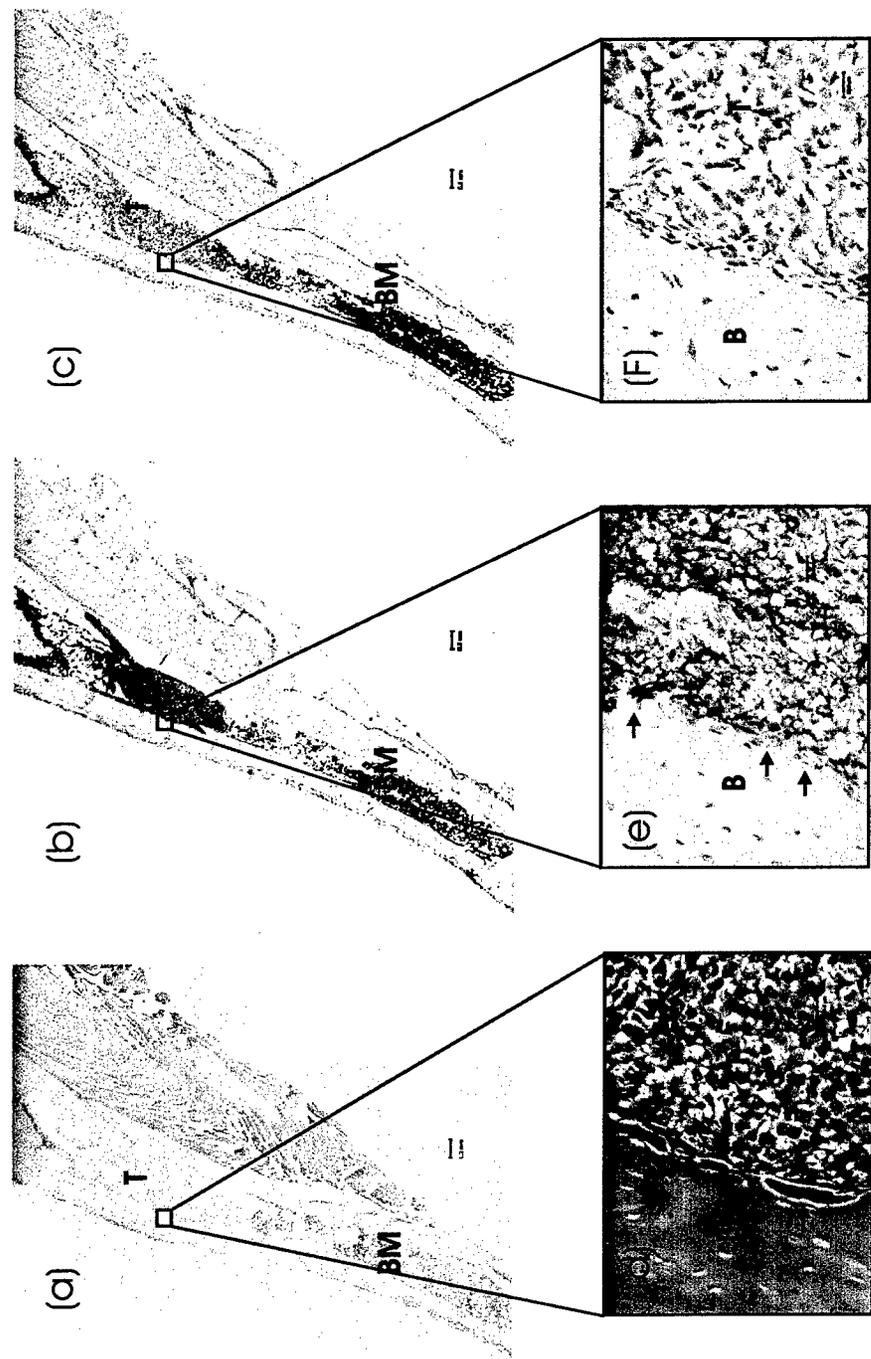


Figure 14. MMP-9 expression in 4T1.2 tumors following intratibial injection into MMP-9 knockout mice. 4T1.2 tumor cells were injected into the proximal marrow space of MMP-9 knockout Balb/c mouse tibias. Tumors were allowed to develop for 11 days prior to harvest. Tibiae were fixed in formalin, and decalcified prior to paraffin embedding. Serial sections were stained for MMP-9 following antigen retrieval by trypsin digestion. (a) H&E stained tibia, demonstrating the tumor mass (T) occupying the upper (proximal) portion of the tibial marrow, adjacent to normal bone marrow (BM). (b) Corresponding serial section stained for MMP-9, showing strong tumor immunoreactivity but absence of specific staining in the surrounding bone. (c) No specific labeling was detected using a control goat pre-immune serum. (d) H&E of tumor-bone interface showing multinucleated osteoclasts within resorption lacunae. (e) Section stained for MMP-9 demonstrating strong immunoreactivity in 4T1.2 tumor cells. (f) Absence of specific staining using control pre-immune serum. *B-cortical bone, T-tumor.* Magnification: 2.5X (a-c), 40X (d-f).

CURRICULUM VITAE

Personal Details:

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Nationality: Canadian/Australian (Permanent Resident)

Languages: English and French (spoken and written)

Education:

- | | |
|--------------------------------|--|
| August 1992 – June 1998 | Doctor of Philosophy (Cancer Research)
Ludwig Institute for Cancer Research
University of Melbourne,
Victoria, Australia |
| September 1995 – October 1995 | Leadership and Professional Development Program
University of Melbourne
School of Graduate Studies/Melbourne Business School
Victoria, Australia |
| September 1989 - July 1991 | Master of Science (Immunology)
University of Sherbrooke
Québec, Canada |
| January 1986 - April 1989 | Bachelor of Science (Biotechnology)
University of Québec in Montreal
Québec, Canada |
| September 1984 - December 1985 | College Diploma in Science
Collège de Montmorency
Québec, Canada |

Present Appointment:

- | | |
|---------------------|--|
| June 2000 – Current | Research Fellow
Peter MacCallum Cancer Institute
St-Andrews Place, East Melbourne
Victoria, Australia 3002 |
|---------------------|--|

Post-Doctoral Fellowships:

April 2003 – June 2004	Post-Doctoral Fellowship	U.S.Army Medical Research & Materiel Command
January 2003 – December 2005	Post-Doctoral Fellowship	NH&MRC Project Grant
January 1999 – December 1999	Post-Doctoral Fellowship	Anti-Cancer Council of Victoria (ACCV)

Post-Graduate Scholarships and Awards:

September 1992 - March 1996	Ph.D. Scholarship:	Commonwealth Scholarship and Fellowship Plan (CSFP)
September 1990 - September 1991	M.Sc. Scholarship:	Fonds Canadiens d'Aide à la Recherche (FCAR)
September 1989 - September 1990	M.Sc. Scholarship	Fonds Canadiens d'Aide à la Recherche (FCAR)
June 1989 - August 1989	M.Sc. Excellence Award	Fonds de Recherche en Santé du Québec (FRSQ)
May 1989 - September 1989	B.Sc. Scholarship	Fonds de Recherche en Santé du Québec (FRSQ)
May 1988 - September 1988	B.Sc. Scholarship	Conseil de Recherche en Science Naturelles et en Génie (CRSNG)

Research Experience:

Current Research Project (April 2003 – Current):

“Breast Tumour/Stromal Cell Interactions in Bone”
Peter MacCallum Cancer Centre

The aim of my current research is to investigate factors that regulate the establishment of breast cancer metastases in bone. Specifically, I am using an orthotopic mouse model of breast cancer metastasis to bone established in Dr. Robin Anderson's lab at Peter MacCallum Cancer Centre which employs a variety of breast cancer cell line that differ in their metastatic potential. Tumor burden in bone and other tissues following injection of tumor lines into the mammary gland is measured using a real time quantitative PCR (RTQ-PCR) method developed in our laboratory. Other approaches to investigate factors involved in bone metastasis include the use of K/O animals (Beta3 integrin, MMP-9, MMP-12 and M-CSF), specific inhibitors or overexpression construct for selected candidate genes and RNA interference technology both in our *in vivo* model and in *in vitro* co-culture assays. Expression of several factors in the tumour/stroma microenvironment is also being investigated by immunohistochemistry and immunofluorescence microscopy with particular emphasis on PTHrP, RANKL, OPG and MMPs.

Post-Doctoral Research July 2000 – March 2003):

“Microenvironmental regulation of the tissue regenerative capacity of keratinocyte stem cells and their progeny”

Peter MacCallum Cancer Centre

Project: The general objective of this research project was to explore the functional role of the mesenchymal microenvironment (with particular emphasis on laminin-10/11, LN-10/11) in regulating the tissue regenerative capacity of keratinocyte stem cells. I have demonstrated the presence of LN-10/11 in the cutaneous basement membrane and identified 2 receptors in keratinocytes. Further I have demonstrated that LN-10/11 has a functional role in promoting adhesion, proliferation and migration of normal and tumourigenic keratinocytes *in vitro*. This work has been published in *Exp. Dermatol.* and was later extended to an *in vitro* organotypic skin regeneration model. In this system, I have provided strong evidence that LN-10/11 is an important regulatory molecule promoting skin regeneration in part by delaying the onset of keratinocyte terminal differentiation and recruiting early differentiating keratinocytes into proliferation. This work was recently submitted for publication in *J. Clin. Invest.*. The role of soluble dermal factors including FGF-7 and FGF-10 and signaling pathways regulating the expression of LN-10/11 chains in keratinocytes was investigated. I have developed an *in vivo* model of skin regeneration amenable to testing the long-term tissue regenerative potential and plasticity of rare keratinocyte populations (e.g. stem cells) while allowing to investigate the role of microenvironmental factors in this process. The model can easily be adapted to other epithelial tissues or used to investigate the process of tumour progression. A manuscript describing the development of the *in vivo* model is currently in preparation. Together, the results generated through my research have important implications for the treatment of various cutaneous pathologies including skin cancer, blistering disorders, severe burns and other chronic skin wounds.

Technical skills acquired: ^{35}S -Met/Cys-metabolic labelling, FACS analysis, immunohistochemistry, *in vitro* and *in vivo* skin regeneration assays.

Post-Doctoral Research (July 1998 – June 2000):

“Purification and characterisation of a colonic autocrine spreading factor”

Ludwig Institute for Cancer Research, Melbourne Branch

Project: This research project followed from my Ph.D. project. The aim was to purify a sufficient amount of an Autocrine Spreading Factor (ASF) from the conditioned medium of a human colon carcinoma cell line (LIM1215) to enable its identification by amino acid sequence analysis and biological/biochemical characterisation. I was able to optimise the purification protocol significantly and identified ASF by mass spectrometry, amino acid analysis and immunoblotting as laminin-10 (LN-10, $\alpha_5\beta_1\gamma_1$ trimer). Further, I demonstrated that LN-10 is a potent adhesion and motility factor for colon cancer cells and that integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ act as receptors for this laminin isoform. Taken together, the results from my post-doctoral research supports the role of autocrine TGF- α and laminin-10 in the metastasis of colon tumours.

Technical skills acquired: *In vitro* cell motility and invasion assays, proteolytic digestion, enzymatic deglycosylation, confocal microscopy.

Ph.D. Research: "Autocrine Factors Regulate Colon Cancer Cells"
Ludwig Institute for Cancer Research/University of Melbourne
Supervisor: Prof. Antony W. Burgess

Project: The aim of the research project was to study the role of autocrine factors in growth regulation using the colon carcinoma cell line LIM1215 as an *in vitro* model. I found that multiple autocrine factors including a mitogen (TGF- α) a survival/anti-apoptotic factor and an extracellular matrix (ECM)-like spreading factor are produced by LIM1215 cells and synergize to regulate their proliferation and morphology *in vitro*. I successfully purified to homogeneity a large ECM protein and work to identify this protein by amino acid sequencing was undertaken as part of my post-doctoral research project. I studied in detail the signalling pathways triggered by TGF- α /EGF stimulation of LIM1215 cells, particularly the mechanism of activation of the ras/raf-1 signalling pathway and its role in mitogenesis.

Technical skills acquired: *In vitro* cell spreading assays, immunofluorescence, ELISA, production and purification of polyclonal antibodies, sub-cellular fractionation, Western blotting, immunoprecipitation, *in vitro* kinase assays, protein purification/HPLC, electrophoresis.

M.Sc. Research: "Characterization of the Cytotoxic Activity of Guinea Pig Foa-Kurloff Cells."
University of Sherbrooke
Supervisor: Prof. Marek Rola-Pleszczynski

Project: Natural Killer (NK) cells play an important role in immunological defense and constitute the first line of resistance against viral and bacterial infection as well as tumor development. Whilst the Guinea pig has been widely used as an animal model to study natural defense against such invasion, little was known about the exact nature of the NK cell in this animal. During my M.Sc., I demonstrated that a subset of cells, previously known as Foa-Kurloff cells, were in fact the Guinea pig counterpart of human NK cells. Furthermore, I studied in detail their mechanism of action against tumor cells and showed that they display two distinct cytotoxic activities previously thought to be mediated by two subsets of cells namely, Natural Killer (NK) and Natural Cytotoxic (NC) cells.

Technical skills acquired: General cell culture, *in vitro* mitogenic and cytotoxic assays, production of monoclonal antibodies, FACS analysis.

Other Roles Undertaken:

Referee for Journals: - Growth Factors

Grant Assessor: -The Royal Women's Hospital Research Advisory Committee
-Raine Medical Research Foundation
-The Leo & Jenny Leukaemia and Cancer Foundation

Supervisor: Co-supervision of research assistants and students

Industry Experience:

March 1997 – February 1998 Pharmaceutical Development Project Planner
Glaxo-Wellcome Australia Ltd
1061 Mountain Hwy, Boronia
VIC, Australia 3155

July 1991- August 1992 Medical Representative
Procter & Gamble Pharmaceuticals Canada inc.
P.O. Box 355 succ A Toronto, Ontario
Canada M5W 1C5

Teaching Experience:

September 1987 – September 1989 Substitute Teacher
École Polyvalente Évariste Leblanc
1750 Mtée Masson, Duvernay Est, Laval
Québec, Canada

I taught mathematics, physics, chemistry and biology to year 7 to 12 students. In addition, I taught regular classes to students with learning difficulties. Responsibilities included the preparation and presentation of lectures, preparation and correction of exams and reports, and supervision of student's academic progress.

Publications:

Primary Research Articles:

1. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1996).** Guinea pig Kurloff (NK-like) cells mediate TNF-dependent cytotoxic activity: analogy with NC effector cells. *Inflammation*, 20:263-280.
2. **Pouliot N., K. Maghni, F. Blanchette, L. Cironi, P. Sirois, J. Stankova and M. Rola-Pleszczynski (1996).** Natural killer and lectin-dependent cytotoxic activity of Kurloff cells: target cell selectivity, conjugate formation and Ca⁺⁺ dependency. *Inflammation*, 20:647-671.
3. Walker F., A. Kato, L. J. Gonez, M. L. Hibbs, N. **Pouliot**, A. Levitzki and A. W. Burgess (1998). Activation of the Ras/MAPK pathway by kinase defective epidermal growth factor receptors results in cell survival but not proliferation. *Molecular and Cellular Biology*, 18:7192-7204.
4. **Pouliot N. and A. W. Burgess (2000).** Multiple autocrine factors including an extracellular matrix protein are required for the proliferation and spreading of human colon carcinoma cells *in vitro*. *Growth Factors*, 18:7192-7204.
5. **Pouliot N., L.M. Connolly, R.L. Moritz, R.J. Simpson and A. W. Burgess (2000).** Colon cancer cell adhesion and spreading on autocrine laminin-10 is mediated by multiple integrin receptors and modulated by EGF receptor stimulation. *Experimental Cell Research*, 261:360-371.
6. **Pouliot N., E.C. Nice and A.W. Burgess (2001).** Laminin-10 mediates basal and EGF-stimulated motility of human colon carcinoma cells via $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins. *Experimental Cell Research*, 266:1-10.
7. **Pouliot N., N.A. Saunders and P. Kaur (2002).** Laminin-10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in promoting proliferation and migration. *Experimental Dermatology*, 11:387-397.
8. Jorissen R.N., F. Walker, N. **Pouliot**, T.P.J. Garrett, C.W. Ward and A.W. Burgess (2003). Epidermal growth factor receptor: mechanisms of activation and signaling. *Experimental Cell Research*, 284:31-53.
9. Zamurs A., N. **Pouliot**, P. Gibson, G. Hocking and E. Nice (2003). Strategies for the purification of laminin-10 for studies on colon cancer metastasis. *Biomedical Chromatography*, 17:201-211.
10. Li A., N. **Pouliot** and P. Kaur (2003). Epithelial tissue regenerative capacity of keratinocyte stem cells and their progeny: intrinsic versus microenvironmental regulation. *Journal of Clinical Investigation*. Submitted for publication.
11. **Pouliot N., R.P. Redvers and P. Kaur (2003).** Development and characterization of an *in vivo* skin regeneration model to assess the long-term regenerative potential of human and murine keratinocyte stem cells. Manuscript in preparation.

Other Publications:

1. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1990).** The cytotoxic activity of the Foa-Kurloff cells. *FASEB J.*, 4:A1892.
2. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1990).** Étude du mécanisme lytique des cellules Foa-Kurloff. *Medecine Science, supplément no2 (99)* p.36A.

Meeting Presentations:

1. **Pouliot N., N.A. Saunders and P. Kaur (2002).** Laminin-10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in regulating keratinocyte cell proliferation, differentiation and migration. Oral presentation at the First Boden Research Conference on Developmental Cutaneous Biology, Stradbroke Island, Queensland, Australia.
2. **Pouliot N., A. Li, J. Karlis and P. Kaur (2002).** In vitro and in vivo tissue regenerative capacity of keratinocyte stem cells and their progeny is determined by their mesenchymal microenvironment. Poster presented at the 2002 Keystone Symposium, Keystone, Colorado, USA
3. **Pouliot N., A. Li, J. Karlis and P. Kaur (2001).** The functional role of laminin-10/11 in human keratinocyte proliferation and skin maturation. Poster presented at the Fourth Peter Mac Symposium on Molecular and Cell Biology of Cancer, University of Melbourne, Victoria, Australia.
4. **Pouliot N. A. Li and P. Kaur (2001).** Expression and function of laminin-10/11 in human skin. Poster presented at the 2001 Lorne Cancer Conference, Lorne, Victoria, Australia
5. **Pouliot N., L.M. Connolly, R.L. Moritz, R.J. Simpson and A. W. Burgess (1999).** Human colon carcinoma cells adhere to and spread on an autocrine secreted laminin-10 via integrin $\alpha_3\beta_1$ and $\alpha_6\beta_4$ receptors. Poster presented at the 1999 Pan-Pacific Connective Tissues Societies Symposium, Queenstown, New Zealand
6. **Pouliot N. and A. W. Burgess (1999).** Characterisation of an autocrine spreading/motility factor stimulating colonic cells. Poster presented at the 1999 Lorne Cancer Conference, Lorne, Victoria, Australia
7. **Pouliot N. and A. W. Burgess (1996).** Extracellular matrix and TGF-alpha are required autocrine factors required for the proliferation of the colon carcinoma cell line LIM1215. Poster presented at the 1996 Keystone Symposium, Keystone, Colorado, USA.
8. **Pouliot N. and A. W. Burgess (1995).** Multiple autocrine factors are involved in the proliferation of the colon carcinoma cell line LIM1215. Poster presented at the 1995 Lorne Cancer Conference, Lorne, Victoria, Australia.
9. **Pouliot N. and A. W. Burgess (1994).** Autocrine survival and spreading factors produced by the colon carcinoma cell line LIM1215. Poster presented at the 1994 Lorne Cancer Conference, Lorne, Victoria Australia.

10. Pouliot N., K. Maghni, P. Sirois, and M. Rola-Pleszczynski (1990). The cytotoxic activity of Foa-Kurloff cells. Poster presented at the 1990 FASEB Meeting, New Orleans, Louisiana, USA.
11. Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1990). Étude du mécanisme lytique des cellules Foa-Kurloff. Poster presented at the 1990 CRCQ Meeting, Quebec, Canada.

References:*

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- References available upon request.