# The Role of Parathyroid Hormone-Related Protein (PTHrP) in Tumour Initiation, Promotion and Metastasis of Breast Cancer

**Jiarong Li**
**McGill University, Montreal, Canada**
**February, 2010**

**A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Philosophy**

**Department of Medicine, Division of Experimental Medicine**

# Abstract

In this study, we have used a well-validated breast cancer animal model to examine the malignant progression process. We have demonstrated that disruption of *Pthrp* dramatically delays the initial and subsequent steps of malignant conversion of the mammary epithelial cell, without affecting the mammary gland development. PTHrP acts as a promoter of oncogenesis and metastasis upstream of a number of critical checkpoints for PyVMT, such as Akt1, Akt2, factor VIII Bcl-2 and cyclin D1, with the most interesting being CXCR4. This suggests a novel role for PTHrP as a facilitator of oncogenes and emphasizes the importance of attempting its targeting for therapeutic purposes.

# Résumé

Dans cette étude, nous avons utilisé un modèle animal bien connu, le PyVMT, pour illustrer les effets de PTHrP sur l'initation du cancer du sein et sur sa progression métastatique. Nous avons démontré que l'ablation du gène *Pthrp* provoque un délai significatif des étapes initiales et des processus subséquents de la conversion maligne de la cellule épitheliale de la glande mammaire, sans affecter le développement normal de la glande mammaire. Nous démontrons que le PTHrP peut promouvoir l'oncogénèse et les phénomènes métastatiques en amont de plusieurs points de contrôle critiques chez le PyVMT, comme les Akt1, Akt2, facteur VIII, Bcl-2 et cycline D1, et le plus intéressant d'entre eux, le CXCR4. Ceci suggère un rôle nouveau pour le PTHrP comme facilitateur d'oncogènes, et renforce le concept de ciblage de l'activité de signal du PTHrP à des fins thérapeutiques.

# Preface

This Ph.D. thesis was written in accordance to the Guideline for submitting a Doctoral or Master's thesis, by the Faculty of Graduate studies and Research, McGill University. The thesis guidelines state:

"As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication ….2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges proceeding and following each manuscript are mandatory….3. In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. The candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent…."

Manuscripts presented in chapter 2-3 inclusive will be submitted for publication

# Acknowledgements

This work would not have been possible without the help of many people over the years. First, I would like to thank Dr. Richard Kremer for allowing me to be a part of his laboratory and for encouraging me in many aspects of my training. I am indebted to him for fostering the beginning of what I hope will be a long academic research career. His enthusiasm, dedicated help and advice, inspiration and support, was invaluable.

I am extremely grateful to Dr. Andrew Karaplis for sharing his wealth of knowledge and experience so freely, as well as guidance and crucial help when most needed.

I would like to gratefully acknowledge the enthusiastic supervision of Dr. Geoffrey Hendy, during my project, for providing valuable insights and encouragement.

I am truly grateful to Dr. Peter Siegel for his inspiration and advice.

I am grateful to Dr. Anne Camirand for her good constructive advice for editing the manuscripts. Our discussions and her great support have been invaluable.

I am also grateful to the current and former members of my committee, Dr. Giovanni DiBattista and Dr.Pnina Brodt for their patience and encouragement.

I would like thank Mr.Vasilios Papavasiliou for generously sharing his animal techniques.

I thank the people of the Calcium Research Laboratory for always being nice and cheerful, and for providing such a joyful atmosphere in the laboratory.

Last, but certainly not least, I would like to thank my wife, Ping Wang, for her support in every aspect of my life. Not only has she always lent an ear, but also she has challenged, encouraged, and helped me to be a better person.

# Table of contents

## ABBREVIATIONS USED IN THIS THESIS

AKT protein family, which members are also called protein kinases B (PKB)

BMPs bone morphogenic proteins

cAMP cyclic 3', 5'-adenosine monophosphate

CDK cyclin-dependent kinase

CSF-1 colony-stimulating factor-1

CTGF: Connective-tissue growth factor

CXCR4: CXC chemokine receptor 4

DAG diacylglycerol

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

ER estrogen receptor

ERK extracellular signal-regulated kinases

FGF fibroblast growth factor

FGFR Fibroblast growth factor receptor

GPCR G-protein coupled receptor.

HER2: Human epidermal growth factor receptor type 2

HHM humoral hypercalcemia of malignancy

IGFs insulin-like growth factors

IL-1β, IL-3, and IL-6 Interleukin-1, 3, 6

IP3 1,4, 5-inositol triphosphate

MAH Malignancy-associated hypercalcemia

MIP-1α :Macrophage Inflammatory Proteins-1α

MMP-1 Matrix metalloproteinase 1

MMP-9 Matrix metalloproteinase 9

loxP (locus of crossover (x) in P1

M-CSF macrophage-colony-stimulating factor

NLS nuclear localization sequence

PDGF p latelet derived growth factors

PDK Phosphoinositide dependent protein kinase

P13K Phosphoinositide 3-kinase

PKA protein kinase A

PKC protein kinase C

PLC β phospholipase C β

PTH Parathyroid hormone

PTHrP parathyroid hormone-related peptide

PTH1R Type 1 PTH/PTHrP Receptor

PyMT: polyomavirous middle T antigen

PTEN: Phosphatase and tensin homologue

RANK receptor activator of nuclear factor

RANKL: Ligand for the receptor activator of nuclear factor-κB

TGF α and β transforming growth factor α and β

TIP39 tuberoinfundibular peptide

TRAP tartrate-resistant acid phosphatase

VEGF, vascular endothelial growth factor;

VEGFR1, vascular endothelial growth factor receptor 1

# Chapter 1: Literature review: PTHrP and Cancer

## 1.1- PTHrP: background, discovery, gene sequence and protein structure.

### 1.1.1- Background:

Malignancy-associated hypercalcemia (MAH) is a well-recognized syndrome that occurs in patients suffering from certain malignant cancers. The classic signs and symptoms of hypercalcemia are confusion, polydipsia, polyuria, constipation, nausea, vomiting and eventually coma. Hypercalcemia associated with malignancy of non-parathyroid tissues frequently occurs during bone invasion by tumor cells, where bone resorption is the direct result of osteoclast action. Osteoclastic bone-degrading activity is stimulated by several cytokines including MIP-1α, TNF-β, IL-1β, IL-3, and IL-6, which are produced locally by the tumor cells (Shibahara, Nomura, Cui, & Noma, 2005; Sugihara, et al., 1998; Suzuki & Yamada, 1994; Vanderschueren, et al., 1994). MAH is commonly associated with malignant tumors of the breast, lung, head and neck, esophagus, cervix, skin or kidney (T. J. Martin & Suva, 1988).

In 1936, Gutman conducted the first large group study of MAH; these patients suffered from myeloma and breast cancer, and most of them presented bone metastases (Wysolmerski & Broadus, 1994). Fuller Albright in 1941 was the first to propose that a PTH-like humoral factor was responsible for the hypercalcemia in patients with renal carcinoma which resolved after irradiation of bone metastasis. In the following years, the concept was accepted and the term 'ectopic PTH syndrome' became widely used to

describe patients with high circulating plasma calcium concentrations, low phosphorus, and few or no bone metastases (Gellhorn & Plimpton, 1956; Grill, Rankin, & Martin, 1998; Svane, 1964). In 1969, a thorough review was made of cases where patients suffered symptoms of hyperparathyroidism yet exhibited malignant tumors of non-parathyroid origin. The conclusions drawn from this work were that some non-parathyroid tumor cells could initiate transcription and translation of the *PTH* gene and secrete PTH (Omenn, Roth, & Baker, 1969). Such cases of "ectopic PTH syndrome" (R. Buckle, 1974) included kidney adenocarcinoma (R. M. Buckle, McMillan, & Mallinson, 1970) and a malignant hepatoblastoma (Tashjian, 1969).

Rare cases were found where non-parathyroid tumours secreted PTH, and many studies demonstrated that the immunological properties of circulating of PTH-like material in hypercalcemic patients with non-parathyroid cancer or primary hyperparathyroidism were distinct from those of the immunoreactive PTH found in the serum of patients with primary hyperparathyroidism (Benson, Riggs, Pickard, & Arnaud, 1974; Riggs, Arnaud, Reynolds, & Smith, 1971). In 1980, Stewart and associates established the first full biochemical characterization for 50 consecutive patients with cancer-associated hypercalcemia, with or without bone metastases. This study delineated characteristic laboratory findings that now define the PTH-like syndrome, and coined the term humoral hypercalcemia of malignancy (HHM): elevated nephrogenous cyclic AMP excretion levels, high serum calcium, low serum phosphorous, marked reduction in 1,25-dihydroxyvitamin D and low or suppressed reactivity with PTH antisera.

### 1.1.2- Discovery

Based on the biochemical characterization of humoral hypercalcemia of malignancy, much effort was devoted to the problem of identifying and isolating the tumor-secreted unknown factor that was responsible for this syndrome. In 1987, three independent groups simultaneously achieved: (1) purification of an active component with a molecular weight of 18 kD from a human lung cancer cell line (BEN) (Moseley, et al., 1987), (2) purification of a 6 kD active component from cultured human renal carcinoma cells (Strewler, et al., 1987), and (3) purification of a 17 kD active component from a human breast tumor biopsy (Burtis, et al., 1987a). Most interesting was the fact that the N-terminal amino acid sequence of these adenylate cyclase-stimulating proteins revealed outstanding homology to PTH, identifying the existence of a PTH-like factor in those cancer cells.

Using the partial amino acid sequence information from these discoveries, oligonucleotide probes were synthesized and used to identify complementary DNAs (cDNAs) encoding BEN cell mRNA (Suva, et al., 1987), mRNA from a human renal carcinoma (Mangin, Ikeda, Dreyer, Milstone, & Broadus, 1988), and mRNA from a renal carcinoma cell line (Thiede, Strewler, Nissenson, Rosenblatt, & Rodan, 1988). Characterization of those clones revealed a gene and peptide sequence similar to that of human PTH. Eight of the initial 13 residues of the mature protein and the final 2 residues in the signal peptide were identical to those of human PTH. The term parathyroid hormone-related protein (PTHrP) derives from this high homology to the N-terminal sequence of PTH.

The elucidation of the PTHrP sequence allowed investigators to synthesize fragments of the peptide and to study their effects in different experimental conditions.

Kemp, at al 1987 demonstrated that the N-terminal fragment of PTHrP (1-34) exhibited PTH-like activity in rat and chicken kidney and produced effects commonly seen in HHM such as increased excretion of cAMP and phosphorus, and reduced urinary calcium excretion (Kemp, et al., 1987). By using radioligand binding techniques, Nickols and co-workers were able to show that amino-terminal fragments of PTH and PTHrP interacted with identical receptors present on rabbit kidney microvessels and tubules (Nickols, Nickols, & Helwig, 1990). Immunoassays were developed and indicated that PTHrP was circulating at high levels in patient with HHM (Burtis, et al., 1990; Kao, Klee, Taylor, & Heath, 1990). These findings provided strong evidence that circulating PTHrP produced by various cancers was the causal agent responsible for hypercalcemia.

### 1.1.3- Gene sequence and protein structure

The *Pthrp* gene is localized to the short arm of chromosome 12 in humans (Mangin, Webb, et al., 1988) whereas the *PTH* gene is on the short arm of chromosome 11 (Mannens, et al., 1987). The short arms of chromosome 11 and chromosome 12 carry a number of *PTH*-related genes which are thought to have originated from a single ancestral chromosome through an ancient gene duplication, translocation or fusion (Comings, 1972; Ingleton, 2002). The localization of *PTH* and *Pthrp* on these chromosomes and the similarity in sequence and organization of the two genes provide indirect evidence for a common evolutionary origin (Mangin, Webb, et al., 1988; Yasuda, Banville, Hendy, & Goltzman, 1989). PTHrP sequences from various mammals, birds (Philbrick, et al., 1996) and sparus sea bream (Flanagan, et al., 2000) are illustrated in figure 1.

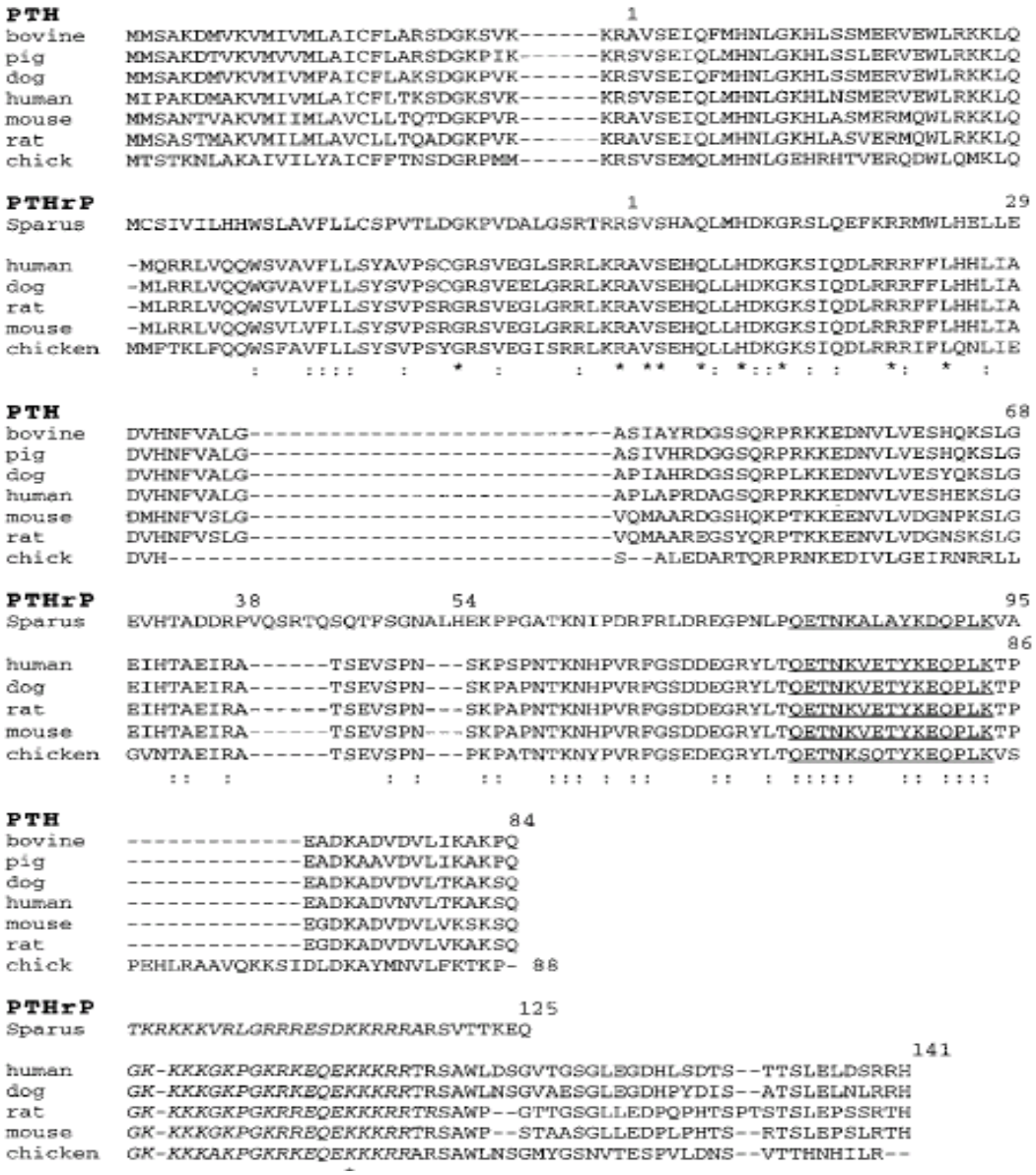



**Figure 1** Multiple sequence alignment of the known PTH and PTHrP peptide molecules. Amino acid identity between all PTH and PTHrP peptides is marked by an asterisk and identity between only PTHrP peptide amino acids is marked by a colon. 1 Marks the start site of the mature PTHrP peptides. The nuclear transporter sequence of mammalian PTHrP, amino acids 70–84, corresponds to *Sparus* PTHrP 79–93 and is underlined. The RNA-binding sequence of mammalian PTHrP, amino acids 87–106, corresponds to residues 96–116 of *Sparus* PTHrP, all of which are shown in italics. Human PTHrP 173 is not included in

the comparisons (Adapted from Flanagan, et al., 2000 General and Comparative Endocrinology **118,** 373–382).

The human *Pthrp* gene (Figure 2) is a complex transcriptional unit which spans more than 15 kb of DNA, with 9 exons and at least 3 promoters. Alternative splicing of the pre-messenger RNAs produces 3 PTHrP isoforms differing at their carboxyl-terminal ends and containing 139, 141, or 173 amino acids. Exon 4 encodes a region common to all peptides, while exons 5 and 6 encode the unique carboxy termini of the other 2 peptides. *PTH* and *Pthrp* genes display an identical pattern of intron/exon organisation in the region of exon 2 and exon 3 (Mangin, Ikeda, Dreyer, & Broadus, 1989, 1990; Mangin, Webb, et al., 1988; Suva, et al., 1987; Yasuda, et al., 1989). In contrast to the human organization, rat and mice *Pthrp* genes are relatively simple with a single promoter homologous to the downstream P3 promoter of the human gene. They produce mature peptides of 141 and 139 amino acids respectively (Karaplis, Yasuda, Hendy, Goltzman, & Banville, 1990; Mangin, Ikeda, & Broadus, 1990). The *Pthrp* gene in the chicken yields a single mature peptide of 139 amino acid (Thiede & Rutledge, 1990).

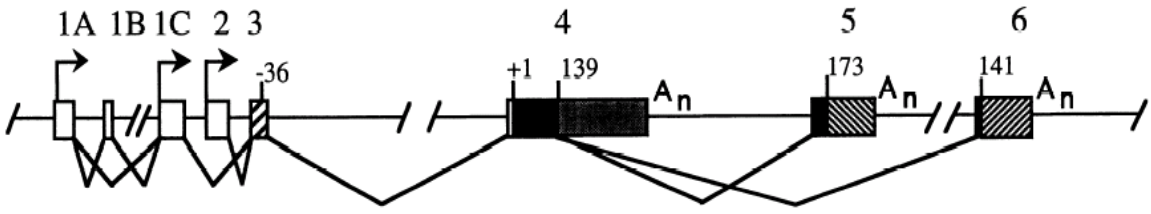



**Figure 2** Structure of human parathyroid hormone-related protein (PTHrP) gene. (Adapted from Philbrick at al 1996 Physiological reviews Vol 76 127-173)

The initial translational human PTHrP products undergo complex processing, including separation of the (-36 to -1) pre-pro sequence and endoproteolysis of the full-length (-36 to 139), (-36 to 141) and (-36 to 173) sequence at multibasic sites. All sequences present an N-terminal signal sequence for endoplasmic reticulum entry and a coding 1-139 peptide (Orloff, et al., 1994; Philbrick, et al., 1996). The amino acid 35-111 region is dramatically conserved in human, rat, mouse and chicken sequences, with the human and rodent amino acid sequences differing in the 1-111 region by only two residues. This high evolutionary conservation suggests important physiological and biological functions. The 35-111 region is rich in putative proteolytic processing sites, with multiple dibasic amino acid groups (Orloff, et al., 1994; Philbrick, et al., 1996).

In 1992 Soifer and co-workers discovered that the N-terminal PTHrP (1-36) peptide resulted from cleavage at the monobasic residue $Arg^{37}$. The purification of a mid-region peptide (38-108) using anti-PTHrP (37-74) fragment affinity columns and reversed phase high pressure liquid chromatography (RP-HPLC) confirmed that $Arg^{37}$ is a monobasic-processing site in the PTHrP precursor (Soifer, et al., 1992). Further evidence of this mid-region fragment beginning at $Ala^{38}$ and comprising the overlapping PTHrP (38-94), (38-95) and (38-101) fragments was confirmed by mass spectrometry analysis (Wu, et al., 1996). Analysis of these three fragments led to the identification of a C-terminal sequence of between 80-106 amino acids which may contain sites for endoproteolysis (Wu, et al., 1996) and a substrate site for prohormone convertases, and indicates that a peptide beginning at position 107 likely exists. Region 107-111 (-Thr-Arg-Ser-Ala-Trp-) or TRSAW is evolutionally highly-conserved, suggesting that a peptide encompassing this region may be physiologically important (Fenton, Kemp,

Hammonds, et al., 1991). Endoproteolytic processing at this site produces the PTHrP (109-138) fragment which has been detected in the circulation of patients with chronic renal failure and HHM (Burtis, et al., 1990; Orloff, Soifer, Fodero, Dann, & Burtis, 1993). In addition, work by Fenton and co-workers has shown that PTHrP (107-139) can inhibit bone resorption and reduce osteoclast activity (Fenton, Kemp, Kent, et al., 1991). Several groups have shown that PTHrP 107-139 may play a role in bone metabolism by influencing osteoblast activity as well (Alonso, et al., 2008; de Gortazar, Alonso, Alvarez-Arroyo, & Esbrit, 2006; De Miguel, et al., 1999; Esbrit, et al., 2000; Seitz, Zhang, Simmons, & Cooper, 1995).

## 1.2 PTHrP physiology

### 1.2.1 PTHrP functional domains

Analysis of the *Pthrp* gene sequence reveals several functional domains (Figure 3), including a signal peptide, a PTH-like N-terminal domain, a mid-region domain that begins at amino acid 38, and a unique carboxy-terminal domain named osteostatin (Cornish, Callon, Nicholson, & Reid, 1997; Fenton, Kemp, Hammonds, et al., 1991).

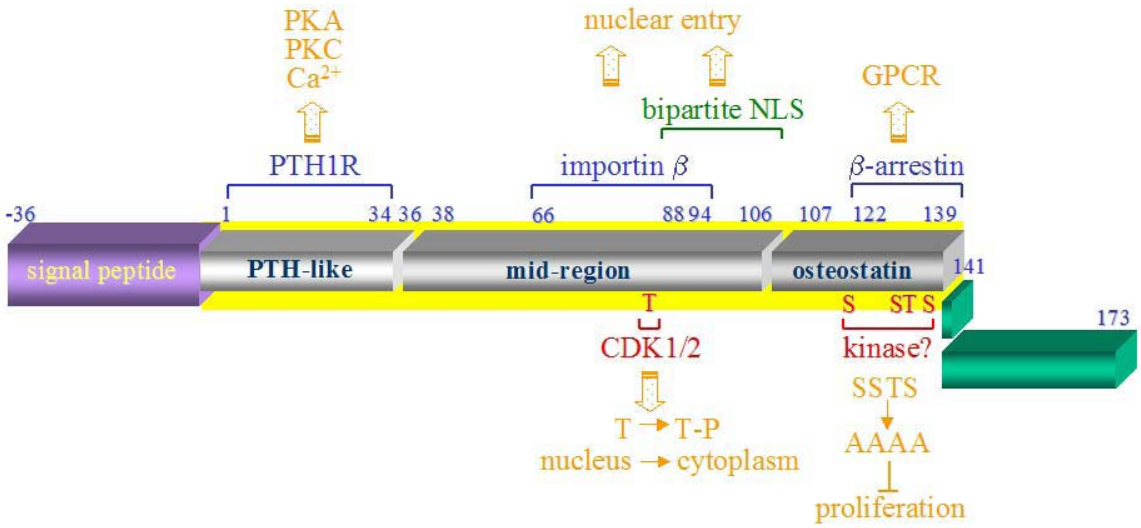



Figure 3. The functional domains of the human PTHrP protein and its interaction partners.

T-P denotes phosphorylation of Thr85. SSTS stands for residues Ser119, Ser130, Thr132 and Ser138 and AAAA denotes their replacements by alanines. CDK = cyclin-dependent kinase, GPCR = G-protein coupled receptor. Green bars show extension of the PTHrP protein in splicing variants –36/141 and –36/173. (Adapted from Dittmer J. *Gene Therapy and Molecular Biology Vol 8, page 451*)

The N-terminal domain of PTHrP (1-36) shares with PTH a homologous sequence that interacts with the PTH/PTHrP type 1 receptor (PTH1R), a class II G-protein coupled receptor (GPCR). Upon binding PTHrP, the PTH1R receptor can either activates the formation of cyclic 3', 5'-adenosine monophosphate (cAMP) by stimulating adenylate cyclase (AC) through Gαs, or activates phospholipase Cβ (PLCβ) through Gαq/11. cAMP then activates protein kinase A (PKA), whereas activated PLCβ stimulates the formation of diacylglycerol (DAG) and 1, 4, 5-inositol triphosphate (IP3). In turn, DAG activates protein kinase C (PKC), and the production of IP3 leading to an increase in the intracellular free $Ca^{++}$ (Blind, Raue, Knappe, Schroth, & Ziegler, 1993; Iida-Klein, et al., 1997). The PTH1R receptor can also stimulate the influx of extra-cellular $Ca^{++}$ through

regulation of calcium channels (Swarthout, D'Alonzo, Selvamurugan, & Partridge, 2002), and activate protein kinase C through a PLC-independent pathway (Takasu, Guo, & Bringhurst, 1999; Whitfield, et al., 2001; Yang, Guo, Divieti, & Bringhurst, 2006). The PTH1R receptor classical transduction pathways can lead to different biological effects in a cell type-specific manner.

The mid-region PTHrP comprises a bipartite nuclear localization sequence (NLS) consisting of residues 88-91 and 102-106. This NLS sequence may allow PTHrP to accumulate in the nucleus (J. E. Henderson, et al., 1995; Massfelder, et al., 1997; Nguyen, He, & Karaplis, 2001) and binds rRNA (Aarts, et al., 2001; Aarts, et al., 1999). This peptide likely enters the nucleus immediately after NLS binding to importin-β1 to form a complex that links to the microtubular trackway, where it is pulled by a dynien motor to a nuclear envelope pore and handed over to the pore channel transport machinery (Cingolani, Bednenko, Gillespie, & Gerace, 2002; Jans, Thomas, & Gillespie, 2003; Lam, Briggs, et al., 1999; Lam, et al., 2002). Although the NLS exact function is still obscure, it is likely of great significance since mice missing the NLS and the C-terminal region of the PTHrP gene present decreased cellular proliferative capacity and increased apoptosis in multiple tissues, retarded growth, early senescence, and malnutrition leading to an early death (Miao, et al., 2008). In addition, *in vitro* studies indicate that the midregion sequence retains a CDK1/CDK2 phosphorylation site at $Thr^{85}$ (Lam, House, et al., 1999), which suggests that translocation of PTHrP is associated with activation of the cell cycle (Lam, et al., 1997; Massfelder, et al., 1997).

A different receptor recognizing the midregion domain may exist as well, since a number of studies have shown its binding to several non-classical PTH targets such as rat

insulinoma cells (Gaich, et al., 1993), squamous carcinoma cells and normal human epidermal keratinocytes (Orloff, et al., 1992). Amino-terminal PTHrP binding increases intracellular calcium without stimulating cAMP altered signal transduction could arise from activation of a receptor qualitatively different from the classical PTH1R receptor. For example, PTHrP (67-86) can stimulate placental calcium transport stimulation in fetal sheep (Care, et al., 1990) and mice (Kovacs, et al., 1996), which suggests that receptors for this sequence are expressed by placental tissue.

The PTHrP C-terminal domain was named osteostatin because this peptide inhibits rat osteoclastic bone resorption with an incredibly low $EC_{50}$ value of $10^{-15}$M (Fenton, Kemp, Hammonds, et al., 1991; Fenton, Kemp, Kent, et al., 1991; Fenton, Martin, & Nicholson, 1994). An *in vivo* study conducted in adult male mice injected with PTHrP 107-139 for five consecutive days revealed a significant reduction in bone resorption (Martinez, et al., 1997) accompanied by a 70% decrease in osteoclast numbers and by a 40% decrease in osteoblast numbers (Cornish, et al., 1997). The inhibitory effect of the C-terminal peptide may operate through its affinity receptor (TRSAW receptor), since rat osteoclasts do not express the PTH1R receptor and, as expected, PTH1R activators such as PTH (1-34) do not affect rat osteoclast activity (Cuthbertson, Kemp, & Barden, 1999). On the other hand, the inhibitory effect of osteostatin may result from interactions between osteostatin and β-arrestin (Conlan, Martin, & Gillespie, 2002). A C-terminal-specific receptor has however not yet been identified.

### 1.2.2- PTHrP receptors

PTHrP binds to, and activates the G-protein coupled receptor (GPCR) for PTH/PTHrP (PTH1R) which is expressed in PTH and PTHrP target cells such as osteoblasts in bone and renal tubular cells. PTH1R in humans and rodents is encoded by a multi-exon gene with potential for alternate splicing and alternate promoter usage (Kong, et al., 1994). The *PTH1R* gene is located on chromosome 3 in humans and possesses 14 exons.

PTH1R has 7 transmembrane spanning domains (Fig 4) (Datta & Abou-Samra, 2009) and has been cloned from opossum kidney, rat bone, and human bone and kidney (Abou-Samra, et al., 1992; Juppner, et al., 1991; Schipani, et al., 1993). GPCRs are among the most important classes of signaling proteins, and allow cells to sense their environment and respond to endogenous hormones and exogenous agents. The GPCR superfamily can be divided into six primary classes (A, B, B, C, D, E and F), although the most common division is into three main families A, B, and C. The families are readily distinguished by comparing their amino acid sequences, wherein family B is characterized by a large extracellular loop and family C has a large, bilobed extracellular Venus-flytrap-like domain. A second major difference between the families concerns the location of the orthosteric binding site and the nature of the orthosteric ligand. All receptors in each class have several common characteristics, e.g., seven-transmembrane domains (7TM), an extracellular N-terminus, an intracellular carboxy terminus, and the ability to functionally couple to heterotrimeric G proteins. Class II GPCRs are structurally characterized by a relatively long N-terminus (~120–140 amino acids) containing a set of six cysteine (Cys) residues connected by three disulphide bonds,

forming a globular domain. These cysteines are completely conserved across class II receptors (B. Martin, et al., 2005). The amino terminal region of PTH (1–34) and PTHrP (1–34) interacts with the J-domain, the functional portion of the receptor that contains the seven transmembrane-spanning helices and the connecting loops (Gardella & Juppner, 2000).

Following receptor–agonist interaction, PTH1R is activated and mediates not only the endocrine actions of PTH, but also the autocrine/paracrine actions of PTHrP. Some of the PTHrP properties were revealed through the generation of transgenic animals expressing PTHrP under the control of various tissue-specific promoters. For example, PTHrP targeted expression showed its important role in the development of skin, pancreas, and breast and in normal tooth eruption (Juppner, 1999). PTH1R knock-out mice present decreased trabecular bone formation and increased cortical bone thickness during fetal development. A crucial discovery was the understanding that Blomstrand chondrodysplasia (an autosomal recessive human disorder characterized by early lethality, accelerated endochondral and intramembranous ossification) is a disorder caused by inactivating *PTH1R* mutations (Jobert, et al., 1998; Karaplis, et al., 1998). In contrast, histomorphometric analysis of bone from a patient with Jansen's metaphyseal chondrodysplasia which is caused by an activating mutation of PTH1R shows loss of cortical bone without any loss of trabecular bone (Schipani, et al., 1999; Schipani, et al., 1996).

A second G protein coupled receptor named PTH receptor 2 (PTH2R) was subsequently identified and is abundant in the brain and pancreas; it binds the N-terminal end of PTH with much higher affinity than it does the N-terminal domain of PTHrP (Usdin, Bonner, Harta, & Mezey, 1996; Usdin, Gruber, & Bonner, 1995). The

hypothalamic tuberoinfundibular peptide (TIP39) was later identified as a high-affinity ligand for PTH2R (Usdin, Hoare, Wang, Mezey, & Kowalak, 1999). A third receptor, PTH3R was identified in zebrafish (Rubin, et al., 1999) and sea bream enterocytes (Rotllant, et al., 2006), and exhibits higher affinity for hPTHrP and fuguPTHrp than for hPTH. PTH3R is not found in mammals and may represent an important phylogenetic step in the development of the PTH/PTHrP system during species evolution (Datta & Abou-Samra, 2009).

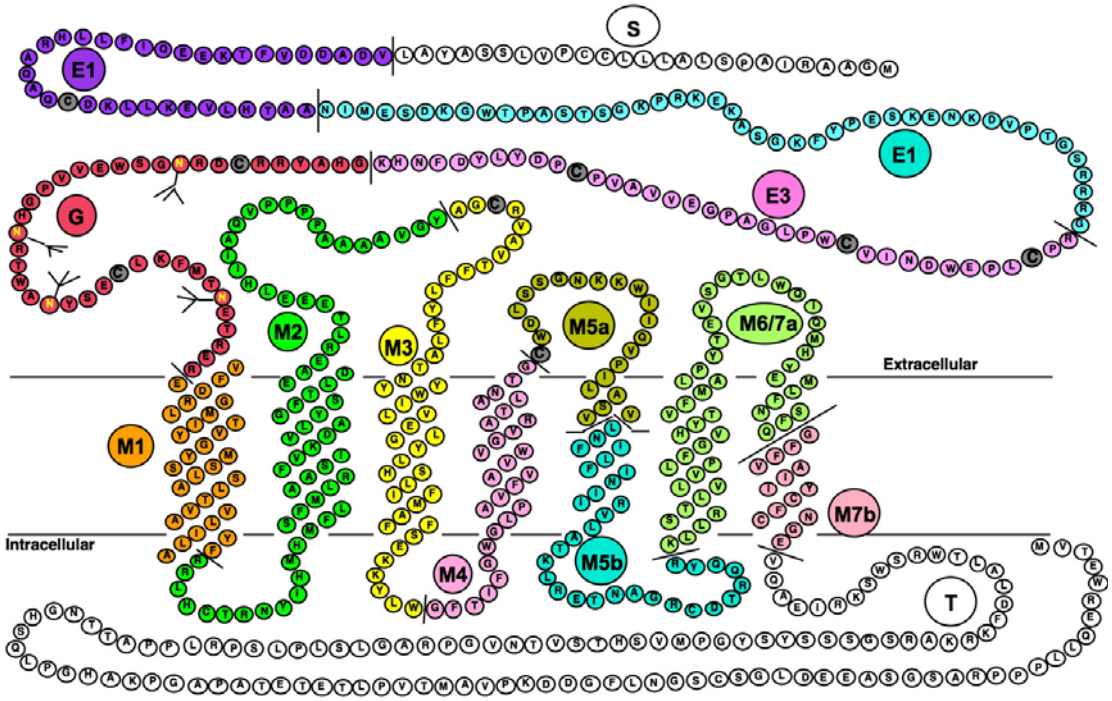



Fig 4

Primary sequence of the rat PTH/PTHrP receptor. Different colors represent sequences encoded by different exons and predicted extracellular, trans-membrane spanning and cytoplasmic domains are shown. Exon nomenclature is as follow: S for exon encoding signal peptide, E1, E2 and E3 for exons encoding the extracellular extension, M1, M2, M3, M4, M5a, M5b, M6/7a and M7b for exons encoding the trans-membrane domains and portions of their connecting loops and T for the exon encoding the cytoplasmic tail. The J domain refers to the trans-membrane domains and the connecting loops.

(Adapted from Datta & Abou-Samra, 2009 Cellular Signalling 1245-54)

### 1.2.3- Normal physiological functions of PTHrP

PTHrP is widely expressed in fetal and adult tissues, including cartilage, bone, breast, skin, skeletal heart and smooth muscle, uterus and placenta, as well as endocrine organs and the central nervous system (Orloff, et al., 1994; Philbrick, et al., 1996; Strewler, 2000). PTHrP is a key regulator for cellular calcium transport and smooth muscle cell contractility, and possesses crucial roles in cell proliferation, development and differentiation (Philbrick, et al., 1996). It is important to note that the known biological properties of PTHrP are not only the results of its interaction with PTH1R and its subsequent signal transduction cascades, but also of PTHrP nuclear translocation (Clemens, et al., 2001; Gensure, Gardella, & Juppner, 2005). PTHrP is known to stimulate or inhibit apoptosis in various settings in a cell- or tissue-specific manner (Amling, et al., 1997; Gagiannis, et al., 2009; Hastings, et al., 2003). These actions are mostly performed in an autocrine/paracrine and intracrine fashion.

The biological actions of PTHrP are particularly important for bone development during endochondral bone formation. Targeted ablation of *Pthrp* results in homozygous$^{-/-}$ mice dying shortly after birth and presenting abnormalities in endochondral bone development (Karaplis, et al., 1994). In contrast, heterozygous *Pthrp*$^{+/-}$ animals are viable but demonstrate a reduction in trabecular bone volume and an early osteoporotic phenotype (Amizuka, et al., 1996). The generation of *PTH1R* knockout mice (Lanske, et al., 1996) shows great similarities of phenotype with *Pthrp*-null mice, in support of PTHrP and PTH1R as important regulators of normal and pathological bone remodelling. Conversely, over expression of PTHrP in chondrocytes leads to a delay in endochondral ossification and bone formation, and mice are born with a completely cartilaginous

endochondral skeleton (Weir, et al., 1996). Mutations resulting in a constitutively active *PTH1R* in humans cause a rare autosomal dominant disorder, Jansen metaphyseal chondrodysplasia, characterized by widespread growth plate abnormalities including delayed mineralization and distorted columns of chondrocytes (Schipani, Kruse, & Juppner, 1995; Schipani, et al., 1996). Targeted over expression of PTH1R in mouse chondrocytes recreated several aspects of the human chondrodylplasia condition (Schipani, et al., 1997). In addition, genetic mouse studies indicate PTHrP regulates hair follicle development, mammary epithelial development, and tooth eruption (Foley, et al., 2001; Philbrick, Dreyer, Nakchbandi, & Karaplis, 1998; Wysolmerski, et al., 1994; Wysolmerski, McCaughern-Carucci, Daifotis, Broadus, & Philbrick, 1995; Wysolmerski, et al., 1998). PTHrP regulation of mammary gland development is discussed in section 1.2.4.

### 1.2.4- PTHrP in mammary gland development

The mammary gland consists of a series of branching tubes that form through a process known as branching morphogenesis. This process of branching growth is similar to the one occurring in other epithelial organs such as lung and salivary glands, and requires communication between epithelial cells and mesenchymal cells. Mammogenesis in the mouse starts around embryonic day 10.5 (E10.5) with the formation of two mammary lines. In the course of one day, the mammary anlagen or placodes (Fig. 5) transform into bulbs of epithelial cells morphologically distinct from the surrounding epidermis. At E12– E13.5, these epithelial buds are visible as elevated knob-like structures either macroscopically or by scanning electron microscopy. During the

following day, the buds sink into the underlying dermis, and by E14.5 they can no longer be detected externally. While further mammary development is temporarily arrested in females, androgen receptor activation in male embryos causes degeneration of the buds between E13.5 and E15.5. In females, further development is resumed at around E15.5 when each bud elongates to form a sprout or cord, invading the underlying fat pad precursor. Each sprout forms a lumen which opens on the surface of the skin and where the nipple forms concurrently by epidermal invagination. At about E16, the first ramifications of the sprouts occur, and by E18.5 the sprouts have developed into small, arborized glands (Veltmaat, Mailleux, Thiery, & Bellusci, 2003).

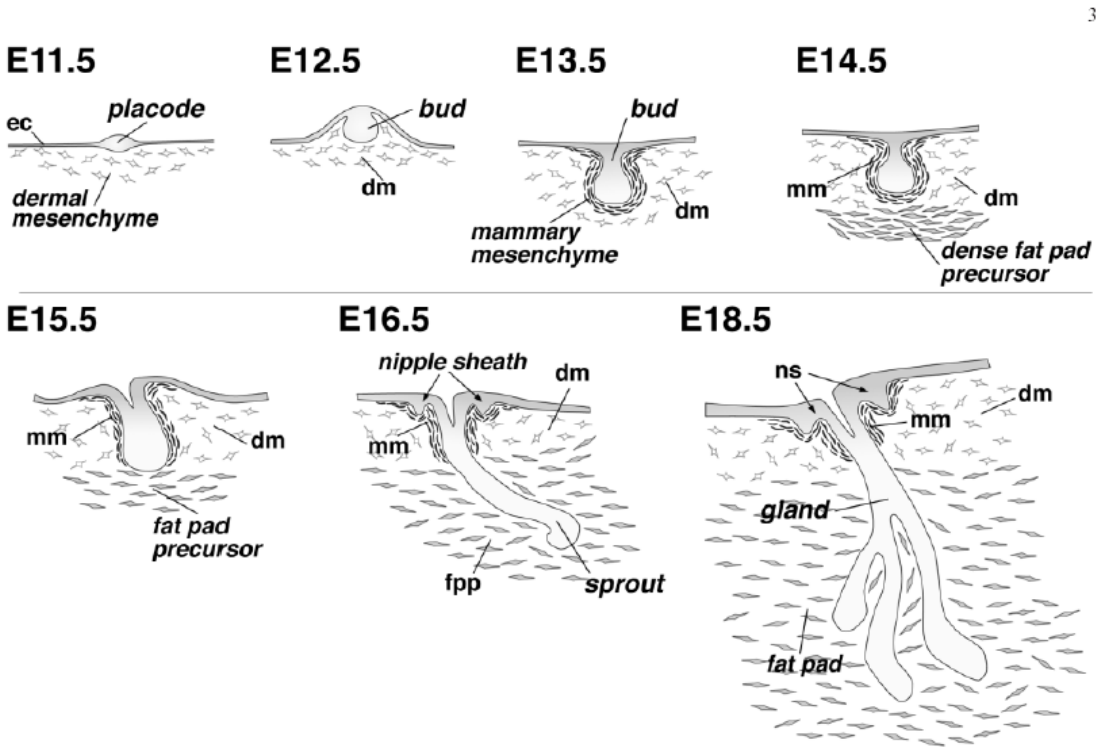



**Fig. 5** Schematic overview of mammogenesis in the mouse embryo (adapted from Veltmaat et al Differentiation 71(1) 1-17 2003)

During post-natal breast development in normal mice, PTHrP appears to be expressed by both luminal and myoepithelial cells of the mammary gland (S. L. Ferrari, Rizzoli, & Bonjour, 1992; Seitz, et al., 1993), while myoepithelial cells and mammary stromal cells express the PTH/PTHrP receptor. Both stromal and myoepithelial cells are important in the branching growth of the mammary gland during sexual maturation and early pregnancy, and PTHrP ligand and receptor are appropriately positioned to participate in this process (S. Ferrari, Rizzoli, Chaponnier, Gabbiani, & Bonjour, 1993; S. L. Ferrari, et al., 1992; Wysolmerski & Stewart, 1998).

*Pthrp*-null mice die soon after birth of severe musculo-skeletal defects (Karaplis, et al., 1994), but *Pthrp* rescue in the chondrocytes of these animals leads to a phenotype compatible with life. This strategy generates a mouse PTHrP-sufficient in chondrocytes, but PTHrP-null in all other sites including breast. These mice are characterized by the absence of normal epithelial-mesenchymal signaling cascade, failure to form mammary mesenchyme, and the resorption of nascent mammary bud (Fig6) (Foley, et al., 2001; Wysolmerski, et al., 1998).

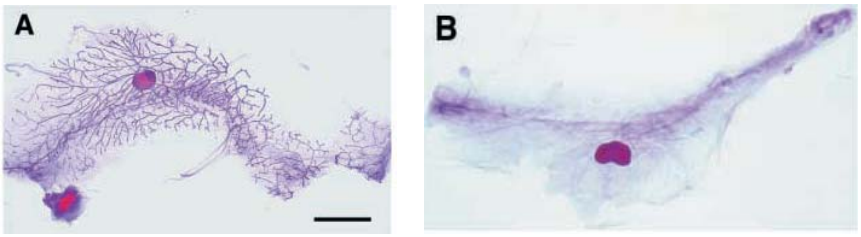



**Fig 6** PTHrP is essential for branching morphogenesis:

The normal gland (A) is characterized by a fully-branched epithelial duct system surrounding the central lymph node. In contrast, the col II-PTHrP/PTHrP-null gland (B) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. (Adopted from Foley, et al., 2001; Wysolmerski, et al., 1998)

## 1.3 PTHrP and cancer biology

### 1.3.1 Breast Cancer

Female breast cancer is a major medical problem with significant public health and societal ramifications. Although breast cancer death rates have declined in recent years, breast cancer remains the most commonly diagnosed cancer and the second leading cause of cancer death in women (Jemal, et al., 2009). According to the Canadian Cancer Society (CCS) 2008 statistics, there were 22,400 new cases/year and 5,300 deaths/year from breast cancer in Canada. The incidence and the mortality of breast cancer were 102.5 cases and 22.3 deaths per 100,000 women respectively. http://www.cancer.ca/Canada-wide/About%20cancer/Cancer%20statistics.aspx?sc_lang=en.

GLOBOCAN 2002 estimated that in 2002, approximately 1,151,298 women were diagnosed with breast cancer, and that an estimated 410,712 died as the result of the disease worldwide http://www-dep.iarc.fr/. Since normal breast growth is regulated by many hormones, growth factors and receptors, it is not surprising that malignant cells derived from breast tissue also express the same hormones, growth factors and receptors. Numerous genes are controlled by complex regulatory networks and involved in the development and progression of breast cancer, and these genes are the key factors determining the characteristics of each tumor.

### 1.3.2 PTHrP and cancer development

Cancer is a class of diseases in which a group of cells displays unrestrained growth and sometimes spreads to other locations in the body via lymph or blood. Cancer is a major public health problem in the world; currently, one in four deaths in the United States is due to cancer (Jemal, et al., 2009). PTHrP was originally discovered in patients with HHM (T. J. Martin & Suva, 1988); approximately 80% of hypercalcemic patients with solid tumours have elevated plasma concentrations of PTHrP as a result of increased secretion by the tumours (Schilling, et al., 1993). However, PTHrP expression has been detected in many tumour types even in the absence of hypercalcemia. For instance, the great majority breast cancer tumor samples show positive staining for PTHrP, and a strong *Pthrp* gene activity in breast tumors is associated with the onset of bone metastases independently of hypercalcemia (Bouizar, Spyratos, & De vernejoul, 1999; Bouizar, Spyratos, Deytieux, de Vernejoul, & Jullienne, 1993; Bucht, et al., 1998; Bundred, et al., 1991; Kohno, Kitazawa, Sakoda, et al., 1994).

Normal prostatic epithelial tissues express low levels of PTHrP, as determined by immunohistochemistry and in situ hybridization. In contrast, overexpression of PTHrP is common in prostate cancer (Iwamura, et al., 1993; Tovar Sepulveda & Falzon, 2002), and many prostate cancer cell lines *in vitro* and metastatic bone lesions *in vivo* express PTHrP. PTHrP stimulates primary prostate tumor growth and protects cells from apoptotic stimuli (Dougherty, et al., 1999). PTHrP expression was found in all major lung cancer cell types (Brandt, Burton, Gazdar, Oie, & Deftos, 1991; Kitazawa, et al., 1991) and was most common in squamous cell lung cancer (Nishigaki, Ohsaki, Toyoshima, & Kikuchi, 1999). While benign colorectal adenomas and non-neoplastic adjacent mucosal epithelia show no detectable PTHrP expression, about 95% of colorectal adenocarcinomas overexpress PTHrP mRNA and protein, and the expression level is higher in poorly-differentiated than in well-differentiated adenocarcinomas (Nishihara, et al., 1999; Nishihara, Kanematsu, Taguchi, & Razzaque, 2007). In a clinical study of 76 patients with various haematological malignancies, 50% of the 14 hypercalcaemic patients had significant elevation in plasma PTHrP concentrations (Kremer, Shustik, Tabak, Papavasiliou, & Goltzman, 1996). Similar observations were made in stomach cancer where PTHrP is expressed in 77% of gastric adenocarcinomas without humoral hypercalcaemia. In contrast, only 5% of adenomas and none of the non-neoplastic epithelium showed PTHrP expression. Similarly, PTHrP expression was more common in moderately-differentiated adenocarcinomas (95.5%) and poorly differentiated adenocarcinomas (100%) than in well-differentiated adenocarcinomas (43 %). Furthermore, PTHrP expression was more intense in the deeply invasive portions than in the mucosal carcinomas (Alipov, et al., 1997).

It is well established that PTHrP is the major causative agent in MAH associated with a broad range of tumours. However, this is only one aspect of the multiple facets of PTHrP in cancer biology. Indeed, the complex growth factor-like properties of PTHrP have shed new light onto potential roles for this peptide in the regulation of tumour growth and invasion. In support of this, PTHrP expression has been shown to be under the control of numerous growth and angiogenic factors such as transforming growth factor (TGF-β,) epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (T. J. Martin, Moseley, & Williams, 1997; Stewart, 2002). Conversely, PTHrP stimulates the expression of many of these factors in various cell types and behaves as an angiogenic factor in endothelial cells (Akino, et al., 2000; Esbrit, et al., 2000; T. J. Martin, et al., 1997). More recently, PTHrP was shown to promote cell invasion *in vitro* (Luparello, Burtis, Raue, Birch, & Gallagher, 1995; Luparello, Sirchia, & Pupello, 2003). These observations clearly suggest a multifunctional role for PTHrP in cancer biology. Its functions include (1) regulation of tumour cells growth, differentiation, and progression; (2) induction of progression of osteolytic bone metastasis, (3) moderation of tumour cell survival factors and interference with apoptosis signalling pathway.

### 1.3.3 PTHrP in breast cancer development

Breast cancer is frequently accompanied by PTHrP-induced hypercalcemia in advanced stages of the disease (Burtis, et al., 1990), and 50–60% of primary human breast cancer tumours overexpress PTHrP (Bundred, et al., 1991; Bundred, et al., 1992; Southby, et al., 1990). Several retrospective studies suggested that breast cancer patients

with PTHrP-positive primary tumours were more likely to develop bone metastases compared to breast cancer patients with PTHrP-negative tumours (Bundred, et al., 1991; Bundred, et al., 1992; Kohno, Kitazawa, Fukase, et al., 1994; Southby, et al., 1990). PTHrP is expressed in 68% of surgically-excised early breast cancers, compared with 100% of bone metastases (Linforth, et al., 2002), and 50% of cases of early breast cancer expressed the PTHrP receptor in contrast to 81% of cases of bone metastases. PTHrP expression without concomitant expression of its PTH1R receptor in primary tumors correlated with a reduced disease-free survival with a mortality rate of 6%, while co-expression of both predicted the worst clinical outcome at 5 years with a mortality rate of 32%, (Linforth, et al., 2002). A similar observation was noted regarding the differential expression of PTHrP isoforms in different stages of breast cancer. The levels of the 1-139 isoform mRNA was much higher in the tumors of patients who later developed metastases than in those of patients who developed no metastases. This mRNA isoform was also more abundant in breast tumors from patients who developed bone metastases than in those of patients who developed metastases in soft tissues. In contrast, the amounts of the 1-141 isoform mRNA in these groups of tumors were similar (Bouizar, et al., 1999).

A prospective study conducted by Dr Kremer's team in patients with malignancy-associated hypercalcemia (including breast cancer patients) indicated that elevated circulating levels of PTHrP is an indicator of poor prognosis and is associated with reduced survival (Fig 7) (Truong, de, Papavasiliou, Goltzman, & Kremer, 2003). Intriguingly however, a retrospective clinical study of breast tumours collected at surgery suggests a better outcome and survival in patients whose primary tumour overexpress PTHrP (M. A. Henderson, et al., 2006; T. J. Martin & Moseley, 2000).

To shed light on this controversy, Sato and co-workers treated animals presenting symptoms similar to those of HHM patients with a humanized anti-PTHrP antibody. The animals showed significant improvement in hypercalcemia and cachexia after antibody treatment (Sato, Onuma, Yocum, & Ogata, 2003). Furthermore, administration of this antibody in nude mice injected intra-cardiac with the human breast cancer cell line MDA-MB-231 reduced the ability of these cells to form bone metastases (Saito, et al., 2005). Animal studies using mice transplanted with human tumors expressing PTHrP suggest that the humanized anti-PTHrP antibody could be an effective and beneficial agent for patients with HHM. *In vitro*, overexpression of PTHrP in the human breast cancer cell line MCF-7 caused an increase in mitogenesis, whereas the inhibition of endogenous PTHrP production resulted in decreased cell proliferation (Falzon & Du, 2000). Enhanced bone tumor growth was also observed when the MCF-7 cells were transfected to overexpress PTHrP. Taken together, these results point to the pro-tumorigenic effects of PTHrP (Nishihara, et al., 2007).

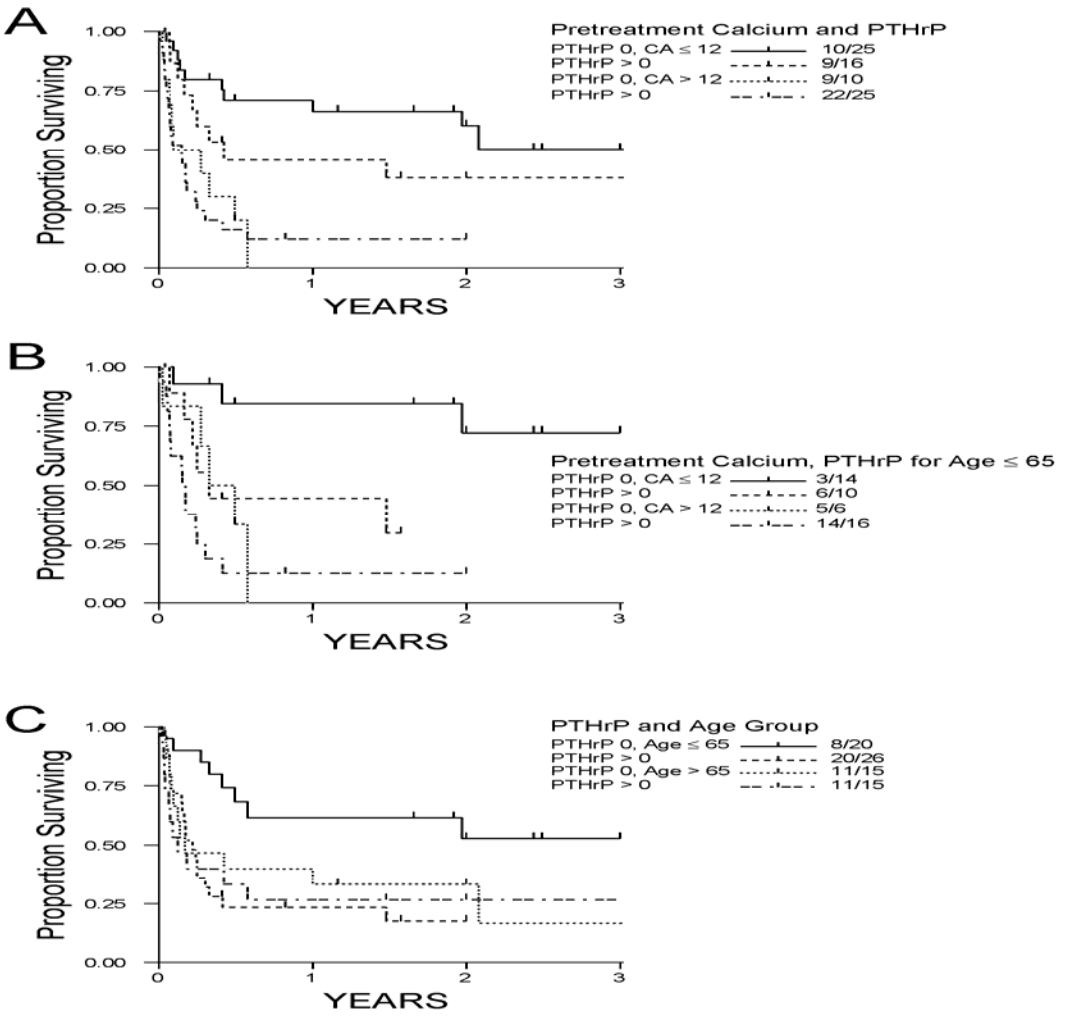



Fig 7: Serum levels of PTHrP and Survival of Patients with MAH

A: Survival in 76 hypercalcemic cancer patients by parathyroid hormone–related peptide (PTHrP) status and pretreatment calcium levels. Numbers shown in the inset are total number of deaths/number of patients at baseline. Number of patients at risk were 40 at 100 days, 22 at 1 year, and 3 at 3 years. B: Survival in 46 hypercalcemic cancer patients ≤65 years old by PTHrP status and pretreatment calcium levels. Number of patients at risk were 26 at 100 days, 13 at 1 year, and 1 at 3 years. C: Survival in hypercalcemic cancer patients by PTHrP status and age group. Number of patients at risk were 41 at 100 days, 22 at 1 year, and 3 at 3 years. CA ≤12 = pretreatment serum calcium levels 10.3 to 12 mg/dL; CA >12 = pretreatment serum calcium levels >12 mg/dL; PTHrP 0 = PTHrP not elevated; PTHrP >0 = PTHrP elevated. (Adopted from, 2003 THE AMERICAN JOURNAL OF MEDICINE Vol 115 119)

1.3.4- Role of PTHrP in breast cancer metastasis to bone

Bone is a unique microenvironment consisting of two physically and biologically distinctive structures. The outer structure is primarily composed of a hard mineralized matrix and the normal mineralization of this bone matrix is contingent upon adequate amounts of vitamin D, calcium, and phosphate (Hadjidakis & Androulakis, 2006). The matrix contains large amounts of growth factors such as type I collagen, insulin-like growth factors (IGFs), transforming growth factor α and β (TGF α and β), fibroblast growth factors (FGF-1 and -2), platelet derived growth factors (PDGFs) and bone morphogenic proteins (BMPs) (Hauschka, Mavrakos, Iafrati, Doleman, & Klagsbrun, 1986).

The bone remodeling cycle involves a series of highly-regulated steps which depend on the interactions of two cell types, the mesenchymal osteoblastic lineage and the hematopoietic osteoclastic lineage. Osteoblasts synthesize some of the growth factors stored in the matrix during bone formation. Most growth factors are released in active form into the marrow when bone is degraded during osteoclastic bone resorption. The resorption areas provide a fertile microenvironment for tumor cell colonization and proliferation (Lipton, 2004; Yoneda, 2000).

The inner structure of bone consists of bone marrow which is multicellular and contains hematopoietic stem cells and stromal cells. Hematopoietic stem cells differentiate into any type of blood and immune cells including macrophages, lymphocytes and osteoclasts. Stromal cells support the differentiation of the

hematopoietic stem cells but importantly, have the ability to differentiate into osteoblasts (Akhtari, Mansuri, Newman, Guise, & Seth, 2008). In addition, physical factors within the bone microenvironment, including low oxygen levels, acidic pH, and high extracellular calcium concentrations may also enhance tumor growth (Kingsley, Fournier, Chirgwin, & Guise, 2007). Furthermore, when the tumor cells stimulate osteoclastic bone resorption, the bone microenvironment becomes more enriched in bone-derived growth factors that enhance survival of the cancer and disrupt normal bone remodeling, resulting in bone destruction.

Metastasis is the spread and growth of tumour cells to distant organs, and represents the most devastating attribute of cancer. The common sites for metastatic spread of breast cancer are bone, lung and liver (fig 8) (Weigelt, Peterse, & van 't Veer, 2005). However, certain cancers will form metastases in specific organs or tissues at higher frequencies than predicted by blood flow patterns alone. For instance breast, prostate, lung cancer and multiple myeloma frequently metastasize to bone. Bone metastases are often broadly classified as osteolytic (proceeding through bone destruction) (Fig 9), or osteoblastic (proceeding through aberrant bone formation) (Fig 10). Osteolytic bone lesions are typical of breast and lung carcinoma as well as hematological malignancies such as multiple myeloma. On the other hand, tumors in bone may stimulate new bone formation resulting in osteoblastic bone metastasis, a situation most often associated with prostate cancer although also encountered in breast cancer. The osteolytic-osteoblastic distinction is not absolute, as many patients with bone metastases have both types of lesions and individual metastatic lesions can contain both osteolytic and osteoblastic

components. A dysregulation of the normal bone remodelling process is encountered in both types of lesions (Guise & Mundy, 1998b).

Breast cancer has the potential to spread to almost any region of the body. The most common metastatic regions are bone, where approximately 25 % of breast tumours will first spread, and lung, where breast cancer metastasizes in 21 % of cases. http://www.cancer.gov/cancertopics/advancedcancer. A survey of 587 patients in terminal stages of breast cancer revealed that 69% had radiological evidence of bone metastases (Coleman & Rubens, 1985). According to the Stanley Paget "seed and soil" hypothesis, tumour cells (seed) invading bone provide growth factors that activate the bone microenvironment (soil), which in turn produces growth factors that feed the tumour cells, creating a vicious cycle of destructive mutual cooperation (Mundy, 2002).

PTHrP has been shown to play a key role in the osteoclastic bone resorption resulting from breast cancer metastasis to bone because it activates local bone turnover and consequently participates actively in the vicious cycle described above (Akhtari, et al., 2008; Guise, et al., 1996; Kakonen & Mundy, 2003). It has to be noted that PTHrP expression by breast cancer at metastatic sites differs dramatically from expression at primary sites. In fact, only 50 percent of primary breast cancers express PTHrP, whereas 92 percent of metastases of breast cancer to bone produce the peptide (Roodman, 2004). The increased local PTHrP stimulates RANKL expression and inhibits OPG secretion by osteoblasts. RANKL binds to its receptor RANK (expressed on osteoclasts) and enhances the differentiation and fusion of active osteoclasts in the presence of the macrophage-colony-stimulating factor. Bone-derived TGF-β, IGFs and FGFs released as a consequence of osteoclastic bone resorption stimulate tumor production of PTHrP via different receptors present in the cancer cells (PKC, MEK, P38 MAPK and Akt

signaling pathway) (Alokail, 2005; Chirgwin & Guise, 2000; Mundy, 2002). Tumour cells might contribute to the vicious cycle by producing growth factors and cytokines which in turn sustain tumor growth (Peyruchaud, 2007). It is however unclear whether the predilection of these cancers for spreading to bone results from the induction of PTHrP in the bone microenvironment, or whether tumors that produce PTHrP are more likely to metastasize to bone (Roodman, 2004). In contrast to its well-characterized role in bone metastasis development, the role of PTHrP in tumour progression outside the skeleton remains controversial (Nishihara, et al., 2007).

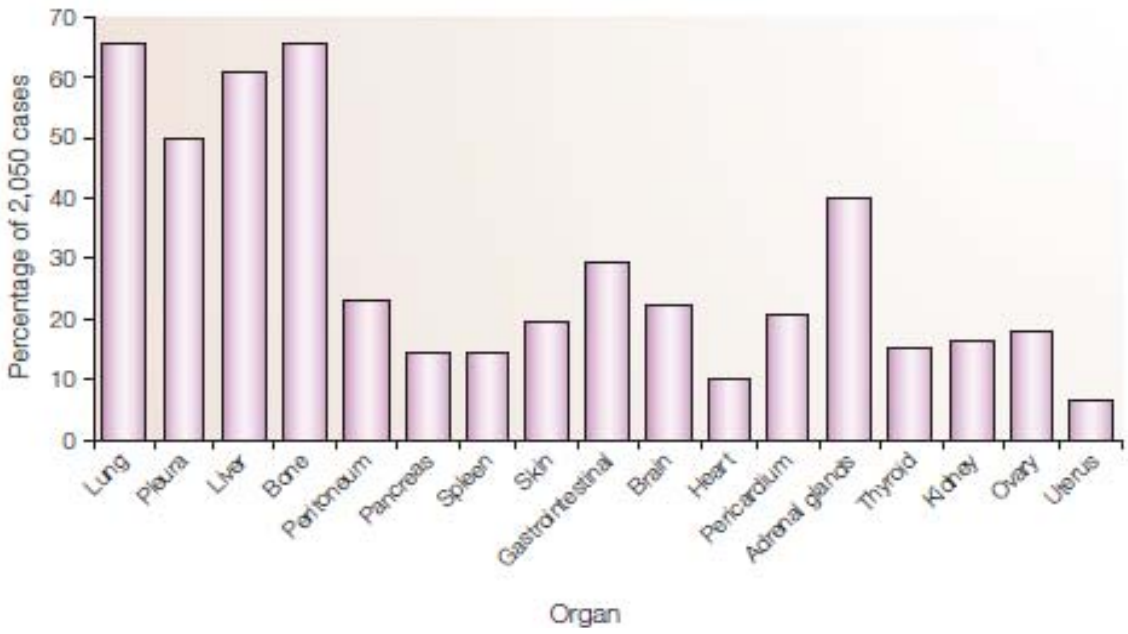



**Fig 8 Most common metastasis sites of breast cancer at autopsy**

(Adapted from Britta Weigelt et al Nature Reviews Cancer 2005)

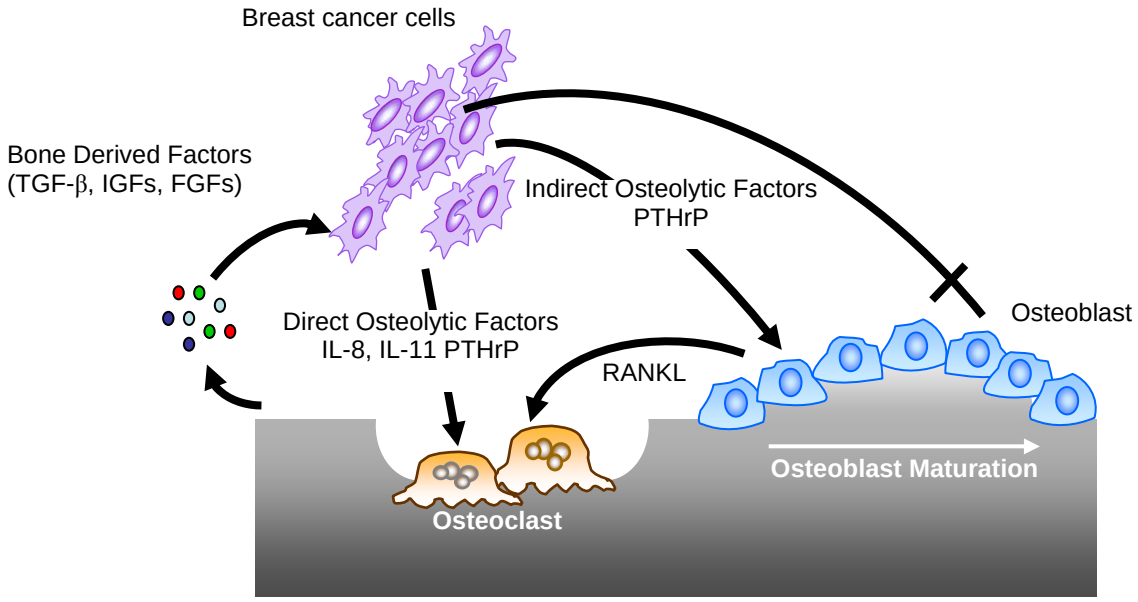



Fig 9 Interactions Driving Osteolytic Metastases
Jiarong Li and Peter Siegel 2007 CIHR Bone training program

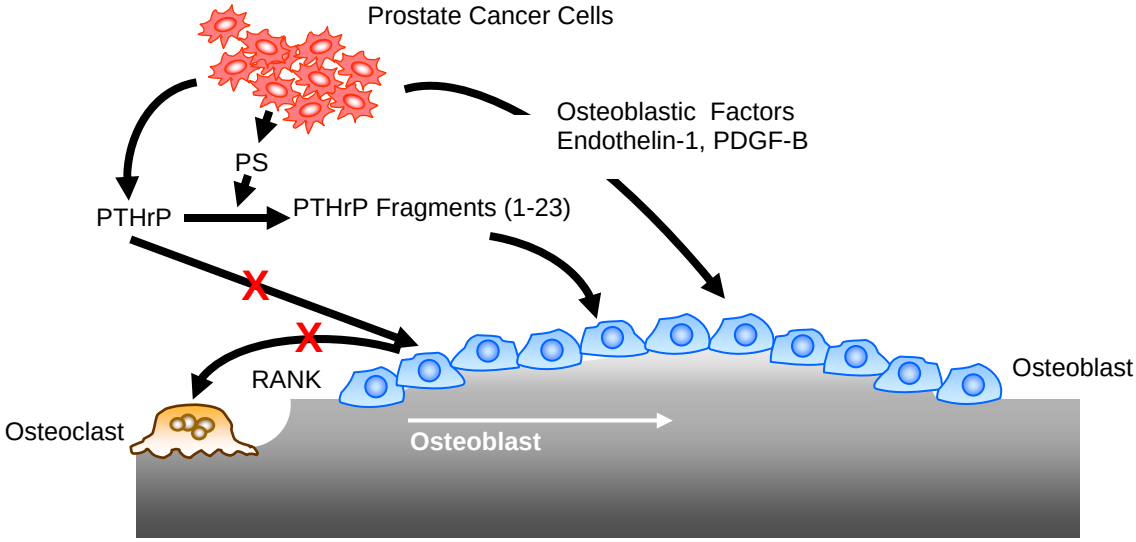



Fig 10 Interactions Driving Osteoblastic Metastases
Jiarong Li and Peter Siegel 2007 CIHR Bone training program

1.4- Animal models used in this study

1.4.1- MMTV-PyMT breast cancer mouse model

In the MMTV-PyMT transgenic mouse model (Guy, Cardiff, & Muller, 1992a), expression of the oncoprotein polyoma middle T antigen (PyMT) is under the control of the mouse mammary tumour virus long terminal repeat (LTR) (Fig 11) and its expression is restricted to the mammary epithelium and absent from myoepithelial and surrounding stromal cells.

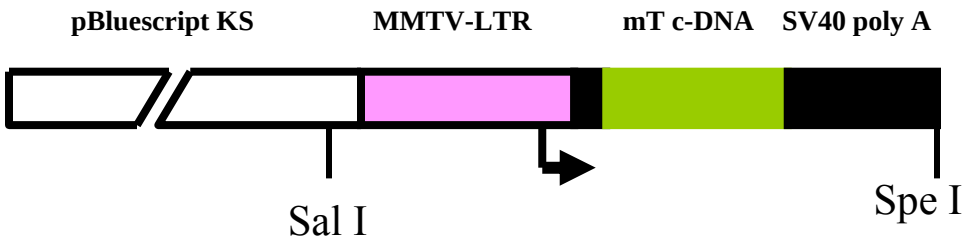



**Fig 11 : Structure of transgene:** The unfilled region represents the sequences within the Bluescript vector backbone, the pink portion contains MMTV LTR, the green region corresponds the cDNA encoding PyV middle T antigen. (Adapted from Guy, Cardiff, & Muller, 1992 Mol Cell Biol *12*, 954-961)

PyMT, a membrane-attached protein, is encoded by the small DNA polyoma virus. PyMT is not normally expressed in human breast tumour cells, but when overexpressed in the mammary epithelium of transgenic mice, it acts as a potent oncogene because its

product (MT oncoprotein) binds various receptors and activates several signal transduction pathways, including those of the Src family kinase, ras and the PI3K pathways, which are frequently activated in human breast cancers (Dilworth, 2002). As shown in figure 12, Src phosphorylates Y315, which in turn activates the p85/p110/PI3K complex, which then activates PDK1 and 2 and results in AKT phosphorylation and activation (Dilworth, 2002). Activation of the PI3K/AKT is thought to mediate cell survival. AKT has three isoforms — AKT1, AKT2 and AKT3 — which are encoded by the genes PKBα, PKBβ and PKBγ, respectively(Scheid & Woodgett, 2001; Vivanco & Sawyers, 2002). The three isoforms share a similar structure: an amino-terminal pleckstrin homology domain, a central serine–threonine catalytic domain, and a small carboxy-terminal regulatory domain. Amplification of AKT1 and AKT2 has been reported in various tumour types(Liu, Cheng, Roberts, & Zhao, 2009). Recent studies in human breast, colorectal and ovarian cancers showed that an activating mutation in the pleckstrin homology domain of AKT1 (E17K) results in growth factor-independent membrane translocation of AKT and increased AKT phosphorylation levels (Carpten, et al., 2007). Mouse mammary tumour virus (MMTV) promoter-driven expression of a constitutively active mutant of PKBα/Akt1, in which the two activatory phosphorylation sites (Thr308/Ser473) were mutated to aspartic acid residues (Akt-DD), did not induce cellular transformation or increase tumour incidence in mammary epithelial cells(Hutchinson, Jin, Cardiff, Woodgett, & Muller, 2001). However, activation AKT1 alone can interfere with the apoptotic process of mammary gland involution and promote tumor progression by providing an important cell survival signal (Hutchinson, et al., 2001). In addition, PyV MT expression leads to SHC/GRB2/Ras/ERK activation. Integration of these multiple signalling pathways ultimately induces cell transformation

and invasion. Previous studies have shown that PTHrP can activate both ras (Aklilu, Park, Goltzman, & Rabbani, 1997) and AKT (Agouni, et al., 2007; Shen & Falzon, 2006) signalling pathways.

Furthermore, the expression of *myc* (an oncogene located in one of the three amplified chromosomal regions found in human primary breast cancer) is increased in PyMT mammary glands (Lin, et al., 2003). In contrast, expression of "metastasis genes" in this model has not been well characterized.

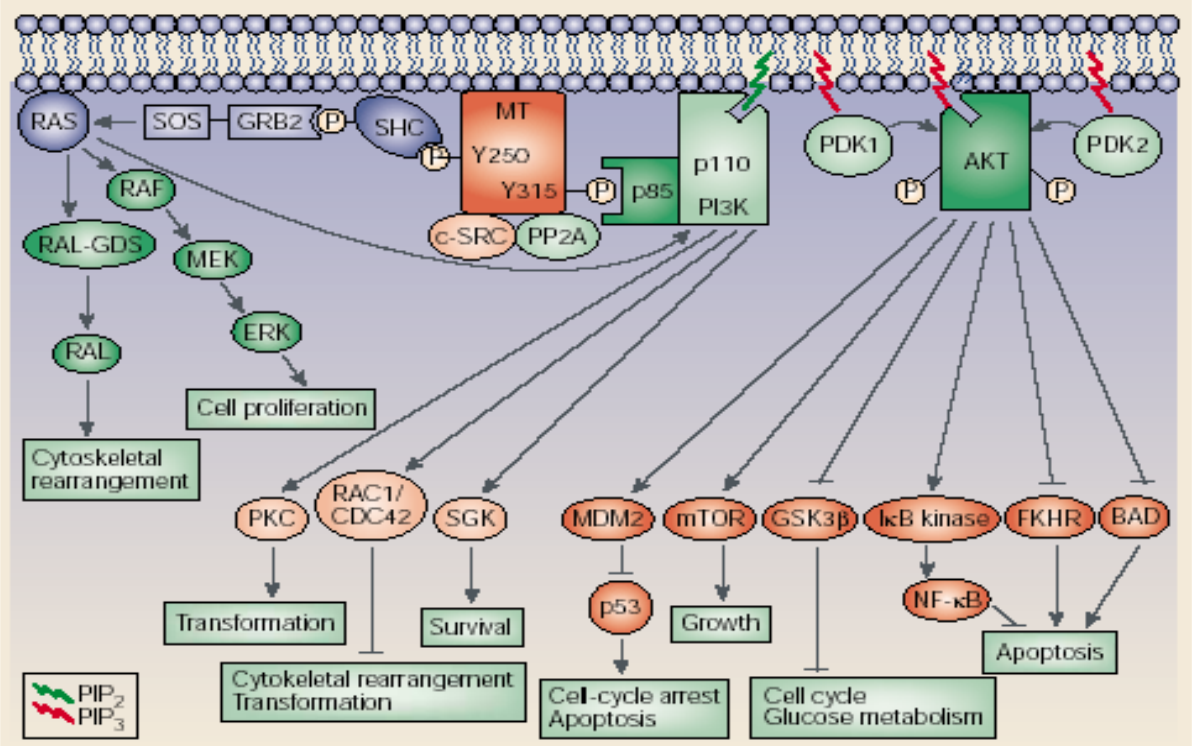



**Fig 12 :** PyMT-stimulated signaling pathways (Dilworth, 2002). There are three major signaling pathways in this model:1.ras/Erk(on the left). Src/PIK3(in the middle) and 3.PDK/Akt (on the right).

Nature Reviews Cancer ***2***, 951-956

1.4 .2- Tumor progression from hyperplasia to metastastic carcinoma

Mammary hyperplasia can be detected in this animal model as early as 4 weeks of age. The hyperplasia then progresses to adenoma in 6 weeks, to early carcinoma in 9 weeks and to late carcinoma 12 weeks, with pulmonary metastasis present in 100% of animals (Figure 13).

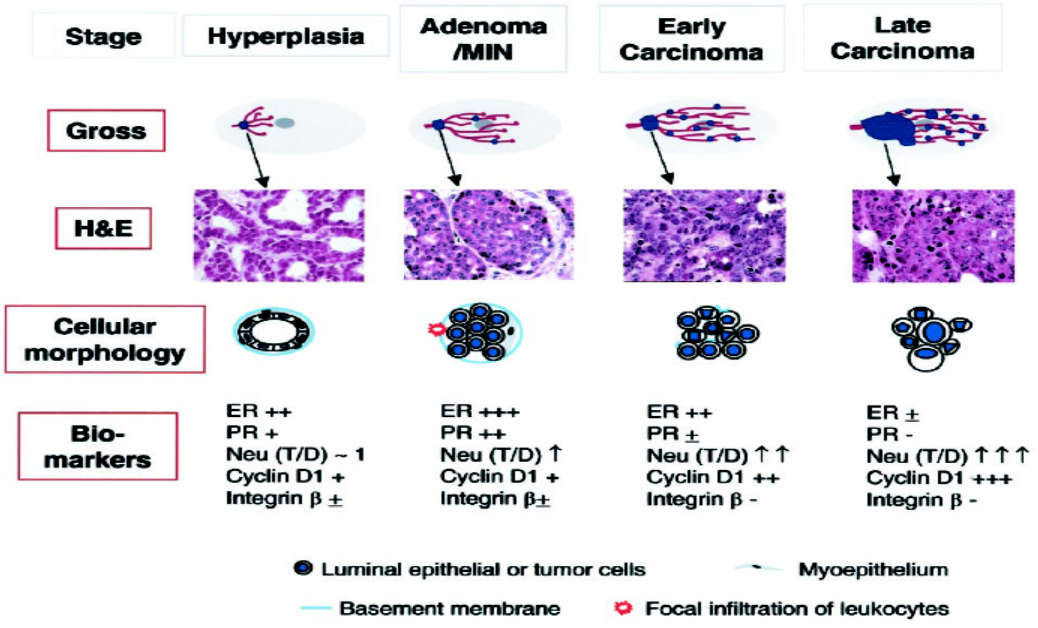
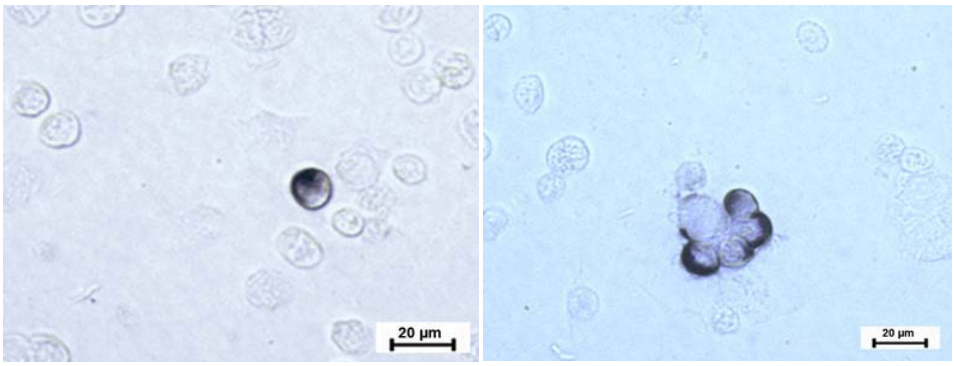



**Figure 13 .** Summary of tumour progression and biomarker expression in PyMT mouse model of breast cancer (Lin, et al., 2003 *Am J Pathol, 163*(5), 2113-2126.)

ER: Estrogen receptor; PR: Progesterone receptor; HER-2/neu: Epidermal growth factor receptor 2

The MMTV-PyMT mouse model of breast cancer is characterized by a high incidence of lung metastasis with highly reproducible progression kinetics. Increased metastatic potential has been shown to depend on the presence of macrophages in primary tumours and on the establishment of a chemoattractant paracrine loop of colony-stimulating factor-1 (CSF-1) and EGF ligands between macrophages and tumour cells (Fantozzi & Christofori, 2006). Although PyMT transgenic mice do not develop bone metastasis *per se*, metastatic cells are found in the bone marrow relatively early and continue to grow in later stages without evidence of bone metastasis (Husemann, et al., 2008) (Fig 14).




Bone marrow at 6 weeks Bone marrow at 11 weeks

Fig 14 Cytokeratin-positive tumour cells in bone marrow at early stages of development in the mouse MMTV-PyVMT model (Husemann, et al., 2008 *Cancer Cell, 13*(1), 58-68)

### 1.4.3- The Cre-loxP system

Cre-loxP is a widely used system for mammalian genome modification through the expression of the Cre recombinase enzyme which causes recombination at specific loxP sites. This system can be utilized to knock genes out in a time- and tissue-specific fashion. The prokaryotic enzyme Cre recombinase is a 38-kDa cyclization recombination enzyme, naturally expressed in the bacteriophage P1 (Sternberg & Hamilton, 1981). This enzyme belongs to the integrase family of recombinases and catalyzes a specific recombination event between 34bp long DNA sequences called loxP (locus of crossover (x) in P1). The recombinase forms a transient DNA-protein covalent linkage to bring the two loxP sites together and mediate a site-specific recombination (Kuhn & Torres, 2002). The loxP sites consist of two 13bp long inverted repeats that are connected by an 8bp non-palindromic spacer sequence which determines the orientation of the site and the outcome of the recombination event (Abremski et al. 1983). If two loxP sites on a linear DNA molecule are oriented in the same direction, the DNA sequence between the two sites will be excised by intramolecular recombination. One of the two loxP sites will remain in the DNA backbone, and the excised sequence together with the second loxP site will form a circular molecule. If the loxP sites are oriented in opposite directions, the "floxed" DNA sequence in between the sites will be simply inverted (Nagy & Mar, 2001; Rossant & Nagy, 1995).

When transgenic mice stably expressing the gene for Cre recombinase under the control of tissue-specific promoters are mated to heterozygous mice carrying the "floxed" allele for the gene of interest, the mating produces a conditional knockout of this gene in the specific tissue. This approach is commonly used when the conventional gene targeting leads to embryonic lethality. Resulting animals that are heterozygous for the loxP-flanked sequence and carry the Cre transgene must now be intercrossed. Among the resulting offspring will be mice homozygous for the loxP-flanked sequence and possessing the Cre transgene. In those animals, the Cre-mediated recombination at the loxP sites will result in the specific removal of the loxP-flanked gene fragment only in those cells where Cre is present. In other words, the tissue-specific promoter drives the expression of Cre and the subsequent recombination, resulting in tissue-specific knockout mice (Kimmel & Faix, 2006; Korets-Smith, et al., 2004; Van Duyne, 2001; Wagner, et al., 2001b; Wagner, et al., 1997) Fig 15

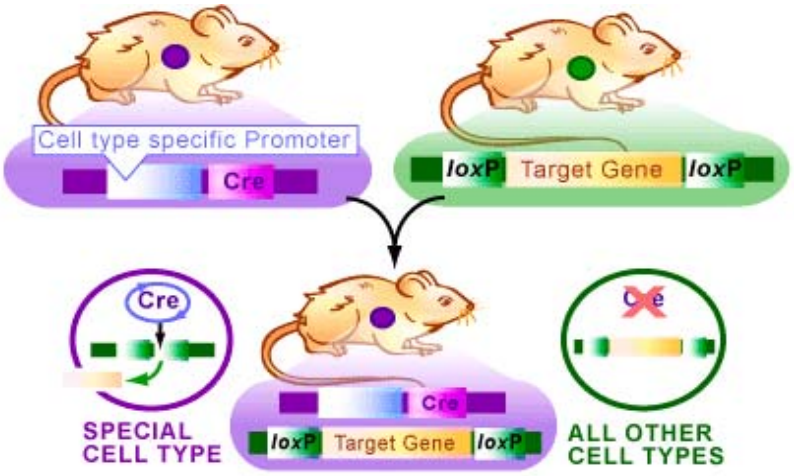



**FIG 15 Cre/*lox* Mouse Breeding.** Mice with the Cre protein expressing in a specific cell type are bred with mice that contain a target gene surrounded by *loxP* sites. When the

mice are bred, the cells carrying Cre will cause those cells to lose the target gene (Alfred Pechisker: The Science Creative Quarterly, Issue Four, 2009)

**Rationale and specific aims of this study**

Mammary gland development begins early in embryonic life, and is under PTHrP control at this stage. A PTHrP deficiency results in severe changes in the adult mammary tissue. It therefore appears that PTHrP has a number of regulatory functions in the mammary gland, at several distinct stages of its development and function. However, the effects of PTHrP disruption in the post-natal period have not yet been explored. We use a model in which PTHrP is specifically excised in mammary epithelial cells using the cre-loxPsystem. In this model, it is important to note that Cre is expressed shortly after birth (around 10 days)(Wagner, et al., 2001b) but not during embryonic development (Andrechek, et al., 2000), making the system suitable for the study of PTHrP roles in mammary development during puberty as well as in tumour initiation arising from the normal mammary epithelium.

PTHrP is expressed in normal epithelial cells but its expression increases in breast cancer and becomes associated with multiple metastatic lesions and reduced survival. It is however still unknown whether PTHrP overexpression is simply a consequence of tumour progression, or whether it is mechanistically linked to the tumour progression process from initiation to metastasis. In order to shed light on this relationship, we

propose to ablate the *Pthrp* gene in mammary epithelial cells and to determine the consequences of this ablation on tumour initiation, growth and metastasis.

# Chapter 2

## Parathyroid hormone–related protein (*Pthrp*) gene knock-out in mammary epithelial cells inhibits tumor initiation and progression *in vivo*.

Parathyroid hormone–related protein (*Pthrp*) gene knock-out in mammary epithelial cells inhibits tumor initiation and progression *in vivo*.

**Authors**: Jiarong Li, Andrew C. Karaplis*, Peter Siegel**, William J. Muller**, Richard Kremer

**Affiliations**: Department of Medicine, McGill University Health Centre, 687 Pine Ave West, Montreal, QC, Canada, H3A1A1, *Lady Davis Institute, Jewish General Hospital, 3755 Côte Ste Catherine, Montréal, QC, Canada, H3T 1E2, and ** McGill Cancer Centre, McGill University, Montreal, QC Canada, H3A 1A1.

**Contact information**: correspondence: richard.kremer@mcgill.ca , phone: (514) 843-1632, fax: (514) 843-1712

**Additional title page footnotes**:

**Running title**: *Pthrp* knockout inhibits breast cancer initiation

**Summary**: An animal model was generated to investigate various implications of PTHrP expression in breast cancer initiation and development. A Cre/loxP system that disrupts the *Pthrp* gene in the mammary epithelium was introduced into the MMTV/PyVMT transgenic mouse model of breast cancer. Differences were visible between $Cre^{+}$ mice (excised *Pthrp*) and controls starting at early stages of hyperplasia, with PTHrP-ablated animals presenting a delay in tumor onset, followed by significant impediment in appearance of palpable tumors and in tumor growth. Mammary epithelium-targeted *Pthrp* disruption was accompanied by a decrease in total AKT1 and an increase in AKT2 protein levels, enhanced programmed cell death, and a diminished proliferative capacity of breast tumor cells *in vitro* and *in vivo* without interference with normal mammary gland development.

**Significance**: Parathyroid hormone-related protein (PTHrP) is a secreted protein possessing autocrine, paracrine and intracrine roles in a wide range of physiological processes. Dysregulated overexpression of PTHrP is associated with oncologic pathologies, and elevated circulating levels of PTHrP cause malignancy-associated hypercalcemia, correlating with the more advanced stages of cancer. Here, we demonstrate that reduction or elimination of PTHrP expression in the mammary epithelium of PyVMT mice significantly delays breast cancer initiation, and disrupts tumor progression by reducing Akt1 expression levels. PTHrP therefore plays a crucial role in transformation of normal cells by the PyVMT oncogene, and potentially by other oncogenes dignaling through AKT. The observation that PTHrP strongly influences the

initial stages of mammary tumorigenesis illustrates the crucial importance of ablating PTHrP signaling in preventing breast cancer development.

**Highlights**:

-Mammary epithelium-targeted *Pthrp* ablation does not affect mammary gland development.

-*Pthrp* gene ablation significantly delays breast tumorigenesis initiation and progression.

-AKT1 kinase total protein levels are reduced and AKT2 increased by *Pthrp* ablation.

-*Pthrp* ablation causes cell cycle inhibition, pro-apoptotic and anti-angiogenic effects.

**Introduction**:

Parathyroid hormone-related protein (PTHrP) is a secreted protein expressed in almost all normal fetal and adult tissues. The name derives from the fact that the N-terminal 13 amino acids of PTHrP are highly homologous with those of parathyroid hormone (PTH), a characteristic which allows PTHrP to act through the type 1 parathyroid hormone receptor (PTH1-R)(Gardella & Juppner, 2001; Gensure, et al., 2005; Jin, et al., 2000). The rest of the PTHrP amino acid sequence is unique, however, and confers to PTHrP many properties resulting from signal transduction cascades and nuclear translocation that are distinct from properties of PTH (Clemens, et al., 2001; Gensure, et al., 2005; Maioli & Fortino, 2004; Miao, et al., 2008). PTHrP acts as an autocrine, paracrine or intracrine factor in a wide range of developmental and physiological processes, it has growth-promoting and anti-apoptotic properties (Maioli & Fortino, 2004) and plays a crucial role in the development of the mammary gland (Foley, et al., 2001; Wysolmerski, et al., 1998). Of special interest is the association of PTHrP with oncologic pathologies; PTHrP involvement has been observed in breast cancers (Bouizar, et al., 1999; Bouizar, et

al., 1993; Bucht, et al., 1998; Kohno, Kitazawa, Fukase, et al., 1994; Shen & Falzon, 2006), and in lung (Brandt, et al., 1991; Kitazawa, et al., 1991; Nishigaki, et al., 1999), prostate (Dougherty, et al., 1999; Iwamura, et al., 1993)((Bhatia, Mula, Weigel, & Falzon, 2009), renal (Agouni, et al., 2007), colorectal (Dihlmann, Kloor, Fallsehr, & von Knebel Doeberitz, 2005; Nishihara, et al., 1999; Nishihara, et al., 2007) and gastric carcinomas (Alipov, et al., 1997; Han, et al., 2008). PTHrP was initially discovered as a paraneoplastic agent produced by a variety of cancer cells, and is associated with malignancy-associated hypercalcemia (MAH) when it is released by tumors into the systemic circulation (Burtis, et al., 1987b; Moseley, et al., 1987; Strewler, et al., 1987; Suva, et al., 1987). Circulating levels of PTHrP generally correlate with the more advanced stages of cancer (Deftos, Barken, Burton, Hoffman, & Geller, 2005; Firkin, Seymour, Watson, Grill, & Martin, 1996; Iwamura, et al., 1999; Kissin, et al., 1993; Kremer, et al., 1996; Nishihara, et al., 1999; Rankin, Grill, & Martin, 1997; Southby, et al., 1990; Truong, et al., 2003). Despite the frequent association of PTHrP dysregulation with many tumor types, a more direct role for PTHrP on cancer development and progression has been difficult to prove, and the role of PTHrP on tumour initiation *in vivo* and on the critical steps of malignant conversion has not yet been addressed.

In order to study the role of PTHrP in mammary tumorigenesis, a targeted disruption of the *Pthrp* gene in an animal model of cancer initiation was required. Since mice homozygous for *Pthrp* gene knock-out (*Pthrp*$^{-/-}$) exhibit perinatal mortality (Karaplis, et al., 1994), we constructed a mammary epithelium-specific knockout animal in the MMTV/PyVMT transgenic mouse model of breast cancer, using mice homozygous for a floxed *Pthrp* allele (two *loxP* sites flanking exon 4 which encodes most of the PTHrP protein) (He, et al., 2001). PyVMT mice are known to closely mimic the four identifiable

stages of tumor progression from pre-malignant to malignant observed in human breast tumors (hyperplasia, adenoma, early and late carcinoma). These stages are followed in the PyVMT animals by a high frequency of distant metastasis, and the morphological characteristics and the expression of biomarkers parallel the cancer process in humans (Lin, et al., 2003). We therefore obtained mice presenting various degrees of ablation for the *Pthrp* gene in the mammary epithelium of a mouse model with complete penetrance and with good recapitulation of human breast carcinomas.

The mammary epithelium-targeted *Pthrp* disruption caused a delay in tumor onset, followed by a significant impediment in appearance of palpable tumors and in tumor growth. Importantly, mammary gland development was unaffected by the specific mammary epithelium-targeted *Pthrp* ablation. We conclude that expression of PTHrP in the mammary epithelium is critical for the initiation of oncogene-induced mammary tumorigenesis, and for tumor growth *in vivo*.

**Results**:

**Conditional ablation of the *Pthrp* gene in mouse mammary epithelium:**

To excise the *Pthrp* gene specifically from mouse mammary epithelium, C57BL6 *Pthrp* floxed mice (He, et al., 2001) were repeatedly backcrossed to FVB (Taketo, et al., 1991) animals to obtain *PTHrP* $^{flox/flox}$ mice in a 99% FVB background, a crucial step to ensure uniformity of tumor onset and to allow comparison between groups of animals. *Pthrp* $^{flox/flox}$ mice were crossed with MMTV/PyVMT (Guy, Cardiff, & Muller, 1992b) or MMTV/Cre (Andrechek, et al., 2000) mice in FVB background to obtain

MMTV/PyVMT; *Pthrp* $^{flox/flox}$ and $^{flox/+}$, and MMTV/Cre; *Pthrp* $^{flox/flox}$ and $^{flox/+}$ mice, respectively. Animals from these resulting groups were then crossed, and mice with the following genotypes were selected: MMTV/PyVMT; *Pthrp* $^{flox/flox}$; $Cre^{+}$ (homozygous); MMTV/PyVMT; *Pthrp* $^{flox/+}$ : $Cre^{+}$ (heterozygous), MMTV/PyVMT; *Pthrp* $^{flox/flox}$ ; $Cre^{-}$ or MMTV/PyVMT; *Pthrp* $^{flox/+}$ ; $Cre^{-}$ (controls)(Supplementary Fig S1 A). Excision of the flox-bordered essential *Pthrp* sequences by the Cre recombinase renders the gene non-functional and the resulting homozygous mice express no PTHrP in the mammary epithelium, while the heterozygous mice present lowered levels of PTHrP expression because they retain one functional *Pthrp* allele. In these animals, the MMTV promoter drives the mammary epithelium-specific expression of the PyMT oncogene and Cre recombinase. Southern blot analysis of tail genomic DNA revealed the *Pthrp* allele status, while PCR analysis confirmed the presence of middle T oncoprotein (MT) and Cre recombinase genes (Supplementary materials, Fig S1 B, C and D).

PTHrP expression in mammary glands from control animals is evident in the ductal epithelium and myoepithelium. In contrast, *Pthrp* $^{flox/flox}$; $Cre^{+}$ animals reveal no immunohistochemical (IHC) staining for PTHrP in cells surrounding the lumen although there is residual activity in myoepithelial cells (Fig. 1 A and B). PTH receptor 1 expression is restricted to stromal cells and shows no differential staining between control and PTHrP-ablated mammary glands (Fig. 1 C and D). Immunofluorescence (IF) staining confirms PTHrP expression in ductal epithelial and myoepithelial cells and reveals a strong reduction in the levels of expression of PTHrP in *Pthrp* $^{flox/flox}$/$Cre^{+}$ mice with respect to MMTV-PyVMT animals (Fig. 1 E to F and I to L).

***Pthrp* ablation does not affect mammary gland development:**

Whole mount analyses of mammary glands from 3, 5, and 7 week-old female *Pthrp* $^{flox/flox}$; *Cre*$^{-}$ control (Fig. 2 A, D and G), *Pthrp* $^{flox/+}$ ; *Cre*$^{+}$ heterozygous (Fig. 2 B, E and H) and *Pthrp* $^{flox/flox}$ ; *Cre*$^{+}$ homozygous (Fig. 2 C, F and I) virgin female animals reveals normal ductal outgrowth during puberty, with no detectable differences between genotypes.

**Reduction in PTHrP expression levels is accompanied by a significant delay in the appearance of palpable tumors**:

In standard PyVMT animals, PTHrP levels in tumors parallels their developmental stage (Figure 3 A; 7 weeks, B; 9 weeks and C; 13 weeks). In contrast, in *Pthrp*-ablated animals, late-developing tumors (13 weeks) show little PTHrP expression (figure 3 J to L) when compared to mice with one or two functional *Pthrp* alleles (Figure 3 G to I and D to F) and there is incremental reduction in PTHrP expression levels from *Pthrp*$^{flox/flox}$; *Cre*$^{-}$ (control) to *Pthrp*$^{flox/+}$; *Cre*$^{+}$ (heterozygous), to *Pthrp*$^{flox/flox}$; *Cre*$^{+}$ (homozygous). When tumor cells are isolated and cultured *in vitro***,** radioimmunometric assays (IRMA) reveal that **t**he levels of PTHrP present in the conditioned medium of *Pthrp*$^{flox/flox}$; Cre$^{-}$ and Cre$^{+}$ tumor-derived cells are 178.7 ± 33.6 and 10.1 ± 2.3 picograms per ml of medium (mean ± SD, n = 13 and 10 mice respectively), suggesting a low level of residual expression of PTHrP in homozygous cells.

P*thrp* ablation causes a significant delay in appearance of palpable tumors in heterozygous (*Pthrp* $^{flox/+}$; Cre$^{+}$) and homozygous (*Pthrp* $^{flox/flox}$; Cre$^{+}$) mice with respect to control Cre$^{-}$ animals. Kaplan-Meier analysis shows that while 100% of control mice

(with normal PTHrP levels) present palpable tumors around day 55, heterozygous and homozygous animals reach this percentage by days 73 and 91 respectively (Figure 3 M).

**PTHrP strongly influences the initial stage of mammary tumorigenesis:**

In *Pthrp* $^{flox/flox}$; Cre$^{-}$ control animals, tumor initiation is histologically detectable in the mammary epithelium as early as 27 days after birth (Figure 4 A) and tumors are established by the 35-day stage (Figure 4 B). In contrast, in the mammary glands of homozygous *Pthrp* $^{flox/flox}$; Cre$^{+}$ animals, the ablation of both *Pthrp* alleles results in a very significant delay in tumor initiation, as histological detection starts around 46 days (Figure 4 C), and tumor seen at 65 days (Figure 4 D) have reached the same size as those observed at 35 days in the control animals.

***Pthrp* ablation slows tumor growth and lowers differentiation status in tumor cells:**

Tumor growth over time is much reduced in homozygous *Pthrp* $^{flox/flox}$; Cre$^{+}$ animals with respect to controls (Figure 5 A), and tumor weight at sacrifice (13 weeks) is 70% lower in animals with complete ablation of *Pthrp* than in controls (Figure 5 B). Hetero- and homozygous *Pthrp* knock-out animals present tumors that are 50% and 75% smaller in size compared with their respective Cre$^{-}$ controls, and there are much fewer tumors (35% and 60% less, respectively) in heterozygous and homozygous mice at 13 weeks (table 1). Ki67 staining is sharply reduced in haploinsufficient or ablated tissues, indicating a decrease in the growing fraction of the tumor cell population concomitant with lowering of PTHrP expression (Figure 5 C to E). The levels of expression of angiogenesis marker factor VIII was also observed to decrease in absence of PTHrP (Figure 5 F to I).

Our results (IRMA assays above, and Fig. 3 E, H, K) as well as other Cre/lox studies (White, et al., 2004) indicate ablation of floxed alleles in only about 90% of cells,

leaving a percentage of cells to potentially express the knock-out protein. In order to overcome the problem of residual PTHrP expression, cells were isolated from *Pthrp*$^{flox/flox}$*; Cre*$^{-}$ tumors, transfected with an adenoCre-GFP virus (or control adeno-GFP), subcultured, then purified by flow cytometry to obtain pure populations of *Cre*$^{+}$ (or control *Cre*$^{-}$) cells with either complete or no ablation of *Pthrp*. Cells from the control or homozygous groups were transplanted into the fourth mammary fat pad of healthy FVB syngeneic mice. PTHrP expression was eliminated in the adenoCre-derived tumors in comparison to adenoGFP controls (Fig. 5 J to M), and tumor appearance was greatly delayed in adenoCre-derived cancers, with tumors reaching 1 $cm^3$ at ~35 weeks compared to ~15 weeks in adenoGFP controls (Fig. 5 N). The fact that a more complete ablation of *Pthrp* residual activity does not eliminate tumor growth suggests a crucial but not exclusive oncogene facilitator role for PTHrP.

**PTH1R is unaffected by *Pthrp* ablation while cyclin D1 expression co-locates to residual cells expressing PTHrP:**

While PTH1R receptor levels are unaffected by *Pthrp* disruption (Fig. 6 A to H and 6 L, see also Fig 1 C and D), expression levels for cyclin D1 decrease along with those of PTHrP (Fig. 6 I to L), and cyclin D1 co-localizes with residual PTHrP staining in isolated cultured tumor cells (Fig. 6 M to P, and Q to T), suggesting a PTHrP-driven cell cycle stimulus in this system.

***Pthrp* disruption effects on sub-cellular signaling and apoptosis:**

Using propidium iodide flow cytometry analysis, it was determined that *Pthrp* knock-out inhibits the G0/G1 to S transition in homozygous tumor cells, with 53.7 ± 1.19 % of *Pthr*$^{pflox/flox}$; *Cre*$^{-}$ and 71.32 ± 3.70 % of *Pthr*$^{pflox/flox}$; *Cre*$^{+}$ tumor cells in G0/G1, and 18.34 ± 1.80 % of *Pthr*$^{pflox/flox}$; *Cre*$^{-}$ and 8.10 ± 4.00 % of *Pthr*$^{pflox/flox}$; *Cre*$^{+}$ cells in S

phase, an observation consistent with the cyclin D1 decrease described above. *Pthrp* ablation is also accompanied by an increase in TUNEL staining in tumors and in tumor-derived cultured cells (Fig. 7 A to D), and by a decrease in Bcl-2 expression (Fig. 7 E) which indicate a higher apoptotic status in heterozygous and homozygous tumor tissue with respect to tumors from control animals.

The absence of PTHrP causes a sharp decrease in AKT1 total protein expression and an increase in total AKT2 in tumor tissues (Fig. 7 E, and F to M). IF staining for total AKT1 protein in *Pthrp* $^{flox/flox}$*; Cre*$^{+}$ cells isolated from tumors co-localizes with that of residual PTHrP (Fig. 7 N to U). SiRNA experiments aimed at reducing the expression of AKT1 in cultured tumor cells (Fig S 2, A and B) demonstrate that tumor cell proliferation after 48 hours is reduced by 38.9 ± 8.0 % through *Pthrp* ablation alone with respect to control cells with normal levels of PTHrP, by 46.7 ± 4.1 % through AKT1 knockdown alone, and by an impressive 80.2 ± 2.2 % in *Pthrp*-ablated cells combined with AKT1 siRNA transfection (Fig. S 2, C). The decrease in AKT1 phosphorylation levels (Ser 473) is also enhanced by PTHrP and AKT1 combined targeting (results not shown).

**Discussion**:

***Pthrp* gene ablation significantly delays breast tumorigenesis initiation**

While evidence has been accumulating for a tumoral growth-promoting effect for PTHrP in many cancers (Dittmer, 2004), there is some controversy as to the value of PTHrP as a prognostic marker especially in breast cancer (Fleming, et al., 2009; M. Henderson, et al., 2001; Linforth, et al., 2002; Nishihara, et al., 2007; Surowiak, et al., 2003), and the implication of PTHrP signalling in breast tumor initiation events needs clarification. The PyVMT model of breast cancer progression where the MT oncogene

drives oncogenic transformation, combined with the use of a Cre-lox conditional deletion system for the *Pthrp* gene was used here to ablate one or both alleles of the gene. The fact that the deletion was targeted specifically to the mammary epithelium allowed us to demonstrate that a local reduction or elimination of PTHrP expression brought dramatic delays in the initiation events of mammary carcinogenesis. In *Pthrp*-ablated animals, hyperplasia was delayed, palpable tumors developed much later, were fewer in number and of much smaller size than in control mice. Malignant foci observed during the progression to more advanced stages arose from a small population of cells that failed to express the MMTVCre and were therefore unable to excise *Pthrp*. But since tumors still eventually develop from uniformly adenoCre-transformed tumor cells, we speculate that PTHrP has a crucial but not exclusive oncogene facilitator role.

***Pthrp* ablation does not affect mammary gland development.**

The ability of *Pthrp* ablation to prevent tumour progression was not associated with a direct effect on mammary gland development. The expression of the *Cre* gene under the control of the MMTV-LTR is detected >6 days post-partum in MMTV-Cre transgenic mice (Wagner, et al., 2001b), causing *Pthrp* ablation to occur after birth. Ductal outgrowth in the mammary glands of both FVB mice and PyMT mice was consequently not affected by *Pthrp* disruption, and mammary gland development was unaffected. *Pthrp*-ablated female mice can, in fact, lactate normally. In a similar MMTV/PyMT mouse model and in MMTV-cNeu mice, the ablation of the *Akt*1 gene inhibited breast cancer progression but caused no interference in mammary ductal outgrowth (Maroulakou, Oemler, Naber, & Tsichlis, 2007). Our observations confirm that PTHrP is not required for mammary development if its ablation occurs after birth, and suggest a possible link between PTHrP and the PI3K/AKT pathway.

**AKT kinase total protein levels are linked to *Pthrp* ablation:**

In our study, the ablation of PTHrP expression in the tumors was accompanied by a significant decrease in the total protein levels of expression of the AKT serine-threonine protein kinase Akt1 isoform, and by an increase in the levels of the Akt2 isoform. AKT plays a crucial role in controlling the balance between cell survival and apoptosis, and its dysregulation is one of the most molecular alterations in human malignancy (Altomare & Testa, 2005; Cantley, 2002). Multiple oncoproteins or tumor suppressors cooperate with AKT in mechanisms which drive oncogenic progression and play a role in the tumor response to treatment. Consequently, many new targeting agents aim at the phosphatidylinositol-3 kinase (PI3K)/AKT pathway (Altomare & Testa, 2005; Fresno Vara, et al., 2004; Testa & Tsichlis, 2005)

Enhanced AKT1 activation and signalling is a well-known driver of human malignancy, with recent reports implicating PTHrP in the activation of AKT1 phosphorylation in renal cancer cell carcinoma, and breast and prostate cancer cell lines(Agouni, et al., 2007; Bhatia, et al., 2009; Shen & Falzon, 2006). However, in the present study, it is the total protein levels for AKT1 and 2 which appear regulated by PTHrP, suggesting a mechanism for tumorigenesis control similar to that reported in colorectal carcinogenesis where the Wnt/β-catenin pathway regulates AKT1 protein levels (Dihlmann, et al., 2005).

In the MMTV/PyMT system used here, the PI3K/AKT pathway is crucial to the transmission of oncogenic signalling from the middle T oncoprotein for development of mammary adenocarcinomas (Fluck & Schaffhausen, 2009; Lin, et al., 2003). As mentioned above, a knock-out of *Akt1* in MMTV/PyMT mice have been shown to interfere with mammary tumor initiation and growth (Maroulakou, et al., 2007).

Conversely, Akt2 ablation accelerates tumor induction and the two isoforms therefore possess opposite oncogenic effects (Fluck & Schaffhausen, 2009; Heron-Milhavet, et al., 2006b; Maroulakou, et al., 2007). In PyMT-*Akt1* knock-out mice, the delay in tumorigenesis was reported to be related to the inhibition of cell proliferation and to promotion of apoptosis due to the loss of Akt1 signalling (Maroulakou, et al., 2007). Similarly, in our study, *Pthrp* ablation increases apoptotic events with respect to controls. *Pthrp* ablation causes an inhibition of S-phase entry analogous to the one observed after the silencing of AKT1 in nontransformed mammalian myoblasts and fibroblasts (Heron-Milhavet, et al., 2006b)

The delay in tumorigenesis observed in PyMT Akt1 knock-out mice was reported to be related to the inhibition of cell proliferation and to promotion of apoptosis due to the loss of Akt1 signalling (Maroulakou, et al., 2007). In our study, *Pthrp* ablation also increases apoptotic events with respect to control samples and emphasizes the position of PTHrP as an upstream controller of AKT. We also noted that *Pthrp* ablation causes an inhibition of S-phase entry analogous to the one observed after silencing Akt1 in nontransformed mammalian myoblasts and fibroblasts (Heron-Milhavet, et al., 2006a), and causes a decrease in KI-67 and cyclin D1 expression, events also observed in *Akt1*-ablated mice (Maroulakou, et al., 2007). Interestingly, our observation that co-targeting *Pthrp* and *Akt1* provides a stronger inhibition in tumor cell proliferation over either blockade alone suggests the existence of PTHrP-independent signalling through AKT, and points to a potential combined therapeutic approach with enhanced efficacy.

### *Pthrp* ablation reduces the levels of angiogenic markers

PTHrP is known to stimulate the expression of many growth factors in various cell types, and behaves as an angiogenic factor in endothelial cells (Akino, et al., 2000; Esbrit, et al., 2000; T. J. Martin, et al., 1997). The levels of expression of the factor VIII marker of angiogenesis are negatively affected by *Pthrp* ablation. It seems likely that angiogenesis is another aspect of mammary tumor initiation that requires PTHrP as a driving factor, and which can be repressed through *Pthrp* ablation.

### Implications of *Pthrp* ablation in cancer therapeutics.

An important aspect of our study was the strong inhibition of the proliferative capacity of *Pthrp*-negative tumour cells. Analysis of cells from *Pthrp*-ablated tumours demonstrated a significant increase in the proportion of quiescent and apoptotic cells with respect to control tumor cells. The doubling time of the *Pthrp*-null tumours *in vivo* was about 14 days compared to 4 days in *Pthrp*-positive tumours, a stunning 3.5- fold decrease in their growth rate. Since PTHrP is expressed in the great majority of breast tumors, its signalling activity constitutes a potential target for breast cancer therapy.

In this study, we have used a well-validated breast cancer animal model to examine the malignant progression process. We have demonstrated that disruption of the *Pthrp* gene dramatically delays the critical initial step of mammary epithelial cell malignant conversion, without affecting mammary gland development. This novel property of PTHrP, coupled with the observation that a number of critical checkpoints downstream of PyMT such as AKT1, AKT2 Bcl-2 and cyclin D1 are affected by *Pthrp* ablation suggest a role for PTHrP as an oncogene-like factor, or at least a facilitator of

oncogenes. Since almost all known oncogenic growth factors, angiogenic factors and cytokines activate AKT after binding to their cognate receptors (Cheng, Lindsley, Cheng, Yang, & Nicosia, 2005), PTHrP, as an efficient upstream AKT control mechanism, becomes a promising therapeutic target to avoid the compensatory pathway activation that can result from blockade of individual downstream signalling molecules (Di Cosimo, 2009; Tabernero, et al., 2008)

**Experimental Procedures**:

**Animals**:

We used homozygous *Pthrp* floxed C57BL6 mice whose genome includes two loxP sites flanking the fourth exon of the *Pthrp* gene which encodes most of the protein (He, et al., 2001). The alterations were introduced into the FVB/NJ background by sequential backcrossing of male *Pthrp* floxed C57BL6 mice with FVB/NJ wild-type females. The $F_1$ generation heterozygous males carrying a floxed ($Pthrp^{flox/+}$) allele were subsequently crossed with FVB/NJ wild-type females to produce a heterozygous $F_2$ ($Pthrp^{flox/+}$) colony. This strategy was repeated until the $F_8$ generation, followed by marker analysis (Jackson laboratories, West Grove, PA) which confirmed animals to have greater than 99% FVB/NJ background.

MMTV/*PyMT* (Guy, et al., 1992a) and MMTV/*Cre* mice (Andrechek, et al., 2000) on a pure FVB/NJ background were kindly supplied by Dr. William Muller

(McGill University Cancer Center), and were crossed with *Pthrp* $^{flox/flox}$ mice in the FVB/NJ background described above. We obtained male MMTV/*PyMT* MTV/*Cre Pthrp* $^{flox/+}$, and female MMTV/*Cre Pthrp* $^{flox/+}$ mice. Crossing these animals produced female MMTV/*PyMT*, MMTV/*Cre Pthrp* $^{flox/flox}$ (homozygous) MMTV/*PyMT*, MMTV/*Cre Pthrp* $^{flox/+}$ (heterozygous), and MMTV/*PyMT Pthrp* $^{flox/+}$ and MMTV/*PyMT Pthrp* $^{flox/flox}$ (controls).

Animals were housed in the Royal Victoria Hospital Central Animal Care Facility, and all experiments were carried out in compliance with regulations of the McGill University institutional animal care committee. All animal surgeries were conducted in accordance with principles and procedures dictated by the highest standards of humane animal care.

**Antibodies:**

The mouse monoclonal anti-PTHrP antibody (1-34, AE-0502) was purchased from IDS Ltd (Hampshire, U.K.). The mouse monoclonal anti-PTH/PTHrP receptor antibody was ordered from Upstate (Temecula, CA). Mouse monoclonal anti-Cre recombinase and anti phospho-Akt1 were purchased from Abcam (Cambridge, MA). Anti-Akt1 (goat) and Akt2 (goat), Ki-67 (goat), cyclin D1 (mouse), Bcl-2 (mouse), actin (mouse), as well as rabbit polyclonal antibodies recognizing the PTH/PTHrP receptor, PTHrP (41-139) C-terminal end, tubulin, anti-rabbit peroxidase conjugates and anti-mouse peroxidase conjugates were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Alexa Fluor 555-conjugated goat anti rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG were from Invitrogen (Burlington, ON, Canada).

**Histology**

Tumors, lungs, livers, kidneys, uterus, spleens, mammary glands, guts and femurs were fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin and sliced in 5-micron thick sections. For immunocytochemistry, tumor cells were grown on sterile glass coverslips overnight in a standard cell incubator at 37º C in DMEM containing 10% FBS, washed briefly with phosphate-buffered saline (PBS), fixed 10 minutes in 1% formalin in PBS, then washed in three changes of PBS and used for histological procedures.

**Whole-mount staining**

The mammary gland fat pad was removed at the nipple and spread onto an ethanol-precleaned glass slide. The fat pad was air-dried and fixed in acidic ethanol 100% for 1 h at room temperature. Lipids were removed with 3 changes of acetone (1 hour each). Tissues were stained with 0.5% Neutral Red (Sigma) in 100% ethanol, then washed twice for 1 hour in 100% ethanol. Tissues were cleared in two changes of xylene (1 hour each) and stored in xylene overnight. The stained and cleared mammary fat pad was mounted under coverslip with Permount (Fisher) and photographed with a slide duplicator.

**Hematoxylin-eosin staining**

Hematoxylin-eosin (HE) staining was conducted according to standard protocols. Briefly, sections were deparaffinized in three changes of xylene, re-hydrated in a graded ethanol series and stained with hematoxylin (Fisher) and eosin (Fisher). Slides were rinsed in water, dehydrated, cleared, and mounted in Permount (Fisher, Fair Lawn, NJ).

**Immunohistochemistry**

Immunohistochemistry staining was performed using the SK-4100 kit from Vector (Burlingame, CA). Endogenous peroxidase activity was quenched by incubating the slides in 3% $H_2O_2$ (Fisher) for 30 minutes, then washing 3 times for 5 minutes with PBST (PBS containing 0.2% Tween-20, Sigma-Aldrich, Oakville, ON). Slides were then incubated in 10% normal blocking serum (Normal Sera for Immunohistochemistry Sigma-Aldrich, Oakville, ON) in PBS for 30 minutes to suppress non-specific IgG binding. Blocking serum was from the same species in which the secondary antibody was raised. The slides were incubated in the presence of the primary antibody at room temperature for 2 hours, then washed 3 times (5 min each) with PBST buffer. The slides were incubated for 1 hour with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature, washed 3 times (5 min each) in PBST, diaminobenzidine (DAB) substrate solution was added and incubated until the desired level of staining was achieved (typically 5 to 15 min). The slides were washed 3 times (5 min each) in PBST and counterstained with hematoxylin. Slides were dehydrated, cleared and mounted as above. Results were analyzed using a Leica DMR microscope and Bioquant Nova Prime software (Bioquant Image Analysis Corporation, Nashville, TN).

**Immunofluorescence staining**

Immunoflurorescence staining was conducted according to manufacturer's instructions (InVitrogen, Burlington, ON), using deparaffinized tissue sections or cells fixed on slides. For tissue sections, antigen unmasking was conducted by heating slides 2 minutes in a microwave oven. Specimens were incubated in 10% normal blocking serum

in PBS for 20 minutes to suppress non-specific IgG binding, washed for 3 min in PBST, and incubated with the primary antibody for 60 minutes. The slides were washed 5 min with PBST and incubated 45 minutes in a dark chamber with the fluorochrome-conjugated secondary antibody (diluted to 1–5 µg/ml in PBS with 1.5%–3% normal blocking serum). Slides were washed with three changes of PBST (5 min each), counterstained in the dark with DAPI (InVitrogen) for 15 minutes, washed with three changes of PBST and mounted under coverslip with aqueous mounting medium (Thermo Electron corp. Pittsburgh, PA). Results were analyzed with an LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging Gmbh, Munich, Germany).

**Western blotting**

Proteins were extracted from tissues and cells with an extraction buffer containing 50mM Tris, 150 mM NaCl and 1 protease inhibitor cocktail tablet (Roche) per 100 ml buffer. Protein concentrations were estimated with the Bradford assay kit (Bio-Rad, Mississauga, ON). Protein samples (30-50 µg) were fractionated by SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with 5% skimmed milk and immunoblotted with primary antibodies. After incubation with an appropriate secondary antibody, the blots were developed by enhanced chemiluminescence using ECL$^{TM}$ Western Blotting System (Baie d'Urfé, QC) and KODAK Film (Carestream Health Inc. Rochester, NY).

**Isolation and Culture of Mouse Breast Tumor cells from Primary Tumors**

Mammary tumors were harvested, washed twice in ice-cold PBS, cut into small pieces and incubated in 2.4 mg/ml collagenase B (Roche, Laval, QC) and dispase II (Roche) in DMEM growth medium containing 1% penicillin-sreptomycin (Invitrogen) at 37$^{o}$C for 2 hours. Floating cells were washed twice with ice-cold PBS, pelleted, resuspended in a mixture of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and further propagated in this medium.

**Infection of Primary Mammary Tumor cells with Adenovirus GFP-Cre Vector.**

A recombinant adenovirus vector containing a green fluorescent protein (GFP)-Cre transgene for the Cre recombinase was a generous gift from Dr. Willam J. Muller (McGill Cancer Center, McGill University). Primary tumor cells were infected according to standard protocols. Briefly, primary tumor cells were grown to 70% confluence and infected by overlaying a solution of $10^{8}$ viral particles/ml in DMEM growth medium for 60-90 min at 37$^{o}$C. The cells were washed twice in PBS, replaced in growth medium and harvested 48-60 hours after infection. Experimental procedures followed regulations of the McGill University Health Center biosafety committee.

**Flow Cytometric Cell Selection and Mammary Gland Injection**

Cells were harvested 48h post-adenovirus infection and processed for positive GFP selection using flow cytometric sorting (BD FACSAria II cell sorter, BD Biosciences, San Jose, CA). Selected GFP-positive cells were injected into 5-week old syngeneic FVB mice (Charles River Laboratories, Senneville, QC). The animals were first anesthetized with ketamine (100mg/ml at 100-200mg/kg), the skin of the fourth

mammary gland was incised and GFP-positive tumor cells ($1x10^6$ cells/gland) in a 50 µl volume of PBS were injected into the fat pad.

**Cell cycle analysis by propidium iodide staining and flow cytometry:**

Cultured cells ($1 \times 10^6$) were harvested, washed and fixed in cold 70% ethanol for 30 minutes on ice. The cells were washed in PBS, resuspended in 500 ul fluorescence activated cell sorting (FACS) buffer and RNAse-treated (100ug/ml). 250 ul of propidium iodide staining solution 50 ug/ml (Sigma P4170) was added, the solution mixed gently and incubated 30 min on ice. After adding 500 ul FACS buffer, the solution was transferred to a FACS glass tube and immediately analyzed for cell cycle using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

**PTHrP assay in conditioned medium**:

Levels of PTHrP secreted into the culture medium by cells of various genotypes were measured using a two-site immunoradiometric assay (IRMA) where sample PTHrP becomes bound to an immobilized antibody and is detected by a second radiolabelled antibody (PTHrP IRMA kit, Diagnostic Systems Labs, Beckman-Coulter, Webster, TX).

**Statistical analysis of tumor progression and tumor growth:**

Homozygous female mice (MMTV/*PyVMT*, MMTV/Cre *Pthrp* $^{flox/flox}$), heterozygous female mice (MMTV/*PyVMT*, MMTV/Cre *Pthrp* $^{flox/+}$), and control mice

MMTV/*PyVMT* Pthrp $^{flox/+}$ and MMTV/*PyVMT Pthrp* $^{flox/flox}$, were examined by palpation twice a week for mammary tumor development. These examinations proceeded until animals were 13 weeks old. Mice that exhibited excessive tumor burden (tumor diameter > 1.5 cm) were euthanized. Numerical data are presented as the mean ± SD. The data were analyzed by ANOVA followed by a Bonferroni post-test to determine the statistical significance of differences. All statistical analyses were performed using Instat Software (GraphPad Software, San Diego, CA), and p ‹0.05 was considered statistically significant. Kaplan-Meier analysis was used to construct curves for primary tumor appearance.

**Acknowledgements**: Grant to R.K.: Operating grant from the Canadian Institutes of Health Research (CIHR), MOP 10839; grant to A.K.: Concept Award from the U.S. Department of Defense Breast Cancer Research Program (BCRP) BC023897.

**Legends for figures**:

**FIGURE 1**: Conditional ablation of the *Pthrp* gene in mouse mammary epithelium:

PTHrP is expressed in the mammary ductal epithelium and myoepithelium from *Pthrp* $^{flox/flox}$; *Cre*$^{-}$ control mice (**A**) while there is no staining for PTHrP in cells surrounding the lumen from *Pthrp* $^{flox/flox}$; *Cre*$^{+}$ animals (**B**). Expression of the receptor PTH1R is restricted to stromal cells and shows no differential staining between control and *Pthrp*-ablated tissues (**C, D**). Red arrows: luminal epithelial cells; yellow arrows: myoepithelial cells; black arrow: stromal cells.

Confirmation by IF of PTHrP expression in control ductal epithelium of control MMTV/PyMT Cre$^{+}$ mice (**E-H**) and absence in luminal cells of *Pthrp* $^{flox/flox}$; *Cre*$^{+}$ tissue (**I-L**).

**FIGURE 2**: *Pthrp* ablation does not affect mammary gland development:

Whole mount staining analysis of mammary glands from 3 (**A-C**), 5 (**D-F**), and 7 week-old (**G–I**) female virgin animals of the three indicated genotypes reveals normal ductal outgrowth during puberty.

**FIGURE 3**: A reduction in PTHrP expression levels is accompanied by a significant delay in the appearance of palpable tumors:

In breast tumors from standard PyMT mice, IHC shows that PTHrP expression increases with developmental stage (**A-C**). At 13 weeks, *Pthrp* $^{flox/flox}$; *Cre*$^{-}$ control tumor tissue show similarly high levels of PTHrP expression (**D to F**). In contrast, *Pthrp* $^{flox/+}$;

*Cre*$^{+}$ heterozygous (**G to I**) and *Pthrp* $^{flox/flox}$; *Cre*$^{+}$ homozygous (**J to L**) tumor express proportionally lower levels of PTHrP.

**M**: Kaplan-Meier analysis of tumor onset shows that 100% of *Cre*$^{-}$ control mice with normal PTHrP levels present tumors by day 55, while *Pthrp*$^{flox/+}$ ; *Cre*$^{+}$ heterozygous and *Pthrp*$^{flox/flox}$ ; *Cre*$^{+}$ homozygous animals reach this percentage by days 75 and 92 respectively.

**FIGURE 4**: PTHrP strongly influences the initial stage of mammary tumorigenesis:

Control animals (*Pthrp* $^{flox/flox}$; *Cre*$^{-}$ ) present mammary gland hyperplasia by 27 days, visible after hematoxylin-eosin staining (**A**), and tumors are established by the 35-day stage (**B**). In contrast, homozygous *Pthrp*-ablated animals (*Pthrp* $^{flox/flox}$; *Cre*$^{+}$) show no obvious hyperplasia until the 46-day stage (**C**), and tumors become established around 65 days (**D**).

**FIGURE 5**: Reduction or ablation of PTHrP expression levels slows tumor growth and lowers differenciation status in tumor cells.

**A and B**: Mice with homozygous *Pthrp* ablation eventually develop tumors but the average tumor volume and weight at sacrifice (13 weeks) is substantially reduced with respect to tumors from control animals. Values shown represent mean ± SD, n = 12 mice for each group. *** $P < 0.001$.

**C to E:** IHC staining for Ki 67 (differentiation) shows that the growing fraction of the tumor cell population decreases with *Pthrp* haploinsufficiency or ablation.

**F to H:** Factor VIII staining shows a decrease in this angiogenesis marker with *Pthrp* haploinsufficiency or ablation, confirmed by Western blot analysis (**I**).

Cells were isolated from *Pthrp*$^{flox/flox}$*; Cre*$^{-}$ tumors, transfected with adeno-GFP (**J**) or adenoCre-GFP virus (**K**), and purified by flow cytometry to obtain pure populations of *Cre*$^{+}$ (or control *Cre*$^{-}$) cells (left panels). When these cells were transplanted into the mammary fat pad of healthy syngeneic mice (right panels **L** and **M**), *Pthrp* expression was eliminated only in *Cre*$^{+}$ cells. **N**: complete adenoviral removal of residual *Pthrp* expression results in further reduction in tumor growth rate but not in complete elimination of tumor growth. Values shown represent mean ± SD, n = 12 mice for each group. *** $P < 0.001$.

**FIGURE 6**: PTH1R expression is unaffected by PTHrP variations, while cyclin D1 expression is linked to PTHrP levels.

In 13-week tumors from *Pthrp* $^{flox/flox}$; *Cre*$^{-}$ control (**A** to **D**) or *Pthrp* $^{flox/flox}$: *Cre*$^{+}$ homozygous mice (**E** to **H**), the expression of PTH1R is independent of PTHrP expression levels. At the same stage, cyclin D1 expression is reduced in tumor tissue from heterozygous and homozygous animals with respect to controls (**I** to **K**). The decrease in cyclin D1 expression with lowered PTHrP levels and the stability of PTH1R levels are confirmed by Western blot (**L**).

IF shows co-localisation of cyclin D1 and residual PTHrP expression in cultured tumor cells (**M** to **T**).

**FIGURE 7**: PTHrP disruption affects cell cycle progression, increases apoptosis and affects AKT1 and AKT2 total protein levels.

**A to D**: TUNEL staining in tumor tissue and in isolated tumor cells increases with *Pthrp* ablation.

**E**: Western blot shows a decrease in total protein levels for Bcl 2 and AKT1, and an increase in total protein levels for AKT2 with *Pthrp* ablation.

**F** to **I**: The absence of PTHrP is accompanied by a decrease in AKT1 total protein expression (**F** and **H**: IHC of tumor tissue, **G** and **I**: IF of cultured tumor cells).

**J** to **M**: The absence of PTHrP is accompanied by an increase in AKT2 total protein expression (**J** and **L**: IHC of tumor tissue, **K** and **M**: IF of cultured tumor cells).

**N** to **U**: IF staining showing co-localization of AKT1 total protein with residual PTHrP staining in tumor-derived cultured cells.

**Supplementary materials**

**PCR analysis and Southern blotting:**

Genotyping was performed by PCR and Southern blotting using tail DNA and ear punches. Primer sequences and PCR protocols were as follows:

for mT oncoprotein:

PCR primers 5' GGA AGC AAG TAC TTC ACA AGG G-3' (F)

5' GCA AAG TCA CTA GGA GCA GGG-3'(R)

for Cre protein:

PCR primers 5' GCT TCT GTC CGT TTG CCG-3' (F)

5' ACT GTG TCC AGA CCA GGC-3' (R)

Southern blot analysis was conducted following digestion of tail tip genomic DNA with *BamH*1, and hybridization with probe B, a 0.65 kb *Sac*I / *Xho*I genomic DNA fragment (He, et al., 2001).

**siRNA transfection for *Akt1* knockdown**:

Mammary tumors were harvested, and cells obtained as per isolation and culture of mouse breast tumor cells protocol described above. Cells were seeded ($5 \times 10^4$) in 6-well plates, and medium without antibiotics was added. 24 hours after plating, the cells were transfected with 12.5 pmol of each siRNA duplex in 5 ul Lipofectamine per well (Lipofectamine RNAiMAX; InVitrogen). The *Akt1* targeting sequences used were from Ambion/Applied Biosciences (s62216): sense: CUC AAG UAC UCA UUC CAG Att, antisense: UCU GGA AUG AGU ACU UGA Ggg, as well as negative control from the

same source. The cells were then maintained in medium containing FBS for 48 or 72 hours. The effect of siRNA on AKT1 protein levels was assayed by Western blot of protein samples separated on 10% acrylamide gels. The effect of *Akt1* silencing on cell proliferation was assayed at various time intervals by hemacytometer cell counting.

**Legends for supplementary figures:**

**Supplementary figure 1**: Targeted ablation of the *Pthrp* gene in the mouse mammary gland epithelium.

**A**: Mating strategy to obtain homozygous, heterozygous and control animals. **B**: Southern blot analysis confirms the targeted recombination of the *Pthrp* floxed allele in mammary gland DNA. 6.2 kb: wild-type allele; 5.2 kb: floxed allele. **C**: PCR analysis of mammary gland DNA for concurrent expression of the mT antigen and (**D**) the Cre recombinase.

**Supplementary figure 2**: Effect of reduction of PTHrP and AKT1 levels on the proliferation of cells isolated from mammary breast tumors. (**A**) Levels of AKT1 protein are lowered by *Akt1* silencing in cells with normal PTHrP expression (*Pthrp* $^{flox/flox}$ *Cre*$^{-}$, 12.5 and 25 pmol) (top row), and to a further extent with the combination of *Pthrp* ablation and *Akt1* silencing (*Pthrp* $^{flox/flox}$ *Cre*$^{+}$, 12.5 and 25 pmol) (middle row). Actin levels are used as loading controls (bottom row). (**B**) Densitometric analysis of AKT1 bands from Western blot showing AKT1 levels as percentage of *Pthrp* $^{flox/flox}$ *Cre*$^{-}$ mock ontrol. (1): mock transfection 48h. (2) siRNA 12.5 pmol 48 h. (3) siRNA 25 pmol 48 h. (4) mock transfection 72 h. (5) siRNA 12.5 pmol 72 h. (6) siRNA 25 pmol 72 h. (**C**) Proliferation of isolated *Pthrp*$^{flox/flox}$ tumor cells is reduced by *Pthrp* ablation alone (*Cre*$^{+}$

mock), by *Akt1* knockdown alone (*Cre*$^-$ AKT1 siRNA), but substantially more by the combined *Pthrp* ablation and Akt1 knockdown (*Cre*$^+$ AKT1 siRNA). Initial cell seeding level indicated by gray bar (representative experiment out of three replicates). ** P values are < 0.05 for comparisons of *Cre*$^-$ mock with *Cre*$^+$ mock, *Cre*$^-$ mock with *Cre*$^-$ si*Akt1*, *Cre*$^+$ mock with *Cre*$^+$ si*Akt1*, and *Cre*$^-$ si*Akt1* with *Cre*$^+$ si*Akt1*. For *Cre*$^-$ mock with *Cre*$^+$ si*Akt1*, $p < 0.0001$.

**References:**

Agouni, A., Sourbier, C., Danilin, S., Rothhut, S., Lindner, V., Jacqmin, D., et al. (2007). Parathyroid hormone-related protein induces cell survival in human renal cell carcinoma through the PI3K Akt pathway: evidence for a critical role for integrin-linked kinase and nuclear factor kappa B. *Carcinogenesis, 28*(9), 1893-1901.

Akino, K., Ohtsuru, A., Kanda, K., Yasuda, A., Yamamoto, T., Akino, Y., et al. (2000). Parathyroid hormone-related peptide is a potent tumor angiogenic factor. *Endocrinology, 141*(11), 4313-4316.

Alipov, G. K., Ito, M., Nakashima, M., Ikeda, Y., Nakayama, T., Ohtsuru, A., et al. (1997). Expression of parathyroid hormone-related peptide (PTHrP) in gastric tumours. *J Pathol, 182*(2), 174-179.

Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., & Muller, W. J. (2000). Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. *Proc Natl Acad Sci U S A, 97*(7), 3444-3449.

Bhatia, V., Mula, R. V., Weigel, N. L., & Falzon, M. (2009). Parathyroid hormone-related protein regulates cell survival pathways via integrin alpha6beta4-mediated activation of phosphatidylinositol 3-kinase/Akt signaling. *Mol Cancer Res, 7*(7), 1119-1131.

Bouizar, Z., Spyratos, F., & De vernejoul, M. C. (1999). The parathyroid hormone-related protein (PTHrP) gene: use of downstream TATA promotor and PTHrP 1-139 coding pathways in primary breast cancers vary with the occurrence of bone metastasis. *J Bone Miner Res, 14*(3), 406-414.

Bouizar, Z., Spyratos, F., Deytieux, S., de Vernejoul, M. C., & Jullienne, A. (1993). Polymerase chain reaction analysis of parathyroid hormone-related protein gene

expression in breast cancer patients and occurrence of bone metastases. *Cancer Res, 53*(21), 5076-5078.

Brandt, D. W., Burton, D. W., Gazdar, A. F., Oie, H. E., & Deftos, L. J. (1991). All major lung cancer cell types produce parathyroid hormone-like protein: heterogeneity assessed by high performance liquid chromatography. *Endocrinology, 129*(5), 2466-2470.

Bucht, E., Rong, H., Pernow, Y., Nordqvist, A. C., Eriksson, E., Rankin, W., et al. (1998). Parathyroid hormone-related protein in patients with primary breast cancer and eucalcemia. *Cancer Res, 58*(18), 4113-4116.

Burtis, W. J., Wu, T., Bunch, C., Wysolmerski, J. J., Insogna, K. L., Weir, E. C., et al. (1987). Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *J Biol Chem, 262*(15), 7151-7156.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science, 296*(5573), 1655-1657.

Clemens, T. L., Cormier, S., Eichinger, A., Endlich, K., Fiaschi-Taesch, N., Fischer, E., et al. (2001). Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. *Br J Pharmacol, 134*(6), 1113-1136.

Deftos, L. J., Barken, I., Burton, D. W., Hoffman, R. M., & Geller, J. (2005). Direct evidence that PTHrP expression promotes prostate cancer progression in bone. *Biochem Biophys Res Commun, 327*(2), 468-472.

Dihlmann, S., Kloor, M., Fallsehr, C., & von Knebel Doeberitz, M. (2005). Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. *Carcinogenesis, 26*(9), 1503-1512.

Dittmer, J. (2004). The importance of PTHrP for cancer development. *Gene Therapy and Molecular Biology, 8*, 451-464.

Dougherty, K. M., Blomme, E. A., Koh, A. J., Henderson, J. E., Pienta, K. J., Rosol, T. J., et al. (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res, 59*(23), 6015-6022.

Esbrit, P., Alvarez-Arroyo, M. V., De Miguel, F., Martin, O., Martinez, M. E., & Caramelo, C. (2000). C-terminal parathyroid hormone-related protein increases vascular endothelial growth factor in human osteoblastic cells. *J Am Soc Nephrol, 11*(6), 1085-1092.

Firkin, F., Seymour, J. F., Watson, A. M., Grill, V., & Martin, T. J. (1996). Parathyroid hormone-related protein in hypercalcaemia associated with haematological malignancy. *Br J Haematol, 94*(3), 486-492.

Fleming, N. I., Trivett, M. K., George, J., Slavin, J. L., Murray, W. K., Moseley, J. M., et al. (2009). Parathyroid hormone-related protein protects against mammary tumor emergence and is associated with monocyte infiltration in ductal carcinoma in situ. *Cancer Res, 69*(18), 7473-7479.

Fluck, M. M., & Schaffhausen, B. S. (2009). Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. *Microbiol Mol Biol Rev, 73*(3), 542-563, Table of Contents.

Foley, J., Dann, P., Hong, J., Cosgrove, J., Dreyer, B., Rimm, D., et al. (2001). Parathyroid hormone-related protein maintains mammary epithelial fate and

triggers nipple skin differentiation during embryonic breast development. *Development, 128*(4), 513-525.

Gardella, T. J., & Juppner, H. (2001). Molecular properties of the PTH/PTHrP receptor. *Trends Endocrinol Metab, 12*(5), 210-217.

Gensure, R. C., Gardella, T. J., & Juppner, H. (2005). Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. *Biochem Biophys Res Commun, 328*(3), 666-678.

Guy, C. T., Cardiff, R. D., & Muller, W. J. (1992a). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol, 12*(3), 954-961.

Guy, C. T., Cardiff, R. D., & Muller, W. J. (1992b). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Molecular and Cellular Biology, 12*(3), 954-961.

Han, Z., Wu, K., Shen, H., Li, C., Han, S., Hong, L., et al. (2008). Akt1/protein kinase B alpha is involved in gastric cancer progression and cell proliferation. *Dig Dis Sci, 53*(7), 1801-1810.

He, B., Deckelbaum, R. A., Miao, D., Lipman, M. L., Pollak, M., Goltzman, D., et al. (2001). Tissue-specific targeting of the pthrp gene: the generation of mice with floxed alleles. *Endocrinology, 142*(5), 2070-2077.

Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., et al. (2001). Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst, 93*(3), 234-237.

Heron-Milhavet, L., Franckhauser, C., Rana, V., Berthenet, C., Fisher, D., Hemmings, B. A., et al. (2006). Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. *Mol Cell Biol, 26*(22), 8267-8280.

Iwamura, M., di Sant'Agnese, P. A., Wu, G., Benning, C. M., Cockett, A. T., Deftos, L. J., et al. (1993). Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res, 53*(8), 1724-1726.

Iwamura, M., Wu, W., Muramoto, M., Ohori, M., Egawa, S., Uchida, T., et al. (1999). Parathyroid hormone-related protein is an independent prognostic factor for renal cell carcinoma. *Cancer, 86*(6), 1028-1034.

Jin, L., Briggs, S. L., Chandrasekhar, S., Chirgadze, N. Y., Clawson, D. K., Schevitz, R. W., et al. (2000). Crystal structure of human parathyroid hormone 1-34 at 0.9-A resolution. *J Biol Chem, 275*(35), 27238-27244.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., et al. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev, 8*(3), 277-289.

Kissin, M. W., Henderson, M. A., Danks, J. A., Hayman, J. A., Bennett, R. C., & Martin, T. J. (1993). Parathyroid hormone related protein in breast cancers of widely varying prognosis. *Eur J Surg Oncol, 19*(2), 134-142.

Kitazawa, S., Fukase, M., Kitazawa, R., Takenaka, A., Gotoh, A., Fujita, T., et al. (1991). Immunohistologic evaluation of parathyroid hormone-related protein in human lung cancer and normal tissue with newly developed monoclonal antibody. *Cancer, 67*(4), 984-989.

Kohno, N., Kitazawa, S., Fukase, M., Sakoda, Y., Kanbara, Y., Furuya, Y., et al. (1994). The expression of parathyroid hormone-related protein in human breast cancer with skeletal metastases. *Surg Today, 24*(3), 215-220.

Kremer, R., Shustik, C., Tabak, T., Papavasiliou, V., & Goltzman, D. (1996). Parathyroid-hormone-related peptide in hematologic malignancies. *Am J Med, 100*(4), 406-411.

Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., et al. (2003). Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol, 163*(5), 2113-2126.

Linforth, R., Anderson, N., Hoey, R., Nolan, T., Downey, S., Brady, G., et al. (2002). Coexpression of parathyroid hormone related protein and its receptor in early breast cancer predicts poor patient survival. *Clin Cancer Res, 8*(10), 3172-3177.

Maioli, E., & Fortino, V. (2004). The complexity of parathyroid hormone-related protein signalling. *Cell Mol Life Sci, 61*(3), 257-262.

Maroulakou, I. G., Oemler, W., Naber, S. P., & Tsichlis, P. N. (2007). Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer Res, 67*(1), 167-177.

Martin, T. J., Moseley, J. M., & Williams, E. D. (1997). Parathyroid hormone-related protein: hormone and cytokine. *J Endocrinol, 154 Suppl*, S23-37.

Moseley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E., Kemp, B. E., Suva, L. J., et al. (1987). Parathyroid hormone-related protein purified from a human lung cancer cell line. *Proc Natl Acad Sci U S A, 84*(14), 5048-5052.

Nishigaki, Y., Ohsaki, Y., Toyoshima, E., & Kikuchi, K. (1999). Increased serum and urinary levels of a parathyroid hormone-related protein COOH terminus in non-small cell lung cancer patients. *Clin Cancer Res, 5*(6), 1473-1481.

Nishihara, M., Ito, M., Tomioka, T., Ohtsuru, A., Taguchi, T., & Kanematsu, T. (1999). Clinicopathological implications of parathyroid hormone-related protein in human colorectal tumours. *J Pathol, 187*(2), 217-222.

Nishihara, M., Kanematsu, T., Taguchi, T., & Razzaque, M. S. (2007). PTHrP and tumorigenesis: is there a role in prognosis? *Ann N Y Acad Sci, 1117*, 385-392.

Rankin, W., Grill, V., & Martin, T. J. (1997). Parathyroid hormone-related protein and hypercalcemia. *Cancer, 80*(8 Suppl), 1564-1571.

Shen, X., & Falzon, M. (2006). PTH-related protein upregulates integrin alpha6beta4 expression and activates Akt in breast cancer cells. *Exp Cell Res, 312*(19), 3822-3834.

Southby, J., Kissin, M. W., Danks, J. A., Hayman, J. A., Moseley, J. M., Henderson, M. A., et al. (1990). Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res, 50*(23), 7710-7716.

Strewler, G. J., Stern, P. H., Jacobs, J. W., Eveloff, J., Klein, R. F., Leung, S. C., et al. (1987). Parathyroid hormonelike protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. *J Clin Invest, 80*(6), 1803-1807.

Surowiak, P., Dziegiel, P., Matkowski, R., Sopel, M., Wojnar, A., Kornafel, J., et al. (2003). Prognostic value of immunocytochemical determination of parathyroid hormone-related peptide expression in cells of mammary ductal carcinoma. Analysis of 7 years of the disease course. *Virchows Arch, 442*(3), 245-251.

Suva, L. J., Winslow, G. A., Wettenhall, R. E., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., et al. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science, 237*(4817), 893-896.

Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., et al. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A, 88*(6), 2065-2069.

Truong, N. U., de, B. E. M. D., Papavasiliou, V., Goltzman, D., & Kremer, R. (2003). Parathyroid hormone-related peptide and survival of patients with cancer and hypercalcemia. *Am J Med, 115*(2), 115-121.

White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., et al. (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell, 6*(2), 159-170.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., & Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development, 125*(7), 1285-1294.

**Figures**

Fig.1

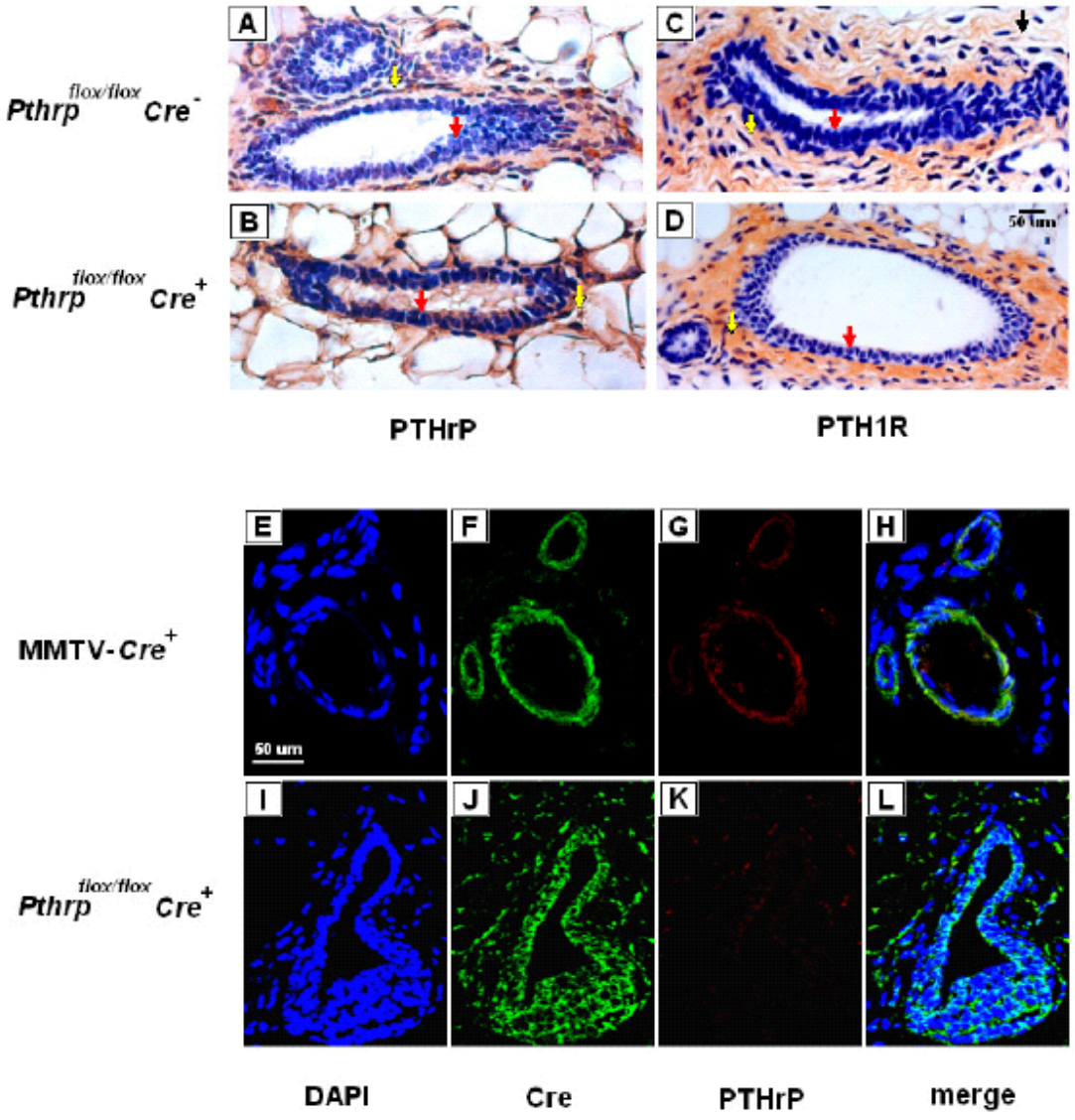


Fig.2

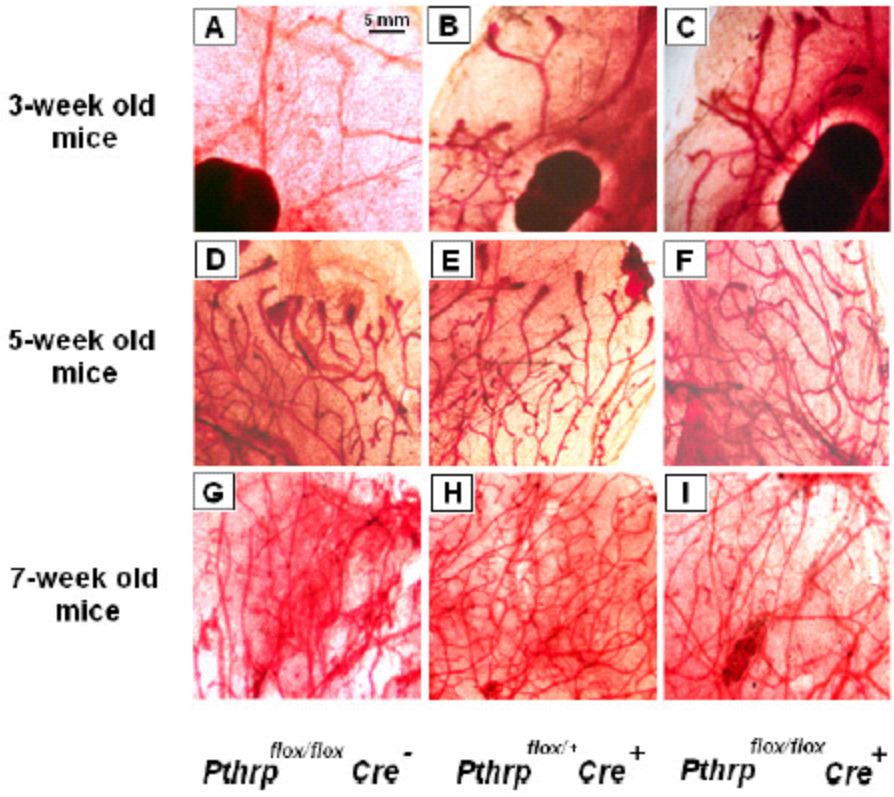

3-week old mice
5-week old mice
7-week old mice
A
5 mm
B
C
D
E
F
G
H
I
Pthrp flox/flox Cre-
Pthrp flox/+ Cre+
Pthrp flox/flox Cre+

Fig.3

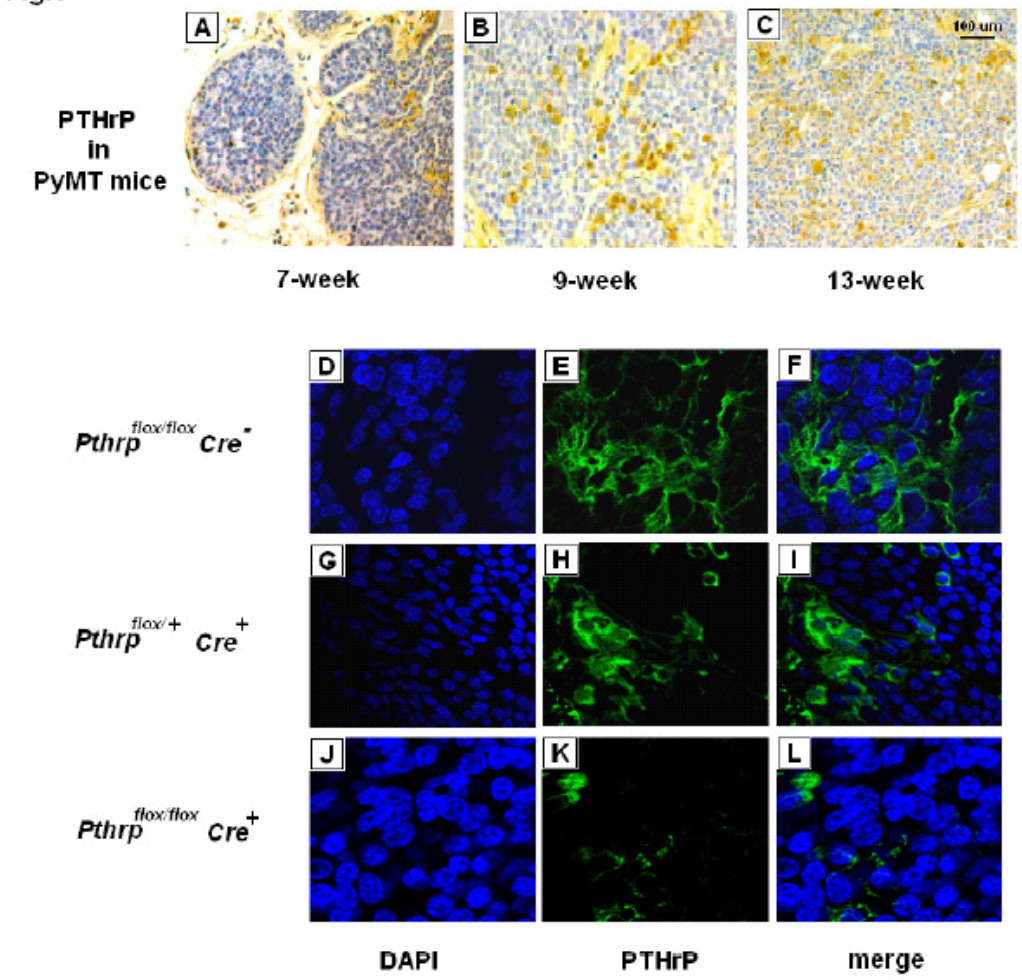

A
B
C
100 um
PTHrP
in
PyMT mice
7-week
9-week
13-week
D
E
F
Pthrp flox/flox Cre-
G
H
I
Pthrp flox/+ Cre+
J
K
L
Pthrp flox/flox Cre+
DAPI
PTHrP
merge
M
Percentage tumor-free mice
Time (days)
flox/+ PthrP Cre-
flox/flox PthrP Cre-
flox/+ PthrP Cre+
flox/flox PthrP Cre+
Controls

Fig.4

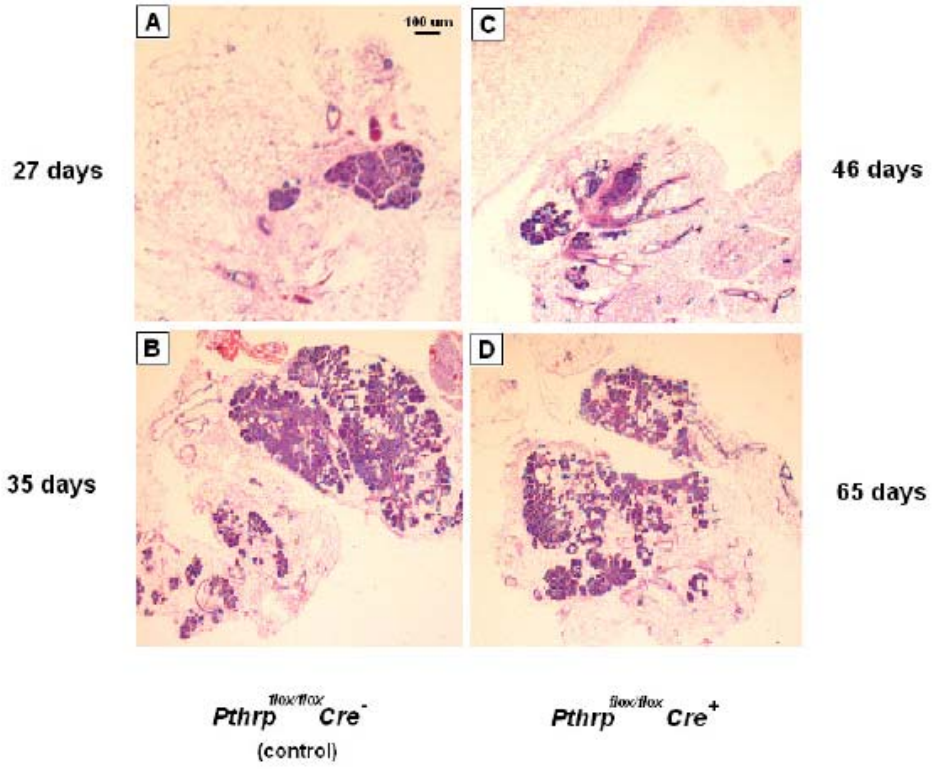

A
100 um
C
27 days
46 days
B
D
35 days
65 days
Pthrp flox/flox Cre-
(control)
Pthrp flox/flox Cre+

Fig.5

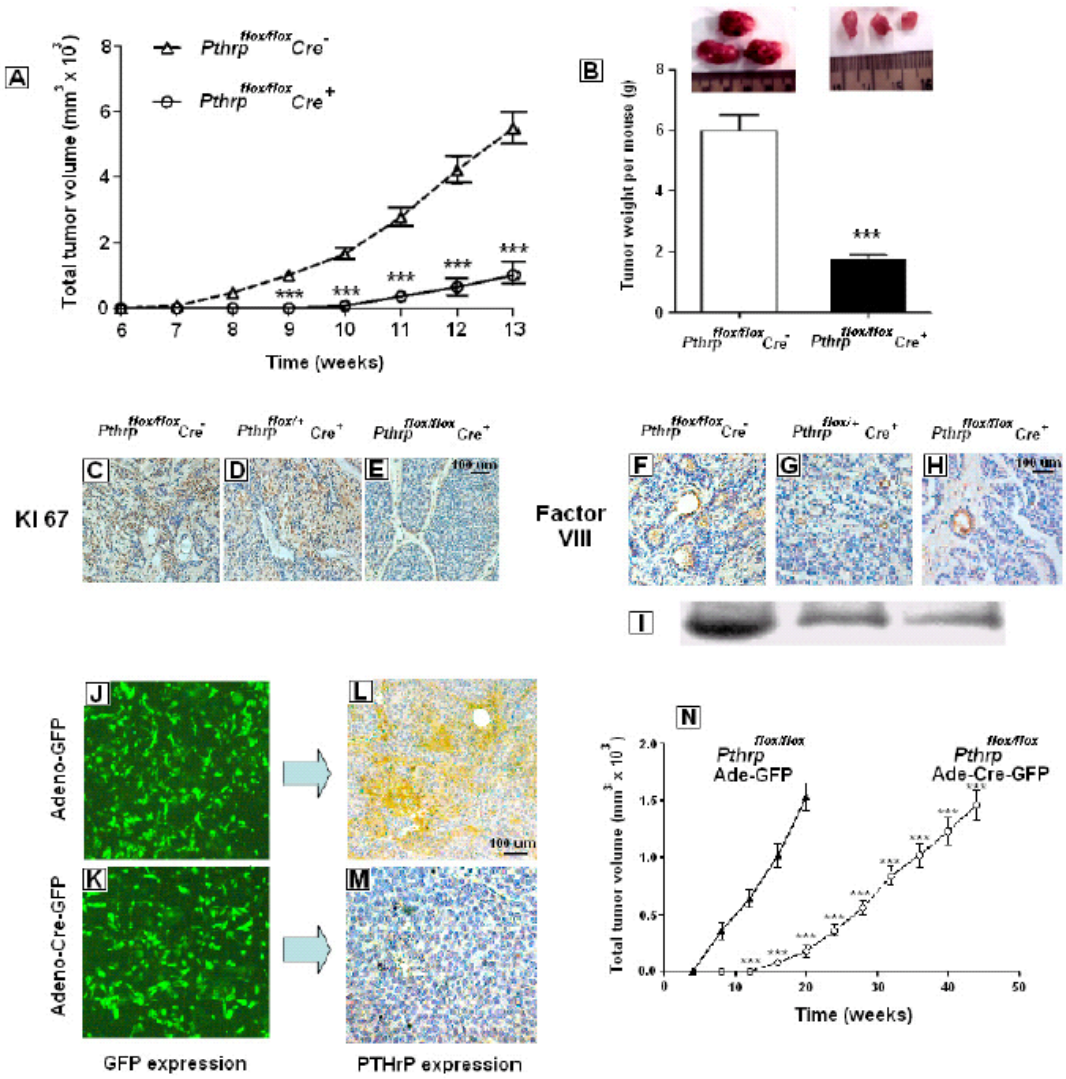

A
Total tumor volume (mm3 x 10)
Pthrp flox/flox Cre-
Pthrp flox/flox Cre+
Time (weeks)
B
Tumor weight per mouse (g)
Pthrp flox/flox Cre-
Pthrp flox/flox Cre+
KI 67
C
D
E
Factor VIII
F
G
H
I
J
K
L
M
Adeno-GFP
Adeno-Cre-GFP
GFP expression
PTHrP expression
N
Pthrp flox/flox Ade-GFP
Pthrp flox/flox Ade-Cre-GFP
Total tumor volume (mm3 x 10)
Time (weeks)

Fig.6

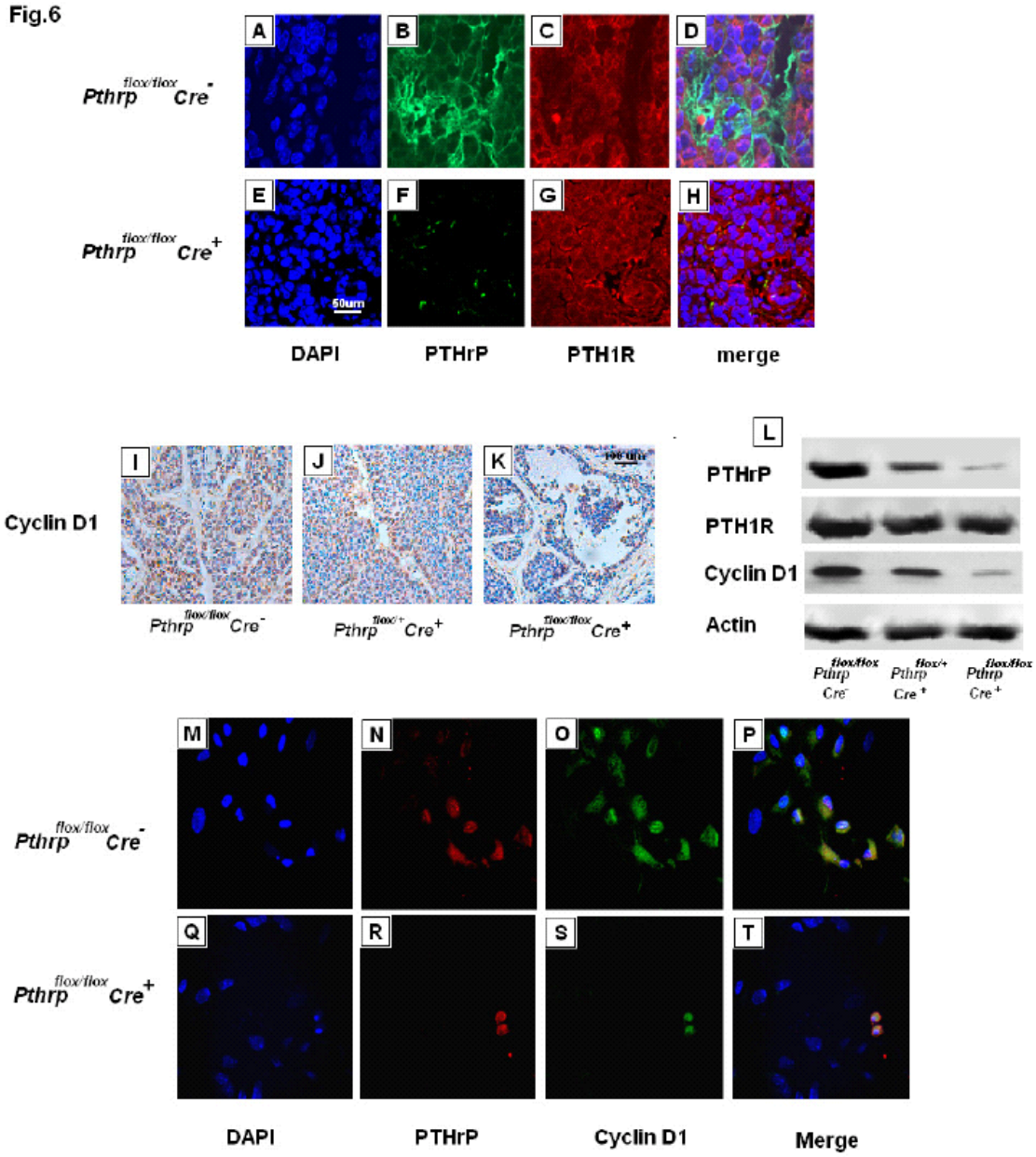

A
B
C
D
E
F
G
H
50um
DAPI
PTHrP
PTH1R
merge
I
J
K
L
Cyclin D1
PTHrP
PTH1R
Cyclin D1
Actin
M
N
O
P
Q
R
S
T
DAPI
PTHrP
Cyclin D1
Merge

Fig.7

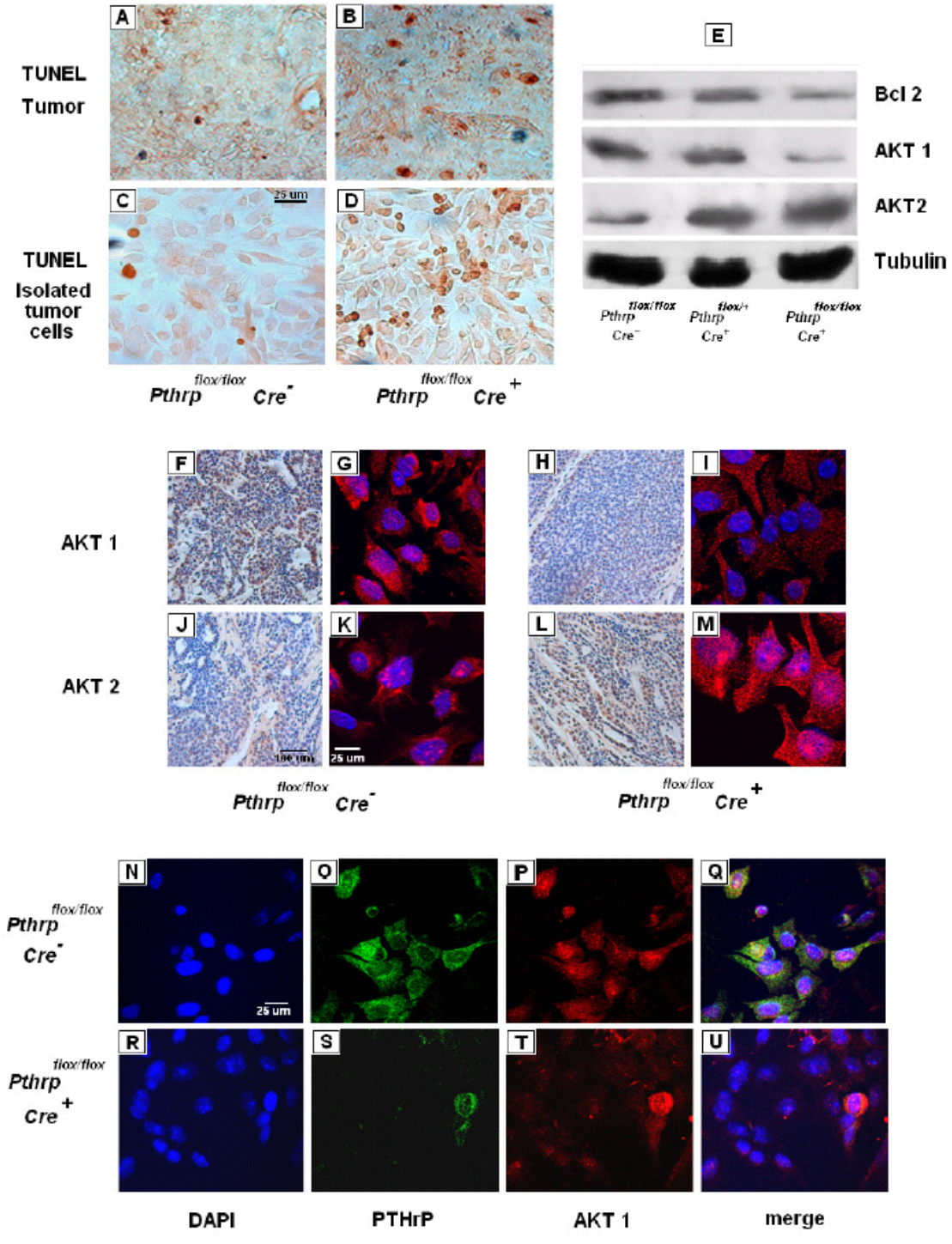
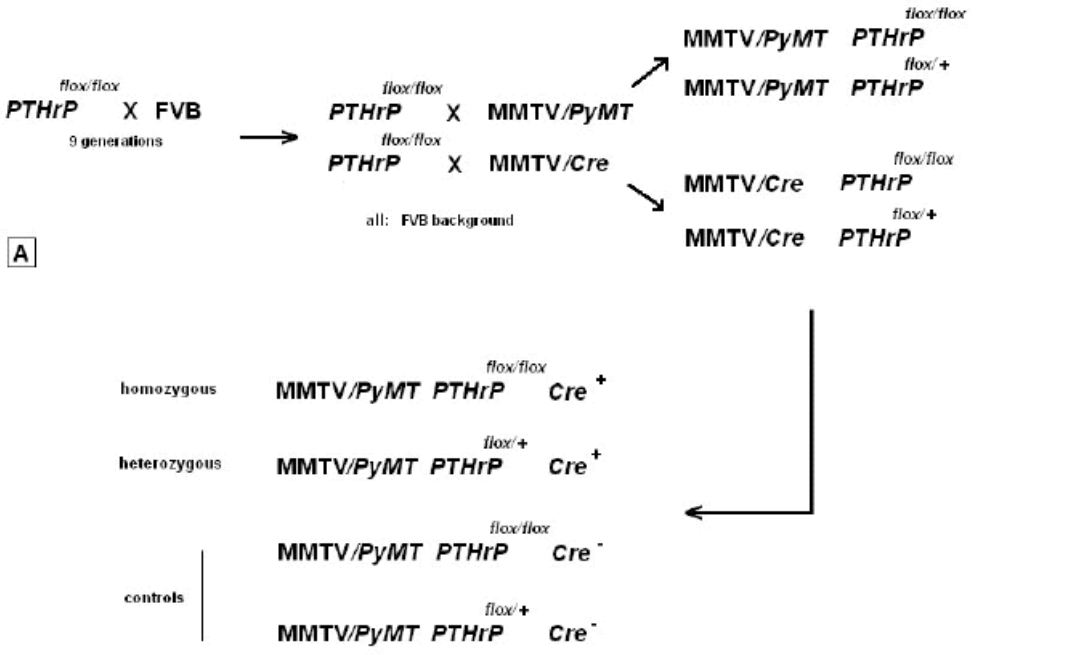

A
B
TUNEL
Tumor
C
D
TUNEL
Isolated
tumor
cells
25 um
Pthrp flox/flox Cre-
Pthrp flox/flox Cre+
E
Bcl 2
AKT 1
AKT2
Tubulin
Pthrp flox/flox Cre-
Pthrp flox/+ Cre+
Pthrp flox/flox Cre+
F
G
H
I
AKT 1
J
K
L
M
AKT 2
25 um
Pthrp flox/flox Cre-
Pthrp flox/flox Cre+
N
O
P
Q
Pthrp flox/flox Cre-
R
S
T
U
Pthrp flox/flox Cre+
25 um
DAPI
PTHrP
AKT 1
merge

Fig S1


flox/flox
PTHrP X FVB
9 generations
flox/flox
PTHrP X MMTV/PyMT
flox/flox
PTHrP X MMTV/Cre
all: FVB background
MMTV/PyMT PTHrP flox/flox
MMTV/PyMT PTHrP flox/+
MMTV/Cre PTHrP flox/flox
MMTV/Cre PTHrP flox/+
A
homozygous
MMTV/PyMT PTHrP flox/flox Cre +
heterozygous
MMTV/PyMT PTHrP flox/+ Cre +
controls
MMTV/PyMT PTHrP flox/flox Cre -
MMTV/PyMT PTHrP flox/+ Cre -

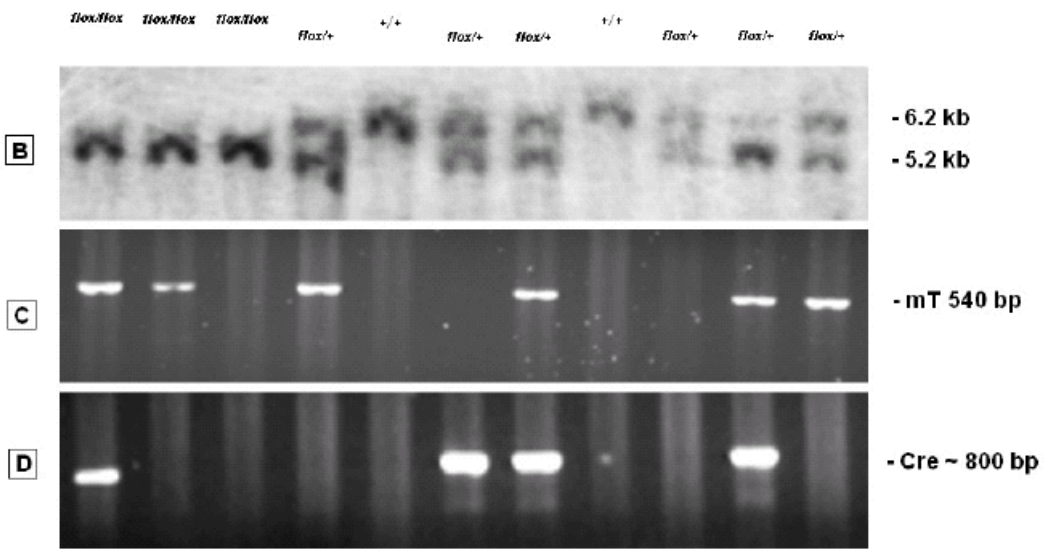
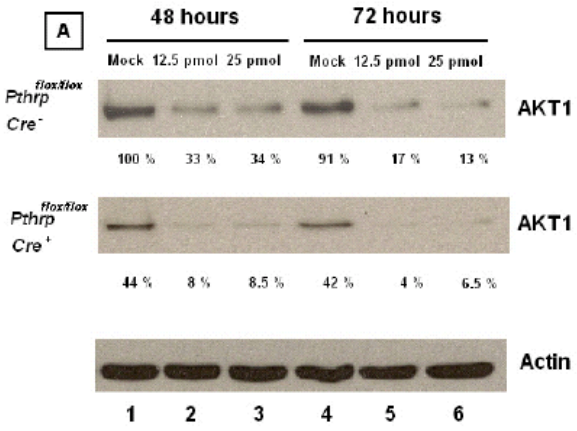
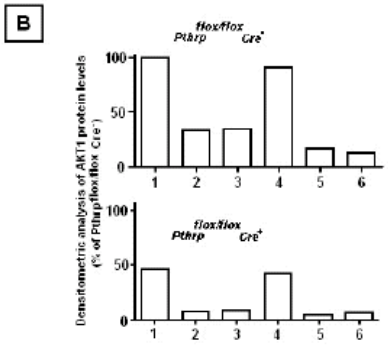
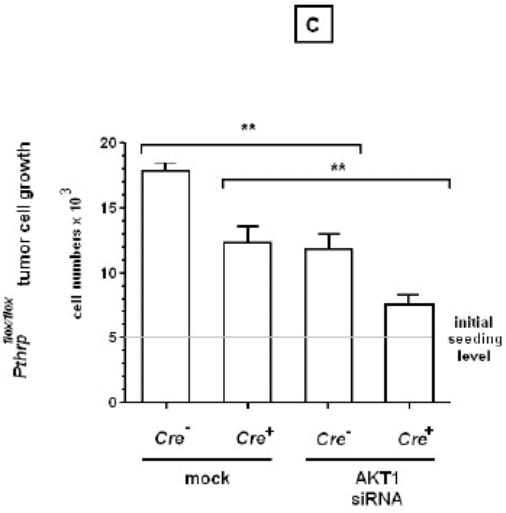

flox/flox
flox/flox
flox/flox
flox/+
+/+
flox/+
flox/+
+/+
flox/+
flox/+
flox/+
B
- 6.2 kb
- 5.2 kb
C
- mT 540 bp
D
- Cre ~ 800 bp

Fig S2


A
48 hours
72 hours
Mock 12.5 pmol 25 pmol
Mock 12.5 pmol 25 pmol
Pthrp flox/flox
Cre-
AKT1
100 % 33 % 34 % 91 % 17 % 13 %
Pthrp flox/flox
Cre+
AKT1
44 % 8 % 8.5 % 42 % 4 % 6.5 %
Actin
1 2 3 4 5 6


B
flox/flox Pthrp Cre-
flox/flox Pthrp Cre+
Densitometric analysis of AKT1 protein levels (% of Pthrp flox/flox Cre-)
100
50
0
1 2 3 4 5 6


C
**
**
Pthrp flox/flox tumor cell growth
cell numbers x 10^3
20
15
10
5
0
initial seeding level
Cre- Cre+ Cre- Cre+
mock
AKT1 siRNA

## Chapter 3

Parathyroid hormone–related protein (*Pthrp*) gene knock-out in mammary epithelial cells inhibits tumor progression and metastasis *in vivo*.

**Rationale and specific aims of this study**

Mammary gland development begins early in embryonic life, and is under PTHrP control at this stage. A PTHrP deficiency results in severe changes in the adult mammary tissue. It therefore appears that PTHrP has a number of regulatory functions in the mammary gland, at several distinct stages of its development and function. However, the effects of PTHrP disruption in the post-natal period have not yet been explored. We use a model in which PTHrP is specifically excised in mammary epithelial cells using the cre-loxPsystem. In this model, it is important to note that Cre is expressed shortly after birth (around 10 days)(Wagner, et al., 2001b) but not during embryonic development (Andrechek, et al., 2000), making the system suitable for the study of PTHrP roles in mammary development during puberty as well as in tumour initiation arising from the normal mammary epithelium.

PTHrP is expressed in normal epithelial cells but its expression increases in breast cancer and becomes associated with multiple metastatic lesions and reduced survival. It is however still unknown whether PTHrP overexpression is simply a consequence of tumour progression, or whether it is mechanistically linked to the tumour progression process from initiation to metastasis. In order to shed light on this relationship, we propose to ablate the *Pthrp* gene in mammary epithelial cells and to determine the consequences of this ablation on tumour initiation, growth and metastasis.

Parathyroid hormone–related protein (*Pthrp*) gene knock-out in mammary epithelial cells inhibits tumor progression and metastasis *in vivo*.

**Authors**: Jiarong Li, Andrew C. Karaplis*, Peter Siegel**, William J. Muller**, Richard Kremer

**Affiliations**: Department of Medicine, McGill University Health Centre, 687 Pine Ave West, Montreal, QC, Canada, H3A1A1, *Lady Davis Institute, Jewish General Hospital, 3755 Côte Ste Catherine, Montréal, QC, Canada, H3T 1E2, and ** McGill Cancer Centre, McGill University, Montreal, QC Canada, H3A 1A1.

**Contact information**: correspondence: richard.kremer@mcgill.ca , phone: (514) 843-1632, fax: (514) 843-1712

**Acknowledgements**: Grant to R.K.: Operating grant from the Canadian Institutes of Health Research (CIHR) MOP 10839; grant to A.K.: Concept Award from the U.S. Department of Defense Breast Cancer Research Program (BCRP) BC023897.

**Running title**: *Pthrp* knockout inhibits breast cancer metastasis

**Abstract:**

In order to investigate the various roles of parathyroid hormone-related protein (PTHrP) in the breast cancer metastatic process, a Cre/loxP system that disrupts the *Pthrp* gene in the mammary epithelium was introduced into the MMTV/PyVMT transgenic mouse model of breast cancer. Our previous work showed that tumors initiate at a much slower rate in *Pthrp*-ablated animals than in control MMTV/PyVMT mice (Li et al, Chapter 2). Here, we demonstrate that cells derived from $Pthrp^{flox/flox}$; $Cre^{+}$ tumors present reduced invasiveness *in vitro* and *in vivo*, and that *Pthrp* ablation reduces the number of tumor cells in peripheral blood, bone marrow and lungs with respect to control animals. Reduction of expression or ablation of the *Pthrp* gene in this system results in the formation of fewer and smaller metastatic lesions through a mechanism involving a decrease in the expression of the chemokine *CXCR4*.

**Introduction:**

Parathyroid hormone-related protein (PTHrP) is a secreted protein expressed in almost all normal fetal and adult tissues where it acts as an autocrine, paracrine or intracrine factor in multiple developmental and physiological processes (Moseley & Gillespie, 1995; Philbrick, et al., 1996; Strewler, 2000). PTHrP has growth-promoting and anti-apoptotic properties, and plays a particularly crucial role in the development of the mammary gland and the skeleton (Karaplis, et al., 1994; Moseley & Gillespie, 1995; Philbrick, et al., 1996; Strewler, 2000; Vortkamp, et al., 1996; Wysolmerski, et al., 1998). PTHrP was discovered as the causative agent of malignancy-associated hypercalcemia (MAH) (Suva, et al., 1987), and dysregulated expression of PTHrP has become associated with the more advanced stages of cancer (Kremer, et al., 1996; Rankin, et al.,

1997; Truong, et al., 2003). Of special interest to us is the association between the increased production of PTHrP by human breast cancer tumors and the development of metastases, as metastasis remains the principal cause of morbidity and mortality in cancer patients.

Metastases are catastrophic consequences of the progression of solid tumors such as breast cancer, and greatly affect patients' quality of life and survival. Breast cancer commonly metastasizes to other organs, with bone and lung as preferred sites (Mundy, 2002; Roodman, 2004; Solomayer, Diel, Meyberg, Gollan, & Bastert, 2000). Skeletal invasions, in particular, are present in close to 70% of patients who die from breast cancer (Coleman & Rubens, 1987) and PTHrP is expressed in more than 90% of these skeletal tumors (Vargas, et al., 1992). Similarly, PTHrP is present in a high percentage of lung tumors of all histological types, with expression increasing from early stage to more advanced and aggressive tumors (Brandt, et al., 1991; Hastings, Araiza, Burton, Bedley, & Deftos, 2004; Hidaka, Nishimura, & Nagao, 1998).

The mechanisms which determine the site specificity for establishment of metastases have not been completely elucidated, but it is now clear that the volume of the blood flow through a certain organ is not the only factor accounting for the successful colonization by tumor cells. There is also the crucial presence of local growth factors which can help the establishment of cancer cells. For example, within the skeletal microenvironment, several growth factors are known to be released by bone cells as a consequence of overproduction of PTHrP by invading tumor cells. PTHrP activates osteoblasts to produce RANKL (receptor activator of nuclear factor-kB ligand), and downregulate osteoprotegerin (OPG). This causes an osteoclast-driven osteolytic release of immobilized transforming growth factor β, insulin-like growth factors I and II,

fibroblast growth factors, bone morphogeneic proteins and calcium. In a "vicious cycle" mechanism, these growth factors provide a feedback stimulation to the invading tumor cells which stimulates further PTHrP production and favours the establishment of cancer within the bone (Goltzman, 1997; Guise, 2000; Guise & Mundy, 1998a; Guise, et al., 1996; Kakonen & Mundy, 2003; Mundy, 2002; Roodman, 2004; Thomas, et al., 1999). A third process favoring the homing and establishment of tumor cells at sites of special avidity has recently been reported and involves the evolutionary-conserved chemokine superfamily whose role it is to regulate the specific targeting of migrating cells within an organism (Muller, et al., 2001; Zlotnik, 2004, 2008). Tumor cells such as those from breast cancer have been found to produce chemokine receptors in a non-random fashion, for ex. the chemokine C-X-C motif receptor 4 (CXCR4) which promotes adhesion to specific cells presenting the appropriate surface ligand (in this case CXCL12, also known as SDF1). The CXCL12/CXCR4 axis is involved in several aspects of tumor progression including angiogengesis, metastasis, and survival (Petit, Jin, & Rafii, 2007; Teicher & Fricker). Many of the organs with the highest expression of CXCL12 correlate with the most common breast cancer metastasis sites (Luker & Luker, 2006). The consequence is not only adhesion of the tumor cell to the ligand-expressing cell, but actin polymerization and pseudopodia formation in cancer cells, resulting in enhanced invasiveness (Muller, et al., 2001). The CXCR4/CXCL12 combination is the most important chemokine mechanism regulating metastatic potential to the bone marrow, lungs, liver and brain (Luker & Luker, 2006; Zlotnik, 2004, 2008). The binding of CXCL12 to CXCR4 stimulates PI3K kinase which then activates the protein kinase AKT, resulting in a variety of responses such as cell survival and /or proliferation, gene transcription. It has been postulated that AKT is a key effector of CXCR4 in breast cancer in vivo (Luker &

Luker, 2006; Teicher & Fricker). Convincingly, neutralization of CXCR4/CXCL12 interactions *in vivo* by antibodies (Muller, et al., 2001), inhibitory peptides (Hatse, Princen, Bridger, De Clercq, & Schols, 2002; Liang, et al., 2004; Liang, et al., 2005), and siRNA (Liang, et al., 2005) attenuates metastases in mouse models, and this chemokine/receptor pair has become a potential target for cancer therapy.

Several retrospective studies suggested that breast cancer patients with PTHrP-positive primary tumors were more likely to develop bone metastases compared to those with PTHrP-negative tumors (Bundred, et al., 1991; Bundred, et al., 1992; Southby, et al., 1990). A prospective study by Kremer's team conducted in patients with MAH including breast cancer patients concluded that elevated serum levels of PTHrP are associated with poor prognosis and reduced survival (Truong, et al., 2003). Although it is well recognized that PTHrP acts as a local cytokine to enhance breast cancer expansion in bone, its role in primary tumor progression remains controversial (Nishihara, et al., 2007) and its function in the spread of tumor cells from the primary tumor to metastatic sites needs to be established. In this paper, we show that *Pthrp* ablation in breast tumor cells reduces invasiveness and metastatic potential, and is accompanied by a significant reduction in the levels of expression of the chemokine CXCR4. This constitutes a novel role for PTHrP, and identifies it as a putative target for countering breast cancer metastases.

**Experimental procedures**:

**Animals**:

We used homozygous *Pthrp*$^{flox/flox}$ C57BL6 mice where two loxP sites flank the *Pthrp* gene exon 4 which encodes the major portion of the PTHrP protein (He, et al., 2001). This genotype was introduced into the FVB/NJ background by sequential backcrossing of *Pthrp*$^{flox/flox}$ C57BL6 males with FVB/NJ females. The F1 heterozygous (*Pthrp*$^{flox/+}$) males were crossed with FVB/NJ females to produce a heterozygous (*Pthrp*$^{flox/+}$) F2 colony, and the strategy was repeated until the F8 generation, when animals were sent to the Jackson Laboratories and confirmed to have a greater than 99% FVB/NJ background. MMTV/PyVMT (Guy, et al., 1992b), and MMTV/Cre mice (Andrechek, et al., 2000) on a pure FVB/NJ background were kindly provided by Dr William J. Muller (Department of Biochemistry, McGill University), and crossed with *Pthrp*$^{flox/flox}$ animals with the same background. We obtained male MMTV/PyVMT MMTV/Cre *Pthrp*$^{flox/+}$ and female MMTV/Cre *Pthrp*$^{flox/+}$ , which were crossed to produce MMTV/PyVMT MMTV/Cre *Pthrp*$^{flox/flox}$ (homozygous), MMTV/PyVMT MMTV/Cre *Pthrp*$^{flox/+}$ (heterozygous), and MMTV/PyVMT *Pthrp*$^{flox/flox}$ and MMTV/PyVMT *Pthrp*$^{flox/+}$ (Cre$^{-}$ controls). Genotyping was performed by PCR and Southern blotting using ear punches and tail DNA obtained at weaning. All animals were housed in the Royal Victoria Central Animal Care Facility, and all experiments were carried out in compliance with the regulations of the McGill University institutional animal care committee.

**Antibodies and reagents**:

The mouse monoclonal antibody against PTHrP (1-34, AE-0502) was purchased from IDS Ltd (U.K.). The monoclonal antibodies against Cre recombinase, CXCR4, and the mT oncogene were from Abcam (Cambridge, MA), and all other antibodies were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 555 conjugated goat anti-rabbit IgG and Alexa Fluor goat anti-mouse IgG were obtained from InVitrogen.

**Immunohistochemistry**:

Immunohistochemistry staining was performed using the SK-4100 kit from Vector (Burlingame, CA). Endogenous peroxidase activity was quenched by incubating the slides in 3% $H_2O_2$ (Fisher) for 30 minutes, then washing 3 times for 5 minutes with PBST (PBS containing 0.2% Tween-20, Sigma-Aldrich, Oakville, ON). Slides were then incubated in 10% normal blocking serum (Normal Sera for Immunohistochemistry Sigma-Aldrich, Oakville, ON) in PBS for 30 minutes to suppress non-specific IgG binding. Blocking serum was from the same species in which the secondary antibody was raised. The slides were incubated in the presence of the primary antibody at room temperature for 2 hours, then washed 3 times (5 min each) with PBST buffer. The slides were incubated for 1 hour with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature, washed 3 times (5 min each) in PBST, diaminobenzidine (DAB) substrate solution was added and incubated until the desired level of staining was achieved (typically 5 to 15 min). The slides were washed 3 times (5 min each) in PBST and counterstained with hematoxylin. Slides were dehydrated, cleared and mounted as above. Results were analyzed using a Leica DMR microscope and Bioquant Nova Prime software (Bioquant Image Analysis Corporation, Nashville, TN).

**Immunofluorescence staining**:

Immunoflurorescence staining was conducted according to InVitrogen's instructions (Burlington, ON), using deparaffinized tissue sections or cells fixed on slides. Antigen unmasking was conducted by heating slides 2 minutes in a microwave

oven. Specimens were incubated in 10% normal blocking serum in PBS for 20 minutes to suppress non-specific IgG binding, washed for 3 min in PBST, and incubated with the primary antibody for 60 minutes. The slides were washed 5 min with PBST and incubated 45 minutes in a dark chamber with the fluorochrome-conjugated secondary antibody (diluted to 1–5 μg/ml in PBS with 1.5%–3% normal blocking serum). Slides were washed with three changes of PBST (5 min each), counterstained in the dark with DAPI (InVitrogen) for 15 minutes, washed with three changes of PBST and mounted under coverslip with aqueous mounting medium (Thermo Electron corp. Pittsburgh, PA). Results were analyzed with an LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging Gmbh, Munich, Germany).

**Histology**:

All samples were fixed with phosphate-buffered saline (PBS)-buffered 4% formaldehyde (v/v) for 24 hours. Tissues were paraffin-embedded, sliced into 5 micron-thick sections and stained with hematoxylin and eosin (H and E). Cultured cells were grown on sterile glass coverslips overnight at 37$^{o}$C, rinsed with PBS, fixed 10 minutes in 1% formalin, rinsed 3 times with PBS and used for staining.

Bone samples were demineralised as follows: the fixed bone tissue was washed in PBS, and incubated with agitation at 4$^{o}$C for 7-10 days in 0.5 M EDTA (pH 7.4), 7.5% glycerol and 15% sucrose. The bones were rinsed several times in PBS containing 7.5% glycerol and 15% sucrose, then rinsed successively in 15% sucrose, 10% sucrose and distilled water. The bones were dehydrated and embedded in methacrylate.

**Western blotting:**

Proteins were extracted from tissues and cells with an extraction buffer containing 50mM Tris, 150 mM NaCl and 1 protease inhibitor cocktail tablet per 100 ml buffer (Roche). Protein concentrations were estimated with the Bradford assay kit (Bio-Rad, Mississauga, ON). Protein samples (30-50 μg) were fractionated by SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with 5% skimmed milk and immunoblotted with primary antibodies. After incubation with an appropriate secondary antibody, the blots were developed by enhanced chemiluminescence using ECL$^{TM}$ Western Blotting System (Baie d'Urfe, QC).and KODAK Film (Carestream Health Inc. Rochester, NY)

**Isolation and culture of mouse breast tumor cells from primary tumors:**

Mammary tumors were harvested, washed twice in ice-cold PBS, cut into small pieces and incubated in 2.4 mg/ml collagenase B (Roche, Laval, QC) and dispase II (Roche) in DMEM growth medium containing 1% penicillin-sreptomycin (InVitrogen) at 37$^{o}$C for 2 hours. Floating cells were washed twice with ice-cold PBS, pelleted, resuspended in a mixture of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and further propagated in this medium.

**Tumor cell isolation and detection from peripheral blood and in the bone marrow**

13 weeks *Pthrp*$^{flox/flox;}$ Cre$^{-}$ (controls) and 18 weeks *Pthrp*$^{flox/flox;}$ Cre$^{+}$ animals were sacrificed when the primary tumors attained similar volumes. 1 ml of fresh whole blood was collected by cardiac puncture .The bone marrow was flushed from the tibias and femurs, triturated through a 25 gauge needle with PBS containing 2% fetal bovine serum

(FBS) to make a single cell suspension , spun at 300 x g for 5 min and the pellet resuspended in 1ml of PBS. Erythrocytes were lysed by adding 3 volumes of RBC 1x lysis buffer (Qiagen, Mississauga, ON) incubated for 30 min and centrifuged at 300 x g for 5 min.The supernatant was discarded , the cell pellet resuspended in 1 ml PBS and the lysis step repeated. Samples were centrifuged, the pellet resuspended in 0.2 ml PBS and spread onto a clearly-defined area of a Cytospin immunoselect adhesion slide (Squarix GmbH, Germany).and tumor cells detected using a pan-cytokeratin monoclonal antibody A45-B/B3 (Epimet epithelial cell detection kit, Micromet AG, Munich, Germany. A Fab fragment of a monoclonal antibody with irrelevant specificity (anti-FITC) conjugated to alkaline phosphatase was used as a negative control.All of the enrichment steps and centrifugations were performed at room temperature.

.

**Matrigel invasion and wound-healing assays**:

Tumor cells were isolated from 13 weeks *Pthrp*$^{flox/flox;}$ Cre$^{-}$ (controls) and 18 weeks *Pthrp*$^{flox/flox;}$ Cre$^{+}$ animals .Tumor cells isolated from tumors or the human MDAMB-435 breast cancer cell line (ATCC, Manassas, VA) were tested with the BD Biocoat Tumor Invasion System (BD, Mississauga, ON). The tumor cells were grown to 80% confluence, then trypsinised and counted. They were plated (1 x $10^5$ cells in 200 µl of serum free DMEM in the upper chamber of rehydrated Matrigel well inserts. 300 µl of DMEM containing 5% FBS (chemoattractant) were added to the lower chamber and the chambers incubated at 37° C for 20-24 h. The medium was aspirated from the lower chamber; 0.5 ml of 4% paraformaldehyde in PBS was added and left for 10 min. The fixative was removed; the wells rinsed with PBS and stained with 1 ml Mayer's

haematoxylin solution for 3 min. The non-invading cells were removed from the top of the transwell with a cotton swab, and the invading cells counted under light microscopy.

For wound healing (motility) assays cells were plated in 6-well plates pre-coated with poly-D-lysine (Milliopore Billerica, Massachusetts) and allowed to reach 90% confluence. The cell carpet was scraped to create a wound with a p200 pipette attached to a sterile tip and the growth of the plated cells into the scraped area monitored microscopically over a period of 24 hours according to the method of Rodriguez et al (Rodriguez L.G, Wu X, Guan J.L Wound-Healing assay in: Methods In Molecular Biology, Vol. 294: Cell Migration: Developmental Methods and protocols. Edited by J-L. Guan. Humana Press Inc. Totowa, NJ P23-29.

**Infection of primary mammary tumor cells with adenovirus GFP-Cre vector.**

A recombinant adenovirus vector containing a green fluorescent protein (GFP)-Cre transgene for the Cre recombinase was a generous gift from Dr. William J. Muller (McGill Cancer Center, McGill University). Primary tumor cells were infected according to standard protocols. Briefly, primary tumor cells were grown 70% confluence, and infected by overlaying a solution of $10^8$ viral particles per ml in DMEM growth medium for 60-90 min at 37$^o$C. The cells were washed twice in PBS, replaced in growth medium and harvested 48-60 hours after infection. Experimental procedures followed regulations of the McGill University Health Center biosafety committee.

**Flow cytometric cell selection and mammary gland injection:**

Cells were harvested 48h post-adenovirus infection and processed for positive GFP selection using flow cytometric sorting (BD FACSAria II cell sorter, BD Biosciences, San Jose, CA). Selected GFP-positive cells were injected into 5-week old syngeneic FVB mice (Charles River Laboratories, Senneville, QC). The animals were first anesthetized with ketamine (100mg/ml) at 100-200mg/kg, the skin of the fourth mammary gland was incised and GFP-positive tumor cells ($0.5 \times 10^6$ cells/gland) in a 50 μl volume of PBS were injected into the fat pad.

**Results**:

The targeted ablation of the *Pthrp* gene in the mammary epithelium of the MMTV-PyMT mouse model of breast cancer was shown by our group to cause a significant delay in the initiation of breast tumors, as well as a reduction in size and numbers for the tumors which eventually develop in the ablated animals with respect to controls (Li et al chap 2). In the present study, we examine the consequences of the same ablation on the subsequent metastatic events which normally occur during the development of breast tumors in the MMTV-PyVMT model system.

**Cells derived from *Pthrp*$^{flox/flox}$; Cre$^{+}$ tumors show reduced motility and migration capacity *in vitro* with respect to control cells with full *Pthrp* expression**:

Growth assays in Matrigel revealed that cells derived from *Pthrp*$^{flox/flox}$; Cre$^{-}$ tumors (control animals, with full *Pthrp* expression) possess more than twice the

invasiveness potential of the cells originating from *Pthrp*$^{flox/flox}$; Cre$^{+}$ mice (ablated *Pthrp*) (Figure 1 a), and migration capacity performance *in vitro* (cell motility after wounding) was qualitatively reduced in tumor-derived cells where *Pthrp* expression had been eliminated, with respect to controls (Figure 1 b).

***Pthrp* ablation reduces numbers of tumor cells in peripheral blood, bone marrow and lungs**:

The number of circulating tumor cells (CTCs) in animals with same size tumors (13 weeks for controls and 18 weeks for *Pthrp*$^{flox/flox;}$ Cre$^{+}$ animals) is lower in mice with reduced or ablated levels of *Pthrp* (Cre$^{+}$) than in Cre$^{-}$ controls (EPIMET stain Figure 2a). In bone marrow, tumor cells are detectable by cytokeratin staining only in control animals with normal PTHrP expression (Figure 2b).

Similarly, an incremental decrease in *Pthrp* expression (control, heterozygous, homozygous mice) reduces the number of hyperplastic sites and metastases in lung tissues (Figure 3 a). When animals of all genotypes are dissected at 13 weeks, 100% (45/45) of control mice (*Pthrp*$^{flox/flox}$; Cre$^{-}$ or *Pthrp*$^{flox/+;}$ Cre$^{-}$ ) present lung metastases, while metastases can be detected in only 47% (8/17) of *Pthrp*$^{flox/+;}$ Cre$^{+}$ heterozygous mice at that stage. There are no lung metastases in *Pthrp*$^{flox/flox}$; Cre$^{+}$ homozygous animals at 13 weeks (0/18), as the eventual metastases which appear in *Pthrp*-ablated mice are not yet detectable at that stage. When animals are dissected at 18 weeks, there are lung metastases in 90% (10/11) of heterozygous mice, and in 40% (12/30) of homozygous animals (Figure 3 b). The control mice are unavailable as they must be euthanized before 18 weeks for humane reasons.

In order to prevent the development of metastases from tumor cells which avoided the knock-out and achieve a more uniform ablated population, tumor cells derived from

*Pthrp*$^{flox/flox}$; Cre$^{-}$ tumors were transfected with adenoGFP or adenoCre GFP and sorted by flow cytometry as described in our previous work (Li et al chap 2). When tumor cells were transfected with an adenoCre GFP or adeno GFP control virus as described above, then purified and implanted (5 x $10^5$ cells) in the fourth MFP of syngeneic mice, 70% (16/23) of control animals (*Pthrp*$^{flox/flox}$; Cre$^{-}$ or *Pthrp*$^{flox/+}$; Cre$^{-}$) presented lung metastases at 8 weeks, while tumors of the same size were present in 55% (6/11) of heterozygous (*Pthrp*$^{flox/+}$; Cre$^{+}$) at 11 weeks, and in 0% (0/22) of homozygous mice (*Pthrp*$^{flox/flox}$; Cre$^{+}$) at 16 weeks (Figure 3 c). Metastases in the homozygous *Pthrp*-ablated mice would likely appear in later stages, but animals injected with tumor cells must be sacrificed when tumor size at injection site reaches 1.5 $cm^3$, preventing observations at higher tumor loads.

It is important to note that when *Pthrp*-ablated mice finally present lung metastases (at 18 weeks), the PTHrP expression in their cells is as abundant as that of lung metastases found in controls (at 13 weeks), indicating that the tumors which eventually develop in homozygous animals are entirely derived from the residual cells that originally escaped the *Pthrp* gene ablation (Figure 3 d).

***Pthrp* ablation reduces the expression of the chemokine receptor *CXCR4***:

Immunofluorescence staining and Western blotting reveal that *Pthrp* ablation greatly reduces the levels of expression of the metastasis marker CXCR4 in the primary tumors of the same size (Figure 4 a, d) suggesting that CXCR4 functions downstream of PTHrP. Immunofluorescence staining for PTHrP and CXCR4 of cells isolated from the primary tumors of control and homozygous animals indicate that CXCR4 expression is exclusively limited to the cells which have escaped *Pthrp* ablation (Figure 4 b). When metastatic lung tumors eventually appear at 18 weeks in *Pthrp*$^{flox/flox:}$ Cre$^{+}$ mice (no

adenoCre transfection), the expression of PTHrP and CXCR4 is comparable to that of controls at 13 weeks (Figure 4 c), indicating the fact that the late lung metastases which develop in homozygous animals are entirely derived from the residual cells that originally escaped the *Pthrp* gene ablation.

**Discussion:**

The targeted ablation of *Pthrp* gene activity from the mammary epithelium in MMTV-PyVMT mice was demonstrated in our previous work to substantially delay breast cancer initiation without affecting mammary gland development (Li et al chap 2). In the present study, we show that the subsequent metastatic stages of breast cancer progression are similarly hindered when PTHrP is not expressed in the mammary epithelium. *Pthrp* ablation substantially reduces the number of tumor cells in peripheral blood, bone marrow and lungs, and a more complete knock-out mediated by adenoCre results in total absence of metastases in the lungs.

**CXCR4 expression is linked to PTHrP levels**:

In the present study, we have observed that not only is *Pthrp* ablation accompanied by a significant decrease in the expression of the chemokine receptor CXCR4, but that the small percentage of residual expression of PTHrP and CXCR4 in ablated tumors co-localizes to the same cells which escaped the knock-out. In the tumors that eventually develop from $Pthrp^{flox/flox;}Cre^{+}$ cells that were not adenoCre-infected, the generalized expression of PTHrP and CXCR4 observed indicates that it is the PTHrP/CXCR4 double-positive cells that are selected to create the invasive

population which develops into the tardy metastases eventually observed in the *Pthrp*$^{flox/flox}$; Cre$^{+}$ derived populations.

The chemokine CXCL12 and its cognate receptor CXCR4 hold crucial roles in primary and metastatic breast cancer and other malignancies such as lung, brain and liver, through their stimulation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway leading to cell survival (Luker & Luker, 2006; Muller, et al., 2001; Zhao, Mueller, DiScipio, & Schraufstatter, 2008). CXCR4/CXCL12 interaction activates angiogenesis, chemotaxis, invasion, and adhesion processes that reinforce the metastatic profile. The levels of CXCR4 in breast tumors are known to increase with cancer progression (Allinen, et al., 2004), and neutralization of CXCR4/CXCL12 interactions *in vivo* by antibodies (Muller, et al., 2001), inhibitory peptides (Hatse, et al., 2002; Liang, et al., 2004; Liang, et al., 2005), and siRNA (Liang, et al., 2005) attenuates metastases in mouse models. Furthermore, CXCR4 has been identified as a signature gene in primary breast cancers expressing the lung and the bone metastasis signature (H. Kang, et al., 2005; Y. Kang, et al., 2003; Minn, et al., 2005).

The upstream controls of the CXCR4 chemokine have not yet been very well defined. CXCR4 expression appears to be regulated through multiple mechanisms involving translational and transcriptional control, and mRNA and protein stability (Luker & Luker, 2006). The only negative regulation elements reported to date are the YY1 factor binding to the CXCR4 promoter to decrease transcription (Lee, et al., 2005), and the AIP4 ligand-dependent degradation which reduces CXCR4 protein stability (Marchese, et al., 2003). Our observation that the targeted removal of PTHrP in the mammary epithelium results in a sharp reduction in levels of the CXCR4 chemokine, coupled with our previous observation that total protein levels for AKT follow a similar

drop (Li et al chap 2), suggest that PTHrP may be an important control for the CXCR4/CXCL12 signalling pathway in breast cancer initiation and metastasis.

C-Src is non-receptor tyrosine protein kinase whose aberrant expression is common in many tumors and is correlated with poor outcome. C-Src has been reported to be required for CXCL12 activation of AKT, and to regulate the competence of breast cancer cells for survival (Zhang, et al., 2009). However, the PTHrP site of action appears to be located upstream of c-Src control, as PTHrP affects both AKT and CXCR4 receptor levels to control metastatic development. This suggests that PTHrP could be targeted in a novel therapeutic approach aimed at reducing breast cancer invasive properties.

**Figure 1**

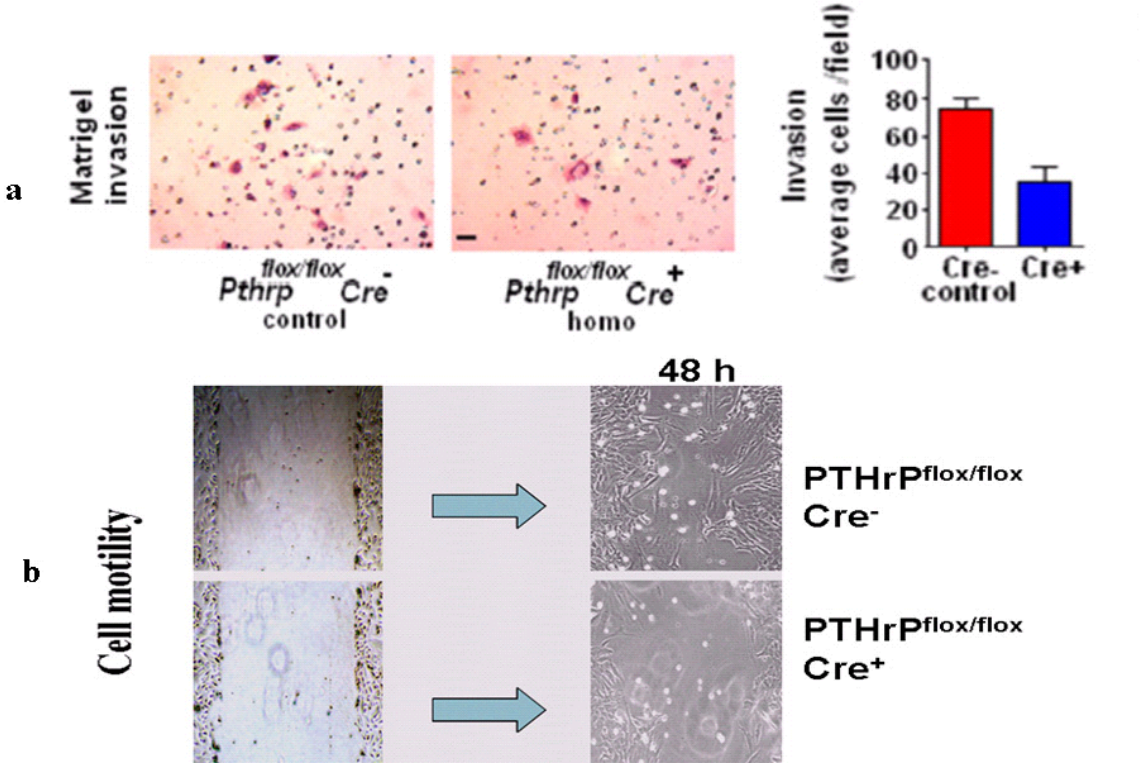



Figure 1: Cells derived from *Pthrp*$^{flox/flox}$; Cre$^{+}$ tumors show reduced invasiveness *in vitro* with respect to controls with full *Pthrp* expression: **(a)** Growth in Matrigel (22 hrs) of tumor cells from *Pthrp*$^{flox/flox}$; Cre$^{-}$ or Cre$^{+}$ tumors, and comparative histogram of cell numbers/field for each genotype. *** $P < 0.001$ (**b**) Cell motility test after wounding (48 hours). Tumor cells were grown to confluence then wounded by scraping; after 48 hours of growth, the motility of *Pthrp*$^{flox/flox}$; Cre$^{-}$ (control) tumor-derived cells is superior to that of Cre$^{+}$ (*Pthrp*-ablated) cells.

# Figure 2

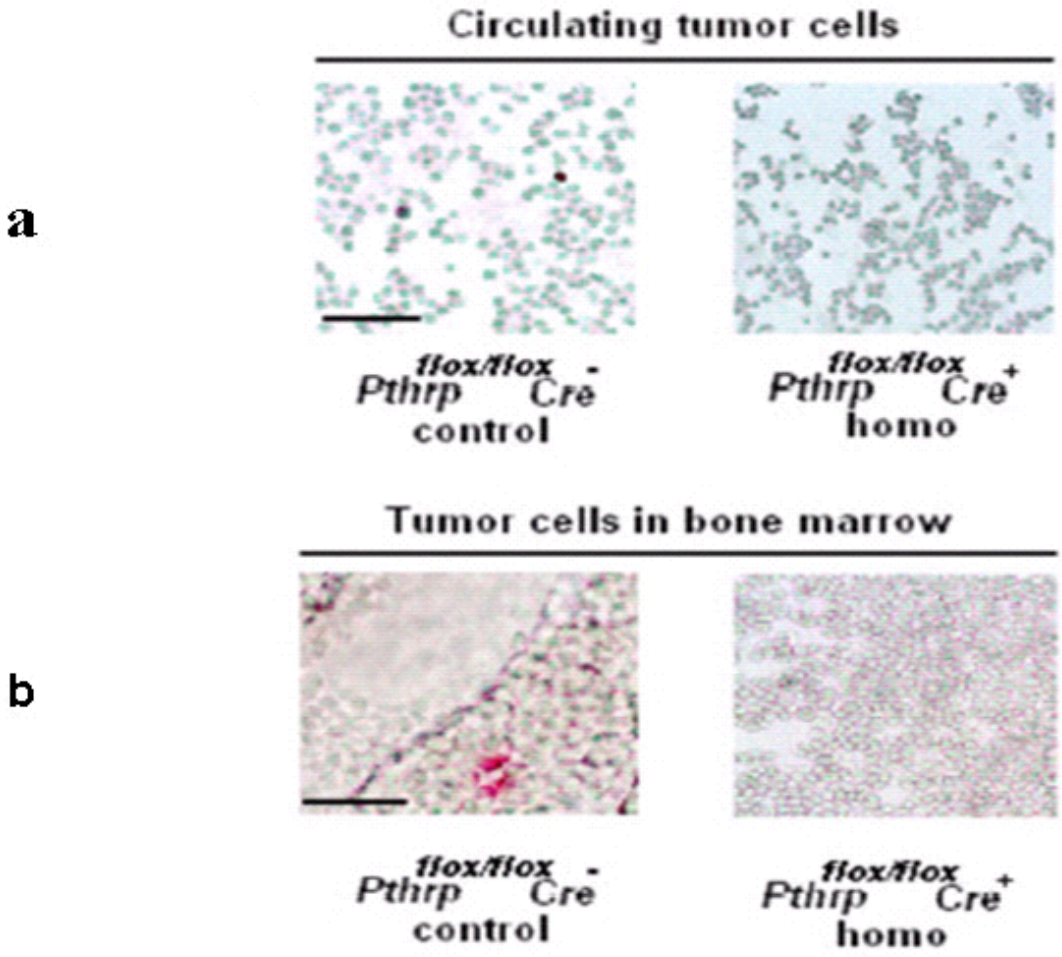



**Figure 2**: **Pthrp ablation reduces numbers of circulating tumor cells in peripheral blood and bone marrow**: (**a**) presence of tumor cells in blood circulation of control ($Cre^-$) animals at 13 weeks and in homozygous animals ($Cre^+$) at 18 weeks (Epicam stain). (b) Cytokeratin stain for presence of cancer cells in bone marrow cytospins.

**Figure 3**

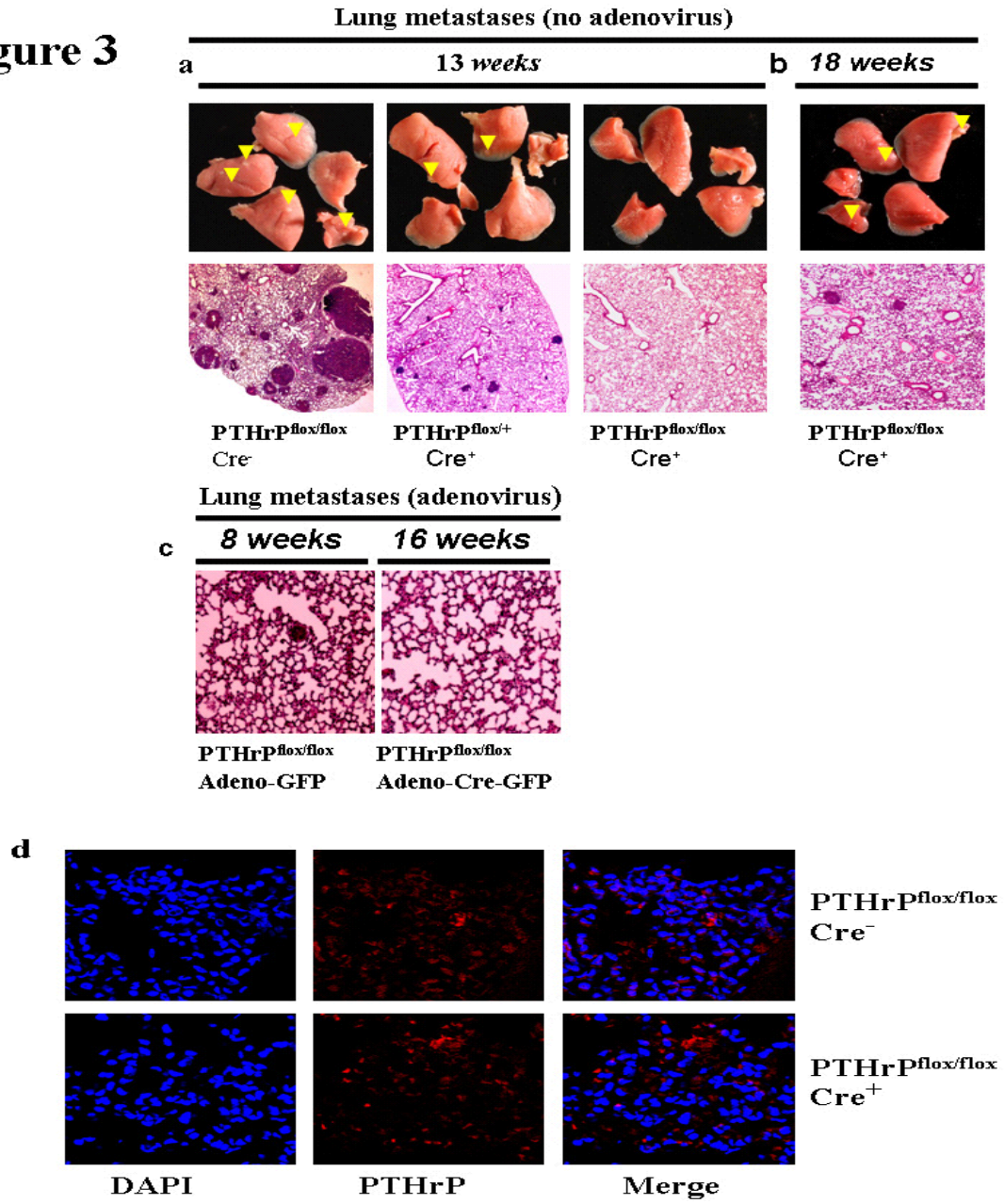



**Figure 3**: **Lung metastases are reduced in absence of PTHrP; lesions that eventually develop derive from residual PTHrP-expressing cells**: (a) Top: Macroscopic appearance of lung metastases at 13 weeks. Bottom: histological micrograph of lung metastases. (b) Top: Macroscopic appearance of lung metastases in homozygous animals at 18 weeks. Bottom: histological micrograph of lung metastases. (c) Histological micrographs of lung metastases appearing in animals injected in the mammary fat pad with adenovirus-transfected tumor cells. (d)IF for PTHrP levels in lung metastases of controls (13 weeks) and homozygous (18 weeks) mice showing generalized expression of PTHrP.

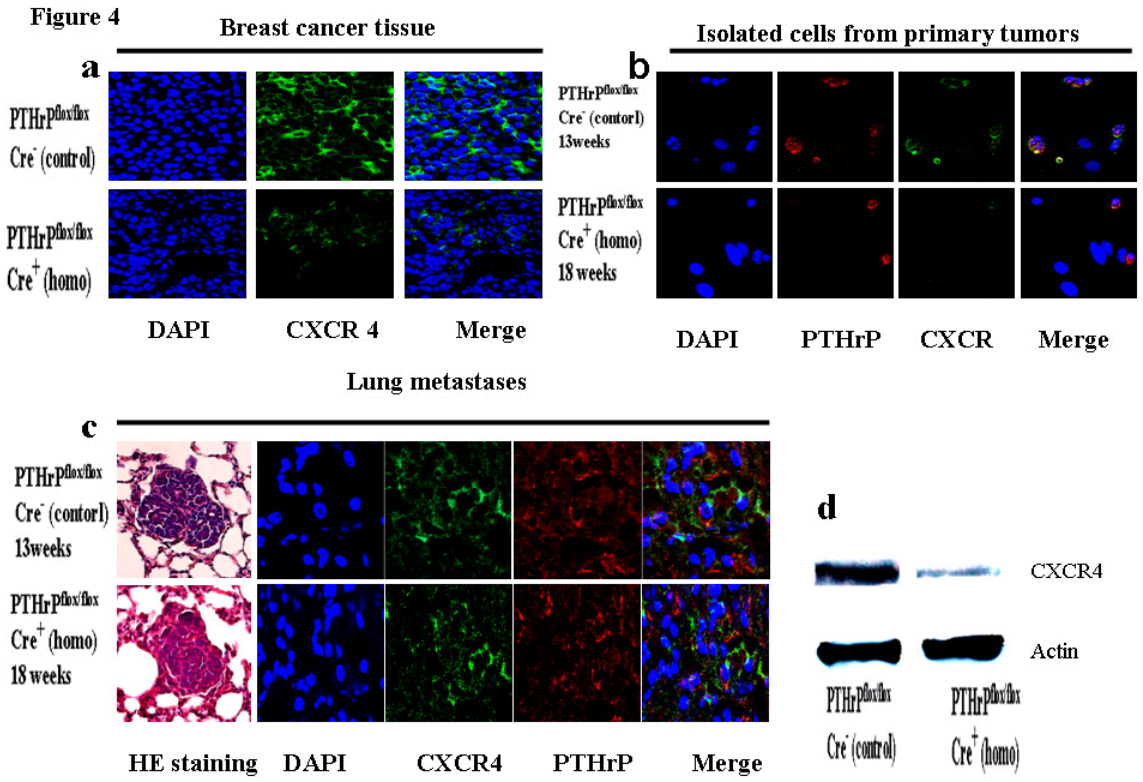



**Figure 4: Primary tumors and lung metastases show different patterns of PTHrP and CXCR4 expression:** (**a**) Confocal image of immunofluorescent staining for DAPI (blue) and CXCR4 (green) in primary breast tumors of controls (13 weeks) and homozygous (18 weeks). (b) Confocal imaging of immunofluorescent staining for DAPI (blue), CXCR4 (green) and PTHrP (red) of cultured cells isolated from primary tumors of control (13 weeks) and homozygous animals (18 weeks). (c) Histological micrographs (single left panels) and confocal images (right panels) of immunofluorescent staining for DAPI (blue), CXCR4 (green) and PTHrP (red) of lung metastases from control (13

weeks) and homozygous mice (18 weeks). (d) Western blot of same tumor extracts showing decrease in CXCR4 expression with *Pthrp* ablation.

**Literature cited**:


Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A., *et al.* (2004). Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell *6*, 17-32.

Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., and Muller, W. J. (2000). Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. Proc Natl Acad Sci U S A *97*, 3444-3449.

Brandt, D. W., Burton, D. W., Gazdar, A. F., Oie, H. E., and Deftos, L. J. (1991). All major lung cancer cell types produce parathyroid hormone-like protein: heterogeneity assessed by high performance liquid chromatography. Endocrinology *129*, 2466-2470.

Bundred, N. J., Ratcliffe, W. A., Walker, R. A., Coley, S., Morrison, J. M., and Ratcliffe, J. G. (1991). Parathyroid hormone related protein and hypercalcaemia in breast cancer. BMJ *303*, 1506-1509.

Bundred, N. J., Walker, R. A., Ratcliffe, W. A., Warwick, J., Morrison, J. M., and Ratcliffe, J. G. (1992). Parathyroid hormone related protein and skeletal morbidity in breast cancer. Eur J Cancer *28*, 690-692.

Coleman, R. E., and Rubens, R. D. (1987). The clinical course of bone metastases from breast cancer. Br J Cancer *55*, 61-66.

Goltzman, D. (1997). Mechanisms of the development of osteoblastic metastases. Cancer *80*, 1581-1587.

Guise, T. A. (2000). Molecular mechanisms of osteolytic bone metastases. Cancer *88*, 2892-2898.

Guise, T. A., and Mundy, G. R. (1998). Cancer and bone. Endocr Rev *19*, 18-54.

Guise, T. A., Yin, J. J., Taylor, S. D., Kumagai, Y., Dallas, M., Boyce, B. F., Yoneda, T., and Mundy, G. R. (1996). Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest *98*, 1544-1549.

Guy, C. T., Cardiff, R. D., and Muller, W. J. (1992). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol *12*, 954-961.

Hastings, R. H., Araiza, F., Burton, D. W., Bedley, M., and Deftos, L. J. (2004). Parathyroid hormone-related protein regulates apoptosis in lung cancer cells through protein kinase A. Am J Physiol Cell Physiol *287*, C1616-1622.

Hatse, S., Princen, K., Bridger, G., De Clercq, E., and Schols, D. (2002). Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. FEBS Lett *527*, 255-262.

He, B., Deckelbaum, R. A., Miao, D., Lipman, M. L., Pollak, M., Goltzman, D., and Karaplis, A. C. (2001). Tissue-specific targeting of the pthrp gene: the generation of mice with floxed alleles. Endocrinology *142*, 2070-2077.

Hidaka, N., Nishimura, M., and Nagao, K. (1998). Establishment of two human small cell lung cancer cell lines: the evidence of accelerated production of parathyroid hormone-related protein with tumor progression. Cancer Lett *125*, 149-155.

Kakonen, S. M., and Mundy, G. R. (2003). Mechanisms of osteolytic bone metastases in breast carcinoma. Cancer *97*, 834-839.

Kang, H., Watkins, G., Parr, C., Douglas-Jones, A., Mansel, R. E., and Jiang, W. G. (2005). Stromal cell derived factor-1: its influence on invasiveness and migration of

breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. Breast Cancer Res *7*, R402-410.

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., Guise, T. A., and Massague, J. (2003). A multigenic program mediating breast cancer metastasis to bone. Cancer Cell *3*, 537-549.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev *8*, 277-289.

Kremer, R., Shustik, C., Tabak, T., Papavasiliou, V., and Goltzman, D. (1996). Parathyroid-hormone-related peptide in hematologic malignancies. Am J Med *100*, 406-411.

Lee, B. C., Lee, T. H., Zagozdzon, R., Avraham, S., Usheva, A., and Avraham, H. K. (2005). Carboxyl-terminal Src kinase homologous kinase negatively regulates the chemokine receptor CXCR4 through YY1 and impairs CXCR4/CXCL12 (SDF-1alpha)-mediated breast cancer cell migration. Cancer Res *65*, 2840-2845.

Liang, Z., Wu, T., Lou, H., Yu, X., Taichman, R. S., Lau, S. K., Nie, S., Umbreit, J., and Shim, H. (2004). Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. Cancer Res *64*, 4302-4308.

Liang, Z., Yoon, Y., Votaw, J., Goodman, M. M., Williams, L., and Shim, H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. Cancer Res *65*, 967-971.

Luker, K. E., and Luker, G. D. (2006). Functions of CXCL12 and CXCR4 in breast cancer. Cancer Lett *238*, 30-41.

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. Dev Cell *5*, 709-722.

Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., Viale, A., Olshen, A. B., Gerald, W. L., and Massague, J. (2005). Genes that mediate breast cancer metastasis to lung. Nature *436*, 518-524.

Moseley, J. M., and Gillespie, M. T. (1995). Parathyroid hormone-related protein. Crit Rev Clin Lab Sci *32*, 299-343.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N.*, et al.* (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature *410*, 50-56.

Mundy, G. R. (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer *2*, 584-593.

Nishihara, M., Kanematsu, T., Taguchi, T., and Razzaque, M. S. (2007). PTHrP and tumorigenesis: is there a role in prognosis? Ann N Y Acad Sci *1117*, 385-392.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E., and Stewart, A. F. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev *76*, 127-173.

Rankin, W., Grill, V., and Martin, T. J. (1997). Parathyroid hormone-related protein and hypercalcemia. Cancer *80*, 1564-1571.

Roodman, G. D. (2004). Mechanisms of bone metastasis. N Engl J Med *350*, 1655-1664.

Smith, M. C., Luker, K. E., Garbow, J. R., Prior, J. L., Jackson, E., Piwnica-Worms, D., and Luker, G. D. (2004). CXCR4 regulates growth of both primary and metastatic breast cancer. Cancer Res *64*, 8604-8612.

Solomayer, E. F., Diel, I. J., Meyberg, G. C., Gollan, C., and Bastert, G. (2000). Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. Breast Cancer Res Treat *59*, 271-278.

Southby, J., Kissin, M. W., Danks, J. A., Hayman, J. A., Moseley, J. M., Henderson, M. A., Bennett, R. C., and Martin, T. J. (1990). Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. Cancer Res *50*, 7710-7716.

Strewler, G. J. (2000). The physiology of parathyroid hormone-related protein. N Engl J Med *342*, 177-185.

Suva, L. J., Winslow, G. A., Wettenhall, R. E., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., and et al. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science *237*, 893-896.

Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T. (1999). Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology *140*, 4451-4458.

Truong, N. U., de, B. E. M. D., Papavasiliou, V., Goltzman, D., and Kremer, R. (2003). Parathyroid hormone-related peptide and survival of patients with cancer and hypercalcemia. Am J Med *115*, 115-121.

Vargas, S. J., Gillespie, M. T., Powell, G. J., Southby, J., Danks, J. A., Moseley, J. M., and Martin, T. J. (1992). Localization of parathyroid hormone-related protein mRNA

expression in breast cancer and metastatic lesions by in situ hybridization. J Bone Miner Res *7*, 971-979.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science *273*, 613-622.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., and Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. Development *125*, 1285-1294.

Zhang, X. H., Wang, Q., Gerald, W., Hudis, C. A., Norton, L., Smid, M., Foekens, J. A., and Massague, J. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell *16*, 67-78.

Zhao, M., Mueller, B. M., DiScipio, R. G., and Schraufstatter, I. U. (2008). Akt plays an important role in breast cancer cell chemotaxis to CXCL12. Breast Cancer Res Treat *110*, 211-222.

Zlotnik, A. (2004). Chemokines in neoplastic progression. Semin Cancer Biol *14*, 181-185.

Zlotnik, A. (2008). New insights on the role of CXCR4 in cancer metastasis. J Pathol *215*, 211-213.

# Chapter 4

# General Discussion

Parathyroid hormone-related protein (PTHrP) has been demonstrated to be a product of many malignant tissues including breast, prostate and lung cancer, where it plays an important role in growth and proliferation regulation. Although there are some controversial reports (Fleming, et al., 2009; M. Henderson, et al., 2001; Surowiak, et al., 2003), patients whose tumors produce high PTHrP levels tend to have higher rates of metastasis and increased or earlier mortality, and PTHrP expression is generally recognised as representing a driving force in cancer progression (Gallwitz, Guise, & Mundy, 2002; Hiraki, et al., 2002; Hutchesson, Bundred, & Ratcliffe, 1995; Liao, et al., 2008; Linforth, et al., 2002; Pecherstorfer, et al., 1994; Shen & Falzon, 2006; Truong, et al., 2003; Yamada, et al., 2008).

The molecular mechanisms responsible for PTHrP cancer-promoting activities have not been completely elucidated and represent a valuable putative target for therapeutic purposes. In the studies presented here, we investigate the biochemical role of PTHrP during two crucial stages of breast cancer progression: the initiation step, and the metastatic spread. The MMTV-PyVMT mouse model has been chosen to harbor the *Pthrp* gene deletion because of the similarity with human breast cancer.

**A- PTHrP in breast tumor initiation**:

The construction of MMTV-PyVMT mice presenting a hetero- or homozygous deletion of the *Pthrp* gene targeted to the mammary epithelium allowed the demonstration that a specific reduction or elimination of PTHrP expression in this set of cells would bring dramatic delays in the initiation events of mammary carcinogenesis. In *Pthrp*-ablated animals, hyperplasia was considerably delayed, and palpable tumors developed much later and were fewer in numbers and of much smaller size than in control mice. The majority of malignant foci observed during the progression to more

advanced stages arose principally from a small population of cells that failed to express the MMTVCre and were therefore unable to excise *Pthrp*.

Mechanistically, the removal of PTHrP expression in the mammary epithelium was accompanied by a significant decrease in the total protein levels of the Akt1 serine-threonine protein kinase isoform, and by an increase in the levels of the Akt2 isoform. The PI3K/AKT pathway is essential to the transmission of oncogenic signalling from the middle T oncoprotein for development of mammary adenocarcinomas in the PyVMT system (Fluck & Schaffhausen, 2009; Lin, et al., 2003). In striking similarity with our observations, a knock-out of Akt1 has been shown to interfere with mammary tumor initiation and growth in MMTV-PyMT mice (Maroulakou, et al., 2007). Conversely, Akt2 ablation is known to accelerate tumor induction and the two AKT isoforms possess opposite oncogenic effects (Fluck & Schaffhausen, 2009; Maroulakou, et al., 2007). It is very likely that PTHrP is situated upstream of AKT in the oncogenic series of events and exerts a role in the translational efficacy of these proteins or is involved in their maintenance or degradation, a novel role for PTHrP. By controlling the expression levels of the two AKT isoforms, PTHrP shifts the cell progression towards an oncogenic direction.

We also observed that *Pthrp* ablation has repercussions in the cell cycle (effects on cyclin D1 and KI-67) and enhances apoptotic events (Bcl-2, TUNEL). *Pthrp* ablation causes an inhibition of S-phase entry analogous to the one observed after silencing Akt1 in nontransformed mammalian myoblasts and fibroblasts (Heron-Milhavet, et al., 2006a). Intracrine PTHrP action on apoptosis has been proposed to proceed by the PI3K/AKT pathway, with AKT as one of the primary mediators of PTHrP-enhanced cell survival,

and with integrin α6β4 providing an essential link between PTHrP and PI3K/Akt (Bhatia, et al., 2009). PTHrP upregulates integrin α6β4 at the transcriptional and-or post transcriptional level in human breast cancer cell lines (Shen & Falzon, 2006), providing another element linking PTHrP, integrins, and the PI3K/AKT pathway. In a mouse model similar to the one used in our study, the disruption of β1-integrin in the mammary epithelium of MMTV-PyVMT mice revealed that β1-integrin function is required for mammary tumor progression but non-essential for mammary ductal outgrowth early stages (White, et al., 2004), effects which are identical to those observed after *Pthrp* ablation in our experiments. Taken together, these observations point to a key upstream role for PTHrP on the PI3K/AKT pathway, with members of the integrin family likely acting as intermediates.

The levels of expression of the factor VIII marker of angiogenesis are negatively affected by *Pthrp* ablation. PTHrP is known to act as an angiogenic factor in endothelial cells (Akino, et al., 2000; Esbrit, et al., 2000; T. J. Martin, et al., 1997), and it seems likely that angiogenesis is another aspect of mammary tumor initiation that requires PTHrP as a driving factor, and which can be repressed through *Pthrp* ablation.

**B- PTHrP in breast tumor metastatic process**:

The targeted ablation of *Pthrp* gene activity from the mammary epithelium in MMTV-PyVMT mice also counters the subsequent stages of breast cancer progression; the metastatic processes to lungs and bones are similarly hindered when PTHrP is not expressed in the mammary epithelium. *Pthrp* ablation substantially reduces the number of tumor cells in peripheral blood, bone marrow and lungs, and a more complete knock-out mediated by adenoCre results in total absence of metastases in the lungs, the

preferred site for metastasis in the MMTV-PyVMT mouse. A crucial observation made here is that *Pthrp* ablation is accompanied by a significant decrease in the expression of the chemokine receptor CXCR4, and that the small percentage of residual expression of PTHrP and CXCR4 in ablated tumors co-localizes to the same cells. In the tumors that eventually develop from $Pthrp^{flox/flox;}Cre^{+}$ cells that were not adenoCre-infected, the generalized expression of PTHrP and CXCR4 indicates that it is the PTHrP and CXCR4 double-positive cells that are selected to create the invasive population which develops into the tardy metastases eventually observed in the $Pthrp^{flox/flox}$; $Cre^{+}$ derived populations. The consequences of this selection for PTHrP/CXCR4 positive tumor cells are dramatic; invasion can now proceed and the metastatic events are unhindered.

The chemokine CXCL12 and its receptor CXCR4 hold crucial roles in primary and metastatic breast cancer and other malignancies such as lung, brain and liver, through their stimulation of the phosphatidylinositol 3-kinase (PI3K) and AKT pathway leading to cell survival (Luker & Luker, 2006; Muller, et al., 2001; Zhao, et al., 2008). CXCR4/CXCL12 interaction activates angiogenesis, chemotaxis, invasion, and adhesion processes that reinforce the metastatic profile. The levels of CXCR4 in breast tumors are known to increase with cancer progression (Allinen, et al., 2004), and neutralization of CXCR4/CXCL12 interactions *in vivo* by antibodies (Muller, et al., 2001), inhibitory peptides (Hatse, et al., 2002; Liang, et al., 2004; Liang, et al., 2005), and siRNA (Liang, et al., 2005) attenuates metastases in mouse models. Furthermore, CXCR4 has been identified as a signature gene in primary breast cancers expressing the lung and the bone metastasis signature (H. Kang, et al., 2005; Y. Kang, et al., 2003; Minn, et al., 2005).

Some upstream controls of the CXCR4 chemokine have been reported; among the negative ones are the YY1 factor which binds to the CXCR4 promoter to decrease

transcription (Lee, et al., 2005), and the AIP4 ligand-dependent degradation which reduces CXCR4 protein stability (Marchese, et al., 2003). Our observation that the targeted removal of PTHrP in the mammary epithelium results in a sharp reduction in levels of the CXCR4 chemokine, coupled with our previous observation that total protein levels for AKT follow a similar drop, suggest that PTHrP may be an important control for the CXCR4/CXCL12 signalling pathway in breast cancer initiation and metastasis.

Again, the PTHrP site of action appears to be located upstream of previously reported controls, as PTHrP affects the levels of both AKT and the CXCR4 receptor to control metastatic development. PTHrP therefore becomes a choice target for novel therapeutic approaches aimed at reducing breast cancer invasive properties.

SUMMARY: In this study, we have used a well-validated breast cancer animal model to examine the malignant progression process. We have demonstrated that disruption of *Pthrp* dramatically delays the initial and subsequent steps of malignant conversion of the mammary epithelial cell, without affecting the mammary gland development. PTHrP acts as a promoter of oncogenesis and metastasis upstream of a number of critical checkpoints for PyVMT, such as Akt1, Akt2, factor VIII Bcl-2 and cyclin D1, with the most interesting being CXCR4. This suggests a novel role for PTHrP as a facilitator of oncogenes and emphasizes the importance of attempting its targeting for therapeutic purposes.

**C-Diagram of the interactions between PTHrP and oncogenic progression signalling molecules**.

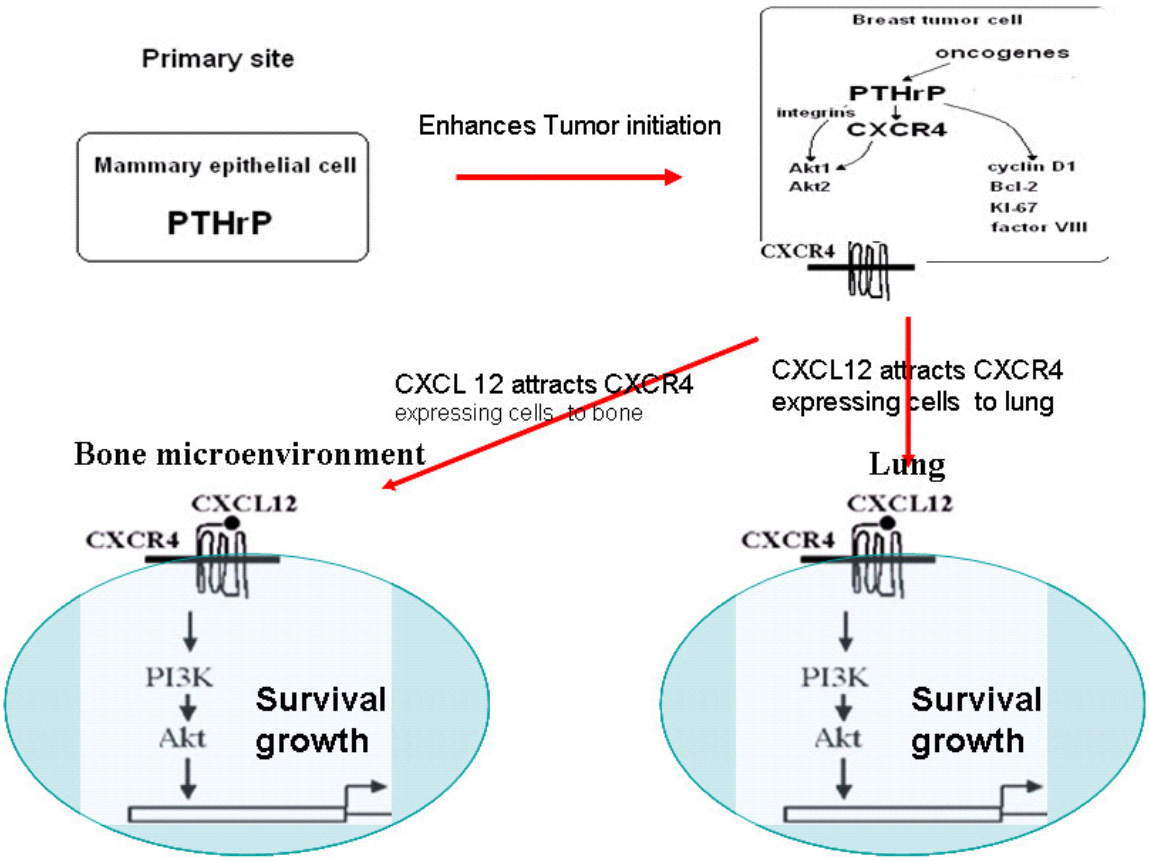



Figure 1 Model showing how PTHrP regulates initiation, proliferation and metastatic spread through CXCR4 and AKT

### D- Limitations of the findings and Suggestions for future directions:

There are obvious differences between human and mouse tumorigenesis, among which are the kinetics of carcinogenesis and the final size of tumors, differences in cell intrinsic features such as the requirements to transform cells, and differences in organ-specific gene expression, in physiology, metabolism, pathology, and in the immune system(Fantozzi & Christofori, 2006). MMTV LTR is active at an early embryonic stage or at low levels in many tissues (Wagner, et al., 1997). MMTV-PyMT and MTV-*Cre* mice expression of the transgene was confined to striated ductal cells of the salivary gland and mammary epithelial cells in virgin mice (Andrechek & Muller, 2000; Guy, et al., 1992a). The MMTV LTR based mouse models have enabled researchers to elucidate many of the genetic and molecular events in breast cancer. However, several limitations associated with MMTV-driven PyMT expression affect our ability to compare these models to the human disease. First, multiple copies of the transgene are randomly integrated into the genome, which affects the pattern of transgene expression between independent lines and may complicate data interpretation(Ursini-Siegel, Schade, Cardiff, & Muller, 2007). Moreover, metastatic dissemination occurs mainly via hematogenous spreading to lungs and lymph nodes in MMTV-PyMT as opposed to the initial spreading of cancer cells to local lymph nodes via the lymphatics in human breast cancer(Fantozzi & Christofori, 2006).

Some of the research directions that would be interesting to explore in the near future:

(a) Achieve the overexpression of PTHrP in normal epithelial cells to investigate the effect on tumor initiation in the presence or absence of an oncogene.

(b) Achieve an *AKT* and *Pthrp* double knock-out, to investigate whether the oncogenic events can persist due to other unknown upstream factors.

(c) Treat *Pthrp* knock-out mice with VEGF inhibitors to see if an additivity effect on angiogenesis and metastasis is detectable.

(d) Achieve a *CXCR4* and *Pthrp* double gene knock-out to confirm the activity of these genes is sufficient for oncogenicity.

(e) Conduct gene array analysis of Pthrp knock-out versus wild-type animals to determine a signature of other genes involved in breast cancer metastasis.

Akino, K., Ohtsuru, A., Kanda, K., Yasuda, A., Yamamoto, T., Akino, Y., et al. (2000). Parathyroid hormone-related peptide is a potent tumor angiogenic factor. *Endocrinology, 141*(11), 4313-4316.

Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., et al. (2004). Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell, 6*(1), 17-32.

Bhatia, V., Mula, R. V., Weigel, N. L., & Falzon, M. (2009). Parathyroid hormone-related protein regulates cell survival pathways via integrin alpha6beta4-mediated activation of phosphatidylinositol 3-kinase/Akt signaling. *Mol Cancer Res, 7*(7), 1119-1131.

Esbrit, P., Alvarez-Arroyo, M. V., De Miguel, F., Martin, O., Martinez, M. E., & Caramelo, C. (2000). C-terminal parathyroid hormone-related protein increases vascular endothelial growth factor in human osteoblastic cells. *J Am Soc Nephrol, 11*(6), 1085-1092.

Fleming, N. I., Trivett, M. K., George, J., Slavin, J. L., Murray, W. K., Moseley, J. M., et al. (2009). Parathyroid hormone-related protein protects against mammary tumor emergence and is associated with monocyte infiltration in ductal carcinoma in situ. *Cancer Res, 69*(18), 7473-7479.

Fluck, M. M., & Schaffhausen, B. S. (2009). Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. *Microbiol Mol Biol Rev, 73*(3), 542-563, Table of Contents.

Gallwitz, W. E., Guise, T. A., & Mundy, G. R. (2002). Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers in vivo. *J Clin Invest, 110*(10), 1559-1572.

Hatse, S., Princen, K., Bridger, G., De Clercq, E., & Schols, D. (2002). Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Lett, 527*(1-3), 255-262.

Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., et al. (2001). Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst, 93*(3), 234-237.

Heron-Milhavet, L., Franckhauser, C., Rana, V., Berthenet, C., Fisher, D., Hemmings, B. A., et al. (2006). Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. *Mol Cell Biol, 26*(22), 8267-8280.

Hiraki, A., Ueoka, H., Bessho, A., Segawa, Y., Takigawa, N., Kiura, K., et al. (2002). Parathyroid hormone-related protein measured at the time of first visit is an indicator of bone metastases and survival in lung carcinoma patients with hypercalcemia. *Cancer, 95*(8), 1706-1713.

Hutchesson, A. C., Bundred, N. J., & Ratcliffe, W. A. (1995). Survival in hypercalcaemic patients with cancer and co-existing primary hyperparathyroidism. *Postgrad Med J, 71*(831), 28-31.

Kang, H., Watkins, G., Parr, C., Douglas-Jones, A., Mansel, R. E., & Jiang, W. G. (2005). Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res, 7*(4), R402-410.

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., et al. (2003). A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell, 3*(6), 537-549.

Lee, B. C., Lee, T. H., Zagozdzon, R., Avraham, S., Usheva, A., & Avraham, H. K. (2005). Carboxyl-terminal Src kinase homologous kinase negatively regulates the chemokine receptor CXCR4 through YY1 and impairs CXCR4/CXCL12 (SDF-1alpha)-mediated breast cancer cell migration. *Cancer Res, 65*(7), 2840-2845.

Liang, Z., Wu, T., Lou, H., Yu, X., Taichman, R. S., Lau, S. K., et al. (2004). Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res, 64*(12), 4302-4308.

Liang, Z., Yoon, Y., Votaw, J., Goodman, M. M., Williams, L., & Shim, H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res, 65*(3), 967-971.

Liao, J., Li, X., Koh, A. J., Berry, J. E., Thudi, N., Rosol, T. J., et al. (2008). Tumor expressed PTHrP facilitates prostate cancer-induced osteoblastic lesions. *Int J Cancer, 123*(10), 2267-2278.

Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., et al. (2003). Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol, 163*(5), 2113-2126.

Linforth, R., Anderson, N., Hoey, R., Nolan, T., Downey, S., Brady, G., et al. (2002). Coexpression of parathyroid hormone related protein and its receptor in early breast cancer predicts poor patient survival. *Clin Cancer Res, 8*(10), 3172-3177.

Luker, K. E., & Luker, G. D. (2006). Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Lett, 238*(1), 30-41.

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., & Benovic, J. L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell, 5*(5), 709-722.

Maroulakou, I. G., Oemler, W., Naber, S. P., & Tsichlis, P. N. (2007). Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer Res, 67*(1), 167-177.

Martin, T. J., Moseley, J. M., & Williams, E. D. (1997). Parathyroid hormone-related protein: hormone and cytokine. *J Endocrinol, 154 Suppl*, S23-37.

Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., et al. (2005). Genes that mediate breast cancer metastasis to lung. *Nature, 436*(7050), 518-524.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature, 410*(6824), 50-56.

Pecherstorfer, M., Schilling, T., Blind, E., Zimmer-Roth, I., Baumgartner, G., Ziegler, R., et al. (1994). Parathyroid hormone-related protein and life expectancy in hypercalcemic cancer patients. *J Clin Endocrinol Metab, 78*(5), 1268-1270.

Shen, X., & Falzon, M. (2006). PTH-related protein upregulates integrin alpha6beta4 expression and activates Akt in breast cancer cells. *Exp Cell Res, 312*(19), 3822-3834.

Surowiak, P., Dziegiel, P., Matkowski, R., Sopel, M., Wojnar, A., Kornafel, J., et al. (2003). Prognostic value of immunocytochemical determination of parathyroid hormone-related peptide expression in cells of mammary ductal carcinoma. Analysis of 7 years of the disease course. *Virchows Arch, 442*(3), 245-251.

Truong, N. U., de, B. E. M. D., Papavasiliou, V., Goltzman, D., & Kremer, R. (2003). Parathyroid hormone-related peptide and survival of patients with cancer and hypercalcemia. *Am J Med, 115*(2), 115-121.

White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., et al. (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell, 6*(2), 159-170.

Yamada, T., Tsuda, M., Ohba, Y., Kawaguchi, H., Totsuka, Y., & Shindoh, M. (2008). PTHrP promotes malignancy of human oral cancer cell downstream of the EGFR signaling. *Biochem Biophys Res Commun, 368*(3), 575-581.

Zhang, X. H., Wang, Q., Gerald, W., Hudis, C. A., Norton, L., Smid, M., et al. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell, 16*(1), 67-78.

Zhao, M., Mueller, B. M., DiScipio, R. G., & Schraufstatter, I. U. (2008). Akt plays an important role in breast cancer cell chemotaxis to CXCL12. *Breast Cancer Res Treat, 110*(2), 211-222.

## General Bibliography


Aarts, M. M., Davidson, D., Corluka, A., Petroulakis, E., Guo, J., Bringhurst, F. R., et al. (2001). Parathyroid hormone-related protein promotes quiescence and survival of serum-deprived chondrocytes by inhibiting rRNA synthesis. *J Biol Chem, 276*(41), 37934-37943.

Aarts, M. M., Levy, D., He, B., Stregger, S., Chen, T., Richard, S., et al. (1999). Parathyroid hormone-related protein interacts with RNA. *J Biol Chem, 274*(8), 4832-4838.

Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., et al. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci U S A, 89*(7), 2732-2736.

Agouni, A., Sourbier, C., Danilin, S., Rothhut, S., Lindner, V., Jacqmin, D., et al. (2007). Parathyroid hormone-related protein induces cell survival in human renal cell carcinoma through the PI3K Akt pathway: evidence for a critical role for integrin-linked kinase and nuclear factor kappa B. *Carcinogenesis, 28*(9), 1893-1901.

Akhtari, M., Mansuri, J., Newman, K. A., Guise, T. M., & Seth, P. (2008). Biology of breast cancer bone metastasis. *Cancer Biol Ther, 7*(1), 3-9.

Akino, K., Ohtsuru, A., Kanda, K., Yasuda, A., Yamamoto, T., Akino, Y., et al. (2000). Parathyroid hormone-related peptide is a potent tumor angiogenic factor. *Endocrinology, 141*(11), 4313-4316.

Aklilu, F., Park, M., Goltzman, D., & Rabbani, S. A. (1997). Induction of parathyroid hormone-related peptide by the Ras oncogene: role of Ras farnesylation inhibitors as potential therapeutic agents for hypercalcemia of malignancy. *Cancer Res, 57*(20), 4517-4522.

Alipov, G. K., Ito, M., Nakashima, M., Ikeda, Y., Nakayama, T., Ohtsuru, A., et al. (1997). Expression of parathyroid hormone-related peptide (PTHrP) in gastric tumours. *J Pathol, 182*(2), 174-179.

Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., et al. (2004). Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell, 6*(1), 17-32.

Alokail, M. S. (2005). Induction of erbB1 and erB2 receptors by PTHrP in breast cancer cells. *AACR Meeting Abstracts, 2005*(1), 1359-.

Alonso, V., de Gortazar, A. R., Ardura, J. A., Andrade-Zapata, I., Alvarez-Arroyo, M. V., & Esbrit, P. (2008). Parathyroid hormone-related protein (107-139) increases human osteoblastic cell survival by activation of vascular endothelial growth factor receptor-2. *J Cell Physiol, 217*(3), 717-727.

Altomare, D. A., & Testa, J. R. (2005). Perturbations of the AKT signaling pathway in human cancer. *Oncogene, 24*(50), 7455-7464.

Amizuka, N., Karaplis, A. C., Henderson, J. E., Warshawsky, H., Lipman, M. L., Matsuki, Y., et al. (1996). Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. *Dev Biol, 175*(1), 166-176.

Amling, M., Neff, L., Tanaka, S., Inoue, D., Kuida, K., Weir, E., et al. (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J Cell Biol, 136*(1), 205-213.

Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., & Muller, W. J. (2000). Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. *Proc Natl Acad Sci U S A, 97*(7), 3444-3449.

Andrechek, E. R., & Muller, W. J. (2000). Tyrosine kinase signalling in breast cancer: tyrosine kinase-mediated signal transduction in transgenic mouse models of human breast cancer. *Breast Cancer Res, 2*(3), 211-216.

Benson, R. C., Jr., Riggs, B. L., Pickard, B. M., & Arnaud, C. D. (1974). Immunoreactive forms of circulating parathyroid hormone in primary and ectopic hyperparathyroidism. *J Clin Invest, 54*(1), 175-181.

Bhatia, V., Mula, R. V., Weigel, N. L., & Falzon, M. (2009). Parathyroid hormone-related protein regulates cell survival pathways via integrin alpha6beta4-mediated activation of phosphatidylinositol 3-kinase/Akt signaling. *Mol Cancer Res, 7*(7), 1119-1131.

Blind, E., Raue, F., Knappe, V., Schroth, J., & Ziegler, R. (1993). Cyclic AMP formation in rat bone and kidney cells is stimulated equally by parathyroid hormone-related protein (PTHrP) 1-34 and PTH 1-34. *Exp Clin Endocrinol, 101*(3), 150-155.

Bouizar, Z., Spyratos, F., & De vernejoul, M. C. (1999). The parathyroid hormone-related protein (PTHrP) gene: use of downstream TATA promotor and PTHrP 1-139 coding pathways in primary breast cancers vary with the occurrence of bone metastasis. *J Bone Miner Res, 14*(3), 406-414.

Bouizar, Z., Spyratos, F., Deytieux, S., de Vernejoul, M. C., & Jullienne, A. (1993). Polymerase chain reaction analysis of parathyroid hormone-

related protein gene expression in breast cancer patients and occurrence of bone metastases. *Cancer Res, 53*(21), 5076-5078.
Brandt, D. W., Burton, D. W., Gazdar, A. F., Oie, H. E., & Deftos, L. J. (1991). All major lung cancer cell types produce parathyroid hormone-like protein: heterogeneity assessed by high performance liquid chromatography. *Endocrinology, 129*(5), 2466-2470.
Bucht, E., Rong, H., Pernow, Y., Nordqvist, A. C., Eriksson, E., Rankin, W., et al. (1998). Parathyroid hormone-related protein in patients with primary breast cancer and eucalcemia. *Cancer Res, 58*(18), 4113-4116.
Buckle, R. (1974). Ectopic PTH syndrome, pseudohyperparathyroidism; hypercalcaemia of malignancy. *Clin Endocrinol Metab, 3*(2), 237-251.
Buckle, R. M., McMillan, M., & Mallinson, C. (1970). Ectopic secretion of parathyroid hormone by a renal adenocarcinoma in a patient with hypercalcaemia. *Br Med J, 4*(5737), 724-726.
Bundred, N. J., Ratcliffe, W. A., Walker, R. A., Coley, S., Morrison, J. M., & Ratcliffe, J. G. (1991). Parathyroid hormone related protein and hypercalcaemia in breast cancer. *Bmj, 303*(6816), 1506-1509.
Bundred, N. J., Walker, R. A., Ratcliffe, W. A., Warwick, J., Morrison, J. M., & Ratcliffe, J. G. (1992). Parathyroid hormone related protein and skeletal morbidity in breast cancer. *Eur J Cancer, 28*(2-3), 690-692.
Burtis, W. J., Brady, T. G., Orloff, J. J., Ersbak, J. B., Warrell, R. P., Jr., Olson, B. R., et al. (1990). Immunochemical characterization of circulating parathyroid hormone-related protein in patients with humoral hypercalcemia of cancer. *N Engl J Med, 322*(16), 1106-1112.
Burtis, W. J., Wu, T., Bunch, C., Wysolmerski, J. J., Insogna, K. L., Weir, E. C., et al. (1987a). Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *Journal of Biological Chemistry, 262*(15), 7151-7156.
Burtis, W. J., Wu, T., Bunch, C., Wysolmerski, J. J., Insogna, K. L., Weir, E. C., et al. (1987b). Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *J Biol Chem, 262*(15), 7151-7156.
Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science, 296*(5573), 1655-1657.
Care, A. D., Abbas, S. K., Pickard, D. W., Barri, M., Drinkhill, M., Findlay, J. B., et al. (1990). Stimulation of ovine placental transport of calcium

and magnesium by mid-molecule fragments of human parathyroid hormone-related protein. *Exp Physiol, 75*(4), 605-608.
Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., et al. (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature, 448*(7152), 439-444.
Cheng, J. Q., Lindsley, C. W., Cheng, G. Z., Yang, H., & Nicosia, S. V. (2005). The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene, 24*(50), 7482-7492.
Chirgwin, J. M., & Guise, T. A. (2000). Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr, 10*(2), 159-178.
Cingolani, G., Bednenko, J., Gillespie, M. T., & Gerace, L. (2002). Molecular basis for the recognition of a nonclassical nuclear localization signal by importin beta. *Mol Cell, 10*(6), 1345-1353.
Clemens, T. L., Cormier, S., Eichinger, A., Endlich, K., Fiaschi-Taesch, N., Fischer, E., et al. (2001). Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. *Br J Pharmacol, 134*(6), 1113-1136.
Coleman, R. E., & Rubens, R. D. (1985). Bone metastases and breast cancer. *Cancer Treatment Reviews, 12*(4), 251-270.
Coleman, R. E., & Rubens, R. D. (1987). The clinical course of bone metastases from breast cancer. *Br J Cancer, 55*(1), 61-66.
Comings, D. E. (1972). Evidence for ancient tetraploidy and conservation of linkage groups in mammalian chromosomes. *Nature, 238*(5365), 455-457.
Conlan, L. A., Martin, T. J., & Gillespie, M. T. (2002). The COOH-terminus of parathyroid hormone-related protein (PTHrP) interacts with beta-arrestin 1B. *FEBS Lett, 527*(1-3), 71-75.
Cornish, J., Callon, K. E., Nicholson, G. C., & Reid, I. R. (1997). Parathyroid hormone-related protein-(107-139) inhibits bone resorption in vivo. *Endocrinology, 138*(3), 1299-1304.
Cuthbertson, R. M., Kemp, B. E., & Barden, J. A. (1999). Structure study of osteostatin PTHrP[Thr107](107-139). *Biochim Biophys Acta, 1432*(1), 64-72.
Datta, N. S., & Abou-Samra, A. B. (2009). PTH and PTHrP signaling in osteoblasts. *Cellular Signalling, 21*(8), 1245-1254.
de Gortazar, A. R., Alonso, V., Alvarez-Arroyo, M. V., & Esbrit, P. (2006). Transient exposure to PTHrP (107-139) exerts anabolic effects through vascular endothelial growth factor receptor 2 in human osteoblastic cells in vitro. *Calcif Tissue Int, 79*(5), 360-369.

De Miguel, F., Martinez-Fernandez, P., Guillen, C., Valin, A., Rodrigo, A., Martinez, M. E., et al. (1999). Parathyroid hormone-related protein (107-139) stimulates interleukin-6 expression in human osteoblastic cells. *J Am Soc Nephrol, 10*(4), 796-803.

Deftos, L. J., Barken, I., Burton, D. W., Hoffman, R. M., & Geller, J. (2005). Direct evidence that PTHrP expression promotes prostate cancer progression in bone. *Biochem Biophys Res Commun, 327*(2), 468-472.

Di Cosimo, S. (2009). Controversies in breast cancer: the mammalian target of rapamycin as a target for breast cancer therapy. *Breast Cancer Res, 11 Suppl 3*, S25.

Dihlmann, S., Kloor, M., Fallsehr, C., & von Knebel Doeberitz, M. (2005). Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells. *Carcinogenesis, 26*(9), 1503-1512.

Dilworth, S. M. (2002). Polyoma virus middle T antigen and its role in identifying cancer-related molecules. *Nat Rev Cancer, 2*(12), 951-956.

Dittmer, J. (2004). The importance of PTHrP for cancer development. *Gene Therapy and Molecular Biology, 8*, 451-464.

Dougherty, K. M., Blomme, E. A., Koh, A. J., Henderson, J. E., Pienta, K. J., Rosol, T. J., et al. (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res, 59*(23), 6015-6022.

Esbrit, P., Alvarez-Arroyo, M. V., De Miguel, F., Martin, O., Martinez, M. E., & Caramelo, C. (2000). C-terminal parathyroid hormone-related protein increases vascular endothelial growth factor in human osteoblastic cells. *J Am Soc Nephrol, 11*(6), 1085-1092.

Falzon, M., & Du, P. (2000). Enhanced growth of MCF-7 breast cancer cells overexpressing parathyroid hormone-related peptide. *Endocrinology, 141*(5), 1882-1892.

Fantozzi, A., & Christofori, G. (2006). Mouse models of breast cancer metastasis. *Breast Cancer Res, 8*(4), 212.

Fenton, A. J., Kemp, B. E., Hammonds, R. G., Jr., Mitchelhill, K., Moseley, J. M., Martin, T. J., et al. (1991). A potent inhibitor of osteoclastic bone resorption within a highly conserved pentapeptide region of parathyroid hormone-related protein; PTHrP[107-111]. *Endocrinology, 129*(6), 3424-3426.

Fenton, A. J., Kemp, B. E., Kent, G. N., Moseley, J. M., Zheng, M. H., Rowe, D. J., et al. (1991). A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts. *Endocrinology, 129*(4), 1762-1768.

Fenton, A. J., Martin, T. J., & Nicholson, G. C. (1994). Carboxyl-terminal parathyroid hormone-related protein inhibits bone resorption by isolated chicken osteoclasts. *J Bone Miner Res, 9*(4), 515-519.

Ferrari, S., Rizzoli, R., Chaponnier, C., Gabbiani, G., & Bonjour, J. P. (1993). Parathyroid hormone-related protein increases cAMP production in mammary epithelial cells. *Am J Physiol, 264*(3 Pt 1), E471-475.

Ferrari, S. L., Rizzoli, R., & Bonjour, J. P. (1992). Parathyroid hormone-related protein production by primary cultures of mammary epithelial cells. *J Cell Physiol, 150*(2), 304-311.

Firkin, F., Seymour, J. F., Watson, A. M., Grill, V., & Martin, T. J. (1996). Parathyroid hormone-related protein in hypercalcaemia associated with haematological malignancy. *Br J Haematol, 94*(3), 486-492.

Flanagan, J. A., Power, D. M., Bendell, L. A., Guerreiro, P. M., Fuentes, J., Clark, M. S., et al. (2000). Cloning of the cDNA for sea bream (Sparus aurata) parathyroid hormone-related protein. *Gen Comp Endocrinol, 118*(3), 373-382.

Fleming, N. I., Trivett, M. K., George, J., Slavin, J. L., Murray, W. K., Moseley, J. M., et al. (2009). Parathyroid hormone-related protein protects against mammary tumor emergence and is associated with monocyte infiltration in ductal carcinoma in situ. *Cancer Res, 69*(18), 7473-7479.

Fluck, M. M., & Schaffhausen, B. S. (2009). Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. *Microbiol Mol Biol Rev, 73*(3), 542-563, Table of Contents.

Foley, J., Dann, P., Hong, J., Cosgrove, J., Dreyer, B., Rimm, D., et al. (2001). Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development, 128*(4), 513-525.

Fresno Vara, J. A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., & Gonzalez-Baron, M. (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treatment Reviews, 30*(2), 193-204.

Gagiannis, S., Muller, M., Uhlemann, S., Koch, A., Melino, G., Krammer, P. H., et al. (2009). Parathyroid hormone-related protein confers chemoresistance by blocking apoptosis signaling via death receptors and mitochondria. *International Journal of Cancer, 125*(7), 1551-1557.

Gaich, G., Orloff, J. J., Atillasoy, E. J., Burtis, W. J., Ganz, M. B., & Stewart, A. F. (1993). Amino-terminal parathyroid hormone-related protein: specific binding and cytosolic calcium responses in rat insulinoma cells. *Endocrinology, 132*(3), 1402-1409.

Gallwitz, W. E., Guise, T. A., & Mundy, G. R. (2002). Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers in vivo. *J Clin Invest, 110*(10), 1559-1572.
Gardella, T. J., & Juppner, H. (2000). Interaction of PTH and PTHrP with their receptors. *Rev Endocr Metab Disord, 1*(4), 317-329.
Gardella, T. J., & Juppner, H. (2001). Molecular properties of the PTH/PTHrP receptor. *Trends Endocrinol Metab, 12*(5), 210-217.
Gellhorn, A., & Plimpton, C. H. (1956). Hypercalcemia in malignant disease without evidence of bone destruction. *Am J Med, 21*(5), 750-759.
Gensure, R. C., Gardella, T. J., & Juppner, H. (2005). Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. *Biochem Biophys Res Commun, 328*(3), 666-678.
Goltzman, D. (1997). Mechanisms of the development of osteoblastic metastases. *Cancer, 80*(8 Suppl), 1581-1587.
Grill, V., Rankin, W., & Martin, T. J. (1998). Parathyroid hormone-related protein (PTHrP) and hypercalcaemia. *Eur J Cancer, 34*(2), 222-229.
Guise, T. A. (2000). Molecular mechanisms of osteolytic bone metastases. *Cancer, 88*(12 Suppl), 2892-2898.
Guise, T. A., & Mundy, G. R. (1998a). Cancer and bone. *Endocr Rev, 19*(1), 18-54.
Guise, T. A., & Mundy, G. R. (1998b). Cancer and bone. *Endocrine Reviews, 19*(1), 18-54.
Guise, T. A., Yin, J. J., Taylor, S. D., Kumagai, Y., Dallas, M., Boyce, B. F., et al. (1996). Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest, 98*(7), 1544-1549.
Guy, C. T., Cardiff, R. D., & Muller, W. J. (1992a). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Molecular and Cellular Biology, 12*(3), 954-961.
Guy, C. T., Cardiff, R. D., & Muller, W. J. (1992b). Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol, 12*(3), 954-961.
Hadjidakis, D. J., & Androulakis, II (2006). Bone remodeling. *Annals of the New York Academy of Sciences, 1092*, 385-396.
Han, Z., Wu, K., Shen, H., Li, C., Han, S., Hong, L., et al. (2008). Akt1/protein kinase B alpha is involved in gastric cancer progression and cell proliferation. *Dig Dis Sci, 53*(7), 1801-1810.
Hastings, R. H., Araiza, F., Burton, D. W., Bedley, M., & Deftos, L. J. (2004). Parathyroid hormone-related protein regulates apoptosis in

lung cancer cells through protein kinase A. *Am J Physiol Cell Physiol, 287*(6), C1616-1622.

Hastings, R. H., Araiza, F., Burton, D. W., Zhang, L., Bedley, M., & Deftos, L. J. (2003). Parathyroid hormone-related protein ameliorates death receptor-mediated apoptosis in lung cancer cells. *Am J Physiol Cell Physiol, 285*(6), C1429-1436.

Hatse, S., Princen, K., Bridger, G., De Clercq, E., & Schols, D. (2002). Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Lett, 527*(1-3), 255-262.

Hauschka, P. V., Mavrakos, A. E., Iafrati, M. D., Doleman, S. E., & Klagsbrun, M. (1986). Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. *Journal of Biological Chemistry, 261*(27), 12665-12674.

He, B., Deckelbaum, R. A., Miao, D., Lipman, M. L., Pollak, M., Goltzman, D., et al. (2001). Tissue-specific targeting of the pthrp gene: the generation of mice with floxed alleles. *Endocrinology, 142*(5), 2070-2077.

Henderson, J. E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B. M., Goltzman, D., et al. (1995). Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol Cell Biol, 15*(8), 4064-4075.

Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., et al. (2001). Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst, 93*(3), 234-237.

Henderson, M. A., Danks, J. A., Slavin, J. L., Byrnes, G. B., Choong, P. F., Spillane, J. B., et al. (2006). Parathyroid hormone-related protein localization in breast cancers predict improved prognosis. *Cancer Res, 66*(4), 2250-2256.

Heron-Milhavet, L., Franckhauser, C., Rana, V., Berthenet, C., Fisher, D., Hemmings, B. A., et al. (2006a). Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. *Mol Cell Biol, 26*(22), 8267-8280.

Heron-Milhavet, L., Franckhauser, C., Rana, V., Berthenet, C., Fisher, D., Hemmings, B. A., et al. (2006b). Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. *Molecular and Cellular Biology, 26*(22), 8267-8280.

Hidaka, N., Nishimura, M., & Nagao, K. (1998). Establishment of two human small cell lung cancer cell lines: the evidence of accelerated

production of parathyroid hormone-related protein with tumor progression. *Cancer Lett, 125*(1-2), 149-155.

Hiraki, A., Ueoka, H., Bessho, A., Segawa, Y., Takigawa, N., Kiura, K., et al. (2002). Parathyroid hormone-related protein measured at the time of first visit is an indicator of bone metastases and survival in lung carcinoma patients with hypercalcemia. *Cancer, 95*(8), 1706-1713.

Husemann, Y., Geigl, J. B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., et al. (2008). Systemic spread is an early step in breast cancer. *Cancer Cell, 13*(1), 58-68.

Hutchesson, A. C., Bundred, N. J., & Ratcliffe, W. A. (1995). Survival in hypercalcaemic patients with cancer and co-existing primary hyperparathyroidism. *Postgrad Med J, 71*(831), 28-31.

Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R., & Muller, W. J. (2001). Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. *Mol Cell Biol, 21*(6), 2203-2212.

Iida-Klein, A., Guo, J., Takemura, M., Drake, M. T., Potts, J. T., Jr., Abou-Samra, A., et al. (1997). Mutations in the second cytoplasmic loop of the rat parathyroid hormone (PTH)/PTH-related protein receptor result in selective loss of PTH-stimulated phospholipase C activity. *J Biol Chem, 272*(11), 6882-6889.

Ingleton, P. M. (2002). Parathyroid hormone-related protein in lower vertebrates. *Comp Biochem Physiol B Biochem Mol Biol, 132*(1), 87-95.

Iwamura, M., di Sant'Agnese, P. A., Wu, G., Benning, C. M., Cockett, A. T., Deftos, L. J., et al. (1993). Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res, 53*(8), 1724-1726.

Iwamura, M., Wu, W., Muramoto, M., Ohori, M., Egawa, S., Uchida, T., et al. (1999). Parathyroid hormone-related protein is an independent prognostic factor for renal cell carcinoma. *Cancer, 86*(6), 1028-1034.

Jans, D. A., Thomas, R. J., & Gillespie, M. T. (2003). Parathyroid hormone-related protein (PTHrP): a nucleocytoplasmic shuttling protein with distinct paracrine and intracrine roles. *Vitam Horm, 66*, 345-384.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., & Thun, M. J. (2009). Cancer statistics, 2009. *CA: A Cancer Journal for Clinicians, 59*(4), 225-249.

Jin, L., Briggs, S. L., Chandrasekhar, S., Chirgadze, N. Y., Clawson, D. K., Schevitz, R. W., et al. (2000). Crystal structure of human parathyroid hormone 1-34 at 0.9-A resolution. *J Biol Chem, 275*(35), 27238-27244.

Jobert, A. S., Zhang, P., Couvineau, A., Bonaventure, J., Roume, J., Le Merrer, M., et al. (1998). Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia. *J Clin Invest, 102*(1), 34-40.

Juppner, H. (1999). Receptors for parathyroid hormone and parathyroid hormone-related peptide: exploration of their biological importance. *Bone, 25*(1), 87-90.

Juppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., et al. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science, 254*(5034), 1024-1026.

Kakonen, S. M., & Mundy, G. R. (2003). Mechanisms of osteolytic bone metastases in breast carcinoma. *Cancer, 97*(3 Suppl), 834-839.

Kang, H., Watkins, G., Parr, C., Douglas-Jones, A., Mansel, R. E., & Jiang, W. G. (2005). Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res, 7*(4), R402-410.

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., et al. (2003). A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell, 3*(6), 537-549.

Kao, P. C., Klee, G. G., Taylor, R. L., & Heath, H., 3rd (1990). Parathyroid hormone-related peptide in plasma of patients with hypercalcemia and malignant lesions. *Mayo Clin Proc, 65*(11), 1399-1407.

Karaplis, A. C., He, B., Nguyen, M. T., Young, I. D., Semeraro, D., Ozawa, H., et al. (1998). Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Blomstrand chondrodysplasia. *Endocrinology, 139*(12), 5255-5258.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., et al. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev, 8*(3), 277-289.

Karaplis, A. C., Yasuda, T., Hendy, G. N., Goltzman, D., & Banville, D. (1990). Gene-encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue. *Mol Endocrinol, 4*(3), 441-446.

Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebeling, P. R., Wettenhall, R. E., Stapleton, D., et al. (1987). Parathyroid hormone-related protein of malignancy: active synthetic fragments. *Science, 238*(4833), 1568-1570.

Kimmel, A. R., & Faix, J. (2006). Generation of multiple knockout mutants using the Cre-loxP system. *Methods Mol Biol, 346*, 187-199.

Kingsley, L. A., Fournier, P. G., Chirgwin, J. M., & Guise, T. A. (2007). Molecular biology of bone metastasis. *Mol Cancer Ther, 6*(10), 2609-2617.

Kissin, M. W., Henderson, M. A., Danks, J. A., Hayman, J. A., Bennett, R. C., & Martin, T. J. (1993). Parathyroid hormone related protein in breast cancers of widely varying prognosis. *Eur J Surg Oncol, 19*(2), 134-142.

Kitazawa, S., Fukase, M., Kitazawa, R., Takenaka, A., Gotoh, A., Fujita, T., et al. (1991). Immunohistologic evaluation of parathyroid hormone-related protein in human lung cancer and normal tissue with newly developed monoclonal antibody. *Cancer, 67*(4), 984-989.

Kohno, N., Kitazawa, S., Fukase, M., Sakoda, Y., Kanbara, Y., Furuya, Y., et al. (1994). The expression of parathyroid hormone-related protein in human breast cancer with skeletal metastases. *Surg Today, 24*(3), 215-220.

Kohno, N., Kitazawa, S., Sakoda, Y., Kanbara, Y., Furuya, Y., Ohashi, O., et al. (1994). Parathyroid Hormone-related Protein in Breast Cancer Tissues: Relationship between Primary and Metastatic Sites. *Breast Cancer, 1*(1), 43-49.

Kong, X. F., Schipani, E., Lanske, B., Joun, H., Karperien, M., Defize, L. H., et al. (1994). The rat, mouse, and human genes encoding the receptor for parathyroid hormone and parathyroid hormone-related peptide are highly homologous. *Biochem Biophys Res Commun, 201*(2), 1058.

Korets-Smith, E., Lindemann, L., Tucker, K. L., Jiang, C., Kabacs, N., Belteki, G., et al. (2004). Cre recombinase specificity defined by the tau locus. *Genesis, 40*(3), 131-138.

Kovacs, C. S., Lanske, B., Hunzelman, J. L., Guo, J., Karaplis, A. C., & Kronenberg, H. M. (1996). Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proc Natl Acad Sci U S A, 93*(26), 15233-15238.

Kremer, R., Shustik, C., Tabak, T., Papavasiliou, V., & Goltzman, D. (1996). Parathyroid-hormone-related peptide in hematologic malignancies. *Am J Med, 100*(4), 406-411.

Kuhn, R., & Torres, R. M. (2002). Cre/loxP recombination system and gene targeting. *Methods Mol Biol, 180*, 175-204.

Lam, M. H., Briggs, L. J., Hu, W., Martin, T. J., Gillespie, M. T., & Jans, D. A. (1999). Importin beta recognizes parathyroid hormone-related

protein with high affinity and mediates its nuclear import in the absence of importin alpha. *J Biol Chem, 274*(11), 7391-7398.

Lam, M. H., House, C. M., Tiganis, T., Mitchelhill, K. I., Sarcevic, B., Cures, A., et al. (1999). Phosphorylation at the cyclin-dependent kinases site (Thr85) of parathyroid hormone-related protein negatively regulates its nuclear localization. *J Biol Chem, 274*(26), 18559-18566.

Lam, M. H., Olsen, S. L., Rankin, W. A., Ho, P. W., Martin, T. J., Gillespie, M. T., et al. (1997). PTHrP and cell division: expression and localization of PTHrP in a keratinocyte cell line (HaCaT) during the cell cycle. *J Cell Physiol, 173*(3), 433-446.

Lam, M. H., Thomas, R. J., Loveland, K. L., Schilders, S., Gu, M., Martin, T. J., et al. (2002). Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules. *Mol Endocrinol, 16*(2), 390-401.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science, 273*(5275), 663-666.

Lee, B. C., Lee, T. H., Zagozdzon, R., Avraham, S., Usheva, A., & Avraham, H. K. (2005). Carboxyl-terminal Src kinase homologous kinase negatively regulates the chemokine receptor CXCR4 through YY1 and impairs CXCR4/CXCL12 (SDF-1alpha)-mediated breast cancer cell migration. *Cancer Res, 65*(7), 2840-2845.

Liang, Z., Wu, T., Lou, H., Yu, X., Taichman, R. S., Lau, S. K., et al. (2004). Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res, 64*(12), 4302-4308.

Liang, Z., Yoon, Y., Votaw, J., Goodman, M. M., Williams, L., & Shim, H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res, 65*(3), 967-971.

Liao, J., Li, X., Koh, A. J., Berry, J. E., Thudi, N., Rosol, T. J., et al. (2008). Tumor expressed PTHrP facilitates prostate cancer-induced osteoblastic lesions. *Int J Cancer, 123*(10), 2267-2278.

Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., et al. (2003). Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol, 163*(5), 2113-2126.

Linforth, R., Anderson, N., Hoey, R., Nolan, T., Downey, S., Brady, G., et al. (2002). Coexpression of parathyroid hormone related protein and its receptor in early breast cancer predicts poor patient survival. *Clin Cancer Res, 8*(10), 3172-3177.

Lipton, A. (2004). Pathophysiology of bone metastases: how this knowledge may lead to therapeutic intervention. *J Support Oncol, 2*(3), 205-213; discussion 213-204, 216-207, 219-220.

Liu, P., Cheng, H., Roberts, T. M., & Zhao, J. J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov, 8*(8), 627-644.

Luker, K. E., & Luker, G. D. (2006). Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Lett, 238*(1), 30-41.

Luparello, C., Burtis, W. J., Raue, F., Birch, M. A., & Gallagher, J. A. (1995). Parathyroid hormone-related peptide and 8701-BC breast cancer cell growth and invasion in vitro: evidence for growth-inhibiting and invasion-promoting effects. *Mol Cell Endocrinol, 111*(2), 225-232.

Luparello, C., Sirchia, R., & Pupello, D. (2003). PTHrP [67-86] regulates the expression of stress proteins in breast cancer cells inducing modifications in urokinase-plasminogen activator and MMP-1 expression. *J Cell Sci, 116*(Pt 12), 2421-2430.

Maioli, E., & Fortino, V. (2004). The complexity of parathyroid hormone-related protein signalling. *Cell Mol Life Sci, 61*(3), 257-262.

Mangin, M., Ikeda, K., & Broadus, A. E. (1990). Structure of the mouse gene encoding parathyroid hormone-related peptide. *Gene, 95*(2), 195-202.

Mangin, M., Ikeda, K., Dreyer, B. E., & Broadus, A. E. (1989). Isolation and characterization of the human parathyroid hormone-like peptide gene. *Proc Natl Acad Sci U S A, 86*(7), 2408-2412.

Mangin, M., Ikeda, K., Dreyer, B. E., & Broadus, A. E. (1990). Identification of an up-stream promoter of the human parathyroid hormone-related peptide gene. *Mol Endocrinol, 4*(6), 851-858.

Mangin, M., Ikeda, K., Dreyer, B. E., Milstone, L., & Broadus, A. E. (1988). Two distinct tumor-derived, parathyroid hormone-like peptides result from alternative ribonucleic acid splicing. *Mol Endocrinol, 2*(11), 1049-1055.

Mangin, M., Webb, A. C., Dreyer, B. E., Posillico, J. T., Ikeda, K., Weir, E. C., et al. (1988). Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. *Proceedings of the National Academy of Sciences of the United States of America, 85*(2), 597-601.

Mannens, M., Slater, R. M., Heyting, C., Geurts van Kessel, A., Goedde-Salz, E., Frants, R. R., et al. (1987). Regional localization of DNA probes on the short arm of chromosome 11 using aniridia-Wilms' tumor-associated deletions. *Human Genetics, 75*(2), 180-187.

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., & Benovic, J. L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell, 5*(5), 709-722.

Maroulakou, I. G., Oemler, W., Naber, S. P., & Tsichlis, P. N. (2007). Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer Res, 67*(1), 167-177.

Martin, B., Lopez de Maturana, R., Brenneman, R., Walent, T., Mattson, M. P., & Maudsley, S. (2005). Class II G protein-coupled receptors and their ligands in neuronal function and protection. *Neuromolecular Med, 7*(1-2), 3-36.

Martin, T. J., & Moseley, J. M. (2000). Mechanisms in the skeletal complications of breast cancer. *Endocr Relat Cancer, 7*(4), 271-284.

Martin, T. J., Moseley, J. M., & Williams, E. D. (1997). Parathyroid hormone-related protein: hormone and cytokine. *J Endocrinol, 154 Suppl*, S23-37.

Martin, T. J., & Suva, L. J. (1988). Parathyroid hormone-related protein: a novel gene product. *Baillieres Clin Endocrinol Metab, 2*(4), 1003-1029.

Martinez, M. E., Garcia-Ocana, A., Sanchez, M., Medina, S., del Campo, T., Valin, A., et al. (1997). C-terminal parathyroid hormone-related protein inhibits proliferation and differentiation of human osteoblast-like cells. *J Bone Miner Res, 12*(5), 778-785.

Massfelder, T., Dann, P., Wu, T. L., Vasavada, R., Helwig, J. J., & Stewart, A. F. (1997). Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. *Proc Natl Acad Sci U S A, 94*(25), 13630-13635.

Miao, D., Su, H., He, B., Gao, J., Xia, Q., Zhu, M., et al. (2008). Severe growth retardation and early lethality in mice lacking the nuclear localization sequence and C-terminus of PTH-related protein. *Proc Natl Acad Sci U S A, 105*(51), 20309-20314.

Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., et al. (2005). Genes that mediate breast cancer metastasis to lung. *Nature, 436*(7050), 518-524.

Moseley, J. M., & Gillespie, M. T. (1995). Parathyroid hormone-related protein. *Crit Rev Clin Lab Sci, 32*(3), 299-343.

Moseley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E., Kemp, B. E., Suva, L. J., et al. (1987). Parathyroid hormone-related

protein purified from a human lung cancer cell line. *Proc Natl Acad Sci U S A, 84*(14), 5048-5052.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature, 410*(6824), 50-56.

Mundy, G. R. (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer, 2*(8), 584-593.

Nagy, A., & Mar, L. (2001). Creation and use of a Cre recombinase transgenic database. *Methods Mol Biol, 158*, 95-106.

Nguyen, M., He, B., & Karaplis, A. (2001). Nuclear forms of parathyroid hormone-related peptide are translated from non-AUG start sites downstream from the initiator methionine. *Endocrinology, 142*(2), 694-703.

Nickols, G. A., Nickols, M. A., & Helwig, J. J. (1990). Binding of parathyroid hormone and parathyroid hormone-related protein to vascular smooth muscle of rabbit renal microvessels. *Endocrinology, 126*(2), 721-727.

Nishigaki, Y., Ohsaki, Y., Toyoshima, E., & Kikuchi, K. (1999). Increased serum and urinary levels of a parathyroid hormone-related protein COOH terminus in non-small cell lung cancer patients. *Clin Cancer Res, 5*(6), 1473-1481.

Nishihara, M., Ito, M., Tomioka, T., Ohtsuru, A., Taguchi, T., & Kanematsu, T. (1999). Clinicopathological implications of parathyroid hormone-related protein in human colorectal tumours. *J Pathol, 187*(2), 217-222.

Nishihara, M., Kanematsu, T., Taguchi, T., & Razzaque, M. S. (2007). PTHrP and tumorigenesis: is there a role in prognosis? *Ann N Y Acad Sci, 1117*, 385-392.

Omenn, G. S., Roth, S. I., & Baker, W. H. (1969). Hyperparathyroidism associated with malignant tumors of nonparathyroid origin. *Cancer, 24*(5), 1004-1011.

Orloff, J. J., Ganz, M. B., Ribaudo, A. E., Burtis, W. J., Reiss, M., Milstone, L. M., et al. (1992). Analysis of PTHRP binding and signal transduction mechanisms in benign and malignant squamous cells. *Am J Physiol, 262*(5 Pt 1), E599-607.

Orloff, J. J., Reddy, D., de Papp, A. E., Yang, K. H., Soifer, N. E., & Stewart, A. F. (1994). Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions. *Endocr Rev, 15*(1), 40-60.

Orloff, J. J., Soifer, N. E., Fodero, J. P., Dann, P., & Burtis, W. J. (1993). Accumulation of carboxy-terminal fragments of parathyroid hormone-related protein in renal failure. *Kidney Int, 43*(6), 1371-1376.

Pecherstorfer, M., Schilling, T., Blind, E., Zimmer-Roth, I., Baumgartner, G., Ziegler, R., et al. (1994). Parathyroid hormone-related protein and life expectancy in hypercalcemic cancer patients. *J Clin Endocrinol Metab, 78*(5), 1268-1270.

Petit, I., Jin, D., & Rafii, S. (2007). The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. *Trends Immunol, 28*(7), 299-307.

Peyruchaud, O. (2007). [Mechanisms of bone metastasis formation]. *J Soc Biol, 201*(3), 229-236.

Philbrick, W. M., Dreyer, B. E., Nakchbandi, I. A., & Karaplis, A. C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc Natl Acad Sci U S A, 95*(20), 11846-11851.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J. J., Yang, K. H., et al. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol Rev, 76*(1), 127-173.

Rankin, W., Grill, V., & Martin, T. J. (1997). Parathyroid hormone-related protein and hypercalcemia. *Cancer, 80*(8 Suppl), 1564-1571.

Riggs, B. L., Arnaud, C. D., Reynolds, J. C., & Smith, L. H. (1971). Immunologic differentiation of primary hyperparathyroidism from hyperparathyroidism due to nonparathyroid cancer. *J Clin Invest, 50*(10), 2079-2083.

Roodman, G. D. (2004). Mechanisms of bone metastasis. *N Engl J Med, 350*(16), 1655-1664.

Rossant, J., & Nagy, A. (1995). Genome engineering: the new mouse genetics. *Nat Med, 1*(6), 592-594.

Rotllant, J., Guerreiro, P. M., Redruello, B., Fernandes, H., Apolonia, L., Anjos, L., et al. (2006). Ligand binding and signalling pathways of PTH receptors in sea bream (Sparus auratus) enterocytes. *Cell Tissue Res, 323*(2), 333-341.

Rubin, D. A., Hellman, P., Zon, L. I., Lobb, C. J., Bergwitz, C., & Juppner, H. (1999). A G protein-coupled receptor from zebrafish is activated by human parathyroid hormone and not by human or teleost parathyroid hormone-related peptide. Implications for the evolutionary conservation of calcium-regulating peptide hormones. *J Biol Chem, 274*(33), 23035-23042.

Saito, H., Tsunenari, T., Onuma, E., Sato, K., Ogata, E., & Yamada-Okabe, H. (2005). Humanized monoclonal antibody against parathyroid hormone-related protein suppresses osteolytic bone metastasis of

human breast cancer cells derived from MDA-MB-231. *Anticancer Res, 25*(6B), 3817-3823.

Sato, K., Onuma, E., Yocum, R. C., & Ogata, E. (2003). Treatment of malignancy-associated hypercalcemia and cachexia with humanized anti-parathyroid hormone-related protein antibody. *Semin Oncol, 30*(5 Suppl 16), 167-173.

Scheid, M. P., & Woodgett, J. R. (2001). PKB/AKT: functional insights from genetic models. *Nat Rev Mol Cell Biol, 2*(10), 760-768.

Schilling, T., Pecherstorfer, M., Blind, E., Leidig, G., Ziegler, R., & Raue, F. (1993). Parathyroid hormone-related protein (PTHrP) does not regulate 1,25-dihydroxyvitamin D serum levels in hypercalcemia of malignancy. *J Clin Endocrinol Metab, 76*(3), 801-803.

Schipani, E., Karga, H., Karaplis, A. C., Potts, J. T., Jr., Kronenberg, H. M., Segre, G. V., et al. (1993). Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTH-related peptide receptor. *Endocrinology, 132*(5), 2157-2165.

Schipani, E., Kruse, K., & Juppner, H. (1995). A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science, 268*(5207), 98-100.

Schipani, E., Langman, C., Hunzelman, J., Le Merrer, M., Loke, K. Y., Dillon, M. J., et al. (1999). A novel parathyroid hormone (PTH)/PTH-related peptide receptor mutation in Jansen's metaphyseal chondrodysplasia. *J Clin Endocrinol Metab, 84*(9), 3052-3057.

Schipani, E., Langman, C. B., Parfitt, A. M., Jensen, G. S., Kikuchi, S., Kooh, S. W., et al. (1996). Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen's metaphyseal chondrodysplasia. *N Engl J Med, 335*(10), 708-714.

Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C. S., Lee, K., et al. (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. *Proc Natl Acad Sci U S A, 94*(25), 13689-13694.

Seitz, P. K., Cooper, K. M., Ives, K. L., Ishizuka, J., Townsend, C. M., Jr., Rajaraman, S., et al. (1993). Parathyroid hormone-related peptide production and action in a myoepithelial cell line derived from normal human breast. *Endocrinology, 133*(3), 1116-1124.

Seitz, P. K., Zhang, R. W., Simmons, D. J., & Cooper, C. W. (1995). Effects of C-terminal parathyroid hormone-related peptide on osteoblasts. *Miner Electrolyte Metab, 21*(1-3), 180-183.

Shen, X., & Falzon, M. (2006). PTH-related protein upregulates integrin alpha6beta4 expression and activates Akt in breast cancer cells. *Exp Cell Res, 312*(19), 3822-3834.

Shibahara, T., Nomura, T., Cui, N. H., & Noma, H. (2005). A study of osteoclast-related cytokines in mandibular invasion by squamous cell carcinoma. *Int J Oral Maxillofac Surg, 34*(7), 789-793.

Soifer, N. E., Dee, K. E., Insogna, K. L., Burtis, W. J., Matovcik, L. M., Wu, T. L., et al. (1992). Parathyroid hormone-related protein. Evidence for secretion of a novel mid-region fragment by three different cell types. *J Biol Chem, 267*(25), 18236-18243.

Solomayer, E. F., Diel, I. J., Meyberg, G. C., Gollan, C., & Bastert, G. (2000). Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. *Breast Cancer Res Treat, 59*(3), 271-278.

Southby, J., Kissin, M. W., Danks, J. A., Hayman, J. A., Moseley, J. M., Henderson, M. A., et al. (1990). Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res, 50*(23), 7710-7716.

Sternberg, N., & Hamilton, D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol, 150*(4), 467-486.

Stewart, A. F. (2002). Hyperparathyroidism, humoral hypercalcemia of malignancy, and the anabolic actions of parathyroid hormone and parathyroid hormone-related protein on the skeleton. *J Bone Miner Res, 17*(5), 758-762.

Strewler, G. J. (2000). The physiology of parathyroid hormone-related protein. *N Engl J Med, 342*(3), 177-185.

Strewler, G. J., Stern, P. H., Jacobs, J. W., Eveloff, J., Klein, R. F., Leung, S. C., et al. (1987). Parathyroid hormonelike protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. *J Clin Invest, 80*(6), 1803-1807.

Sugihara, A., Maeda, O., Tsuji, M., Tsujimura, T., Nakata, Y., Akedo, H., et al. (1998). Expression of cytokines enhancing the osteoclast activity, and parathyroid hormone-related protein in prostatic cancers before and after endocrine therapy: an immunohistochemical study. *Oncol Rep, 5*(6), 1389-1394.

Surowiak, P., Dziegiel, P., Matkowski, R., Sopel, M., Wojnar, A., Kornafel, J., et al. (2003). Prognostic value of immunocytochemical

determination of parathyroid hormone-related peptide expression in cells of mammary ductal carcinoma. Analysis of 7 years of the disease course. *Virchows Arch, 442*(3), 245-251.

Suva, L. J., Winslow, G. A., Wettenhall, R. E., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., et al. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science, 237*(4817), 893-896.

Suzuki, K., & Yamada, S. (1994). Ascites sarcoma 180, a tumor associated with hypercalcemia, secretes potent bone-resorbing factors including transforming growth factor alpha, interleukin-1 alpha and interleukin-6. *Bone Miner, 27*(3), 219-233.

Svane, S. (1964). HYPERCALCEMIA IN MALIGNANT DISEASE WITHOUT EVIDENCE OF BONE DESTRUCTION. A CASE SIMULATING ACUTE HYPERPARATHYROIDISM. *Acta Med Scand, 175*, 353-357.

Swarthout, J. T., D'Alonzo, R. C., Selvamurugan, N., & Partridge, N. C. (2002). Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene, 282*(1-2), 1-17.

Tabernero, J., Rojo, F., Calvo, E., Burris, H., Judson, I., Hazell, K., et al. (2008). Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. *Journal of Clinical Oncology, 26*(10), 1603-1610.

Takasu, H., Guo, J., & Bringhurst, F. R. (1999). Dual signaling and ligand selectivity of the human PTH/PTHrP receptor. *J Bone Miner Res, 14*(1), 11-20.

Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., et al. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A, 88*(6), 2065-2069.

Tashjian, A. H., Jr. (1969). Animal cell cultures as a source of hormones. *Biotechnol Bioeng, 11*(2), 109-126.

Teicher, B. A., & Fricker, S. P. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clinical Cancer Research, 16*(11), 2927-2931.

Testa, J. R., & Tsichlis, P. N. (2005). AKT signaling in normal and malignant cells. *Oncogene, 24*(50), 7391-7393.

Thiede, M. A., & Rutledge, S. J. (1990). Nucleotide sequence of a parathyroid hormone-related peptide expressed by the 10 day chicken embryo. *Nucleic Acids Res, 18*(10), 3062.

Thiede, M. A., Strewler, G. J., Nissenson, R. A., Rosenblatt, M., & Rodan, G. A. (1988). Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: evidence for the

alternative splicing of a single-copy gene. *Proceedings of the National Academy of Sciences of the United States of America, 85*(13), 4605-4609.

Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., et al. (1999). Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology, 140*(10), 4451-4458.

Tovar Sepulveda, V. A., & Falzon, M. (2002). Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. *Regul Pept, 105*(2), 109-120.

Truong, N. U., de, B. E. M. D., Papavasiliou, V., Goltzman, D., & Kremer, R. (2003). Parathyroid hormone-related peptide and survival of patients with cancer and hypercalcemia. *Am J Med, 115*(2), 115-121.

Ursini-Siegel, J., Schade, B., Cardiff, R. D., & Muller, W. J. (2007). Insights from transgenic mouse models of ERBB2-induced breast cancer. *Nat Rev Cancer, 7*(5), 389-397.

Usdin, T. B., Bonner, T. I., Harta, G., & Mezey, E. (1996). Distribution of parathyroid hormone-2 receptor messenger ribonucleic acid in rat. *Endocrinology, 137*(10), 4285-4297.

Usdin, T. B., Gruber, C., & Bonner, T. I. (1995). Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. *J Biol Chem, 270*(26), 15455-15458.

Usdin, T. B., Hoare, S. R., Wang, T., Mezey, E., & Kowalak, J. A. (1999). TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. *Nature Neuroscience, 2*(11), 941-943.

Van Duyne, G. D. (2001). A structural view of cre-loxp site-specific recombination. *Annu Rev Biophys Biomol Struct, 30*, 87-104.

Vanderschueren, B., Dumon, J. C., Oleffe, V., Heymans, C., Gerain, J., & Body, J. J. (1994). Circulating concentrations of interleukin-6 in cancer patients and their pathogenic role in tumor-induced hypercalcemia. *Cancer Immunol Immunother, 39*(5), 286-290.

Vargas, S. J., Gillespie, M. T., Powell, G. J., Southby, J., Danks, J. A., Moseley, J. M., et al. (1992). Localization of parathyroid hormone-related protein mRNA expression in breast cancer and metastatic lesions by in situ hybridization. *J Bone Miner Res, 7*(8), 971-979.

Veltmaat, J. M., Mailleux, A. A., Thiery, J. P., & Bellusci, S. (2003). Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation. *Differentiation, 71*(1), 1-17.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer, 2*(7), 489-501.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., & Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science, 273*(5275), 613-622.

Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R., & Hennighausen, L. (2001a). Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Research, 10*(6), 545-553.

Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R., & Hennighausen, L. (2001b). Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res, 10*(6), 545-553.

Wagner, K. U., Wall, R. J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., et al. (1997). Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res, 25*(21), 4323-4330.

Weigelt, B., Peterse, J. L., & van 't Veer, L. J. (2005). Breast cancer metastasis: markers and models. *Nat Rev Cancer, 5*(8), 591-602.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R., & Broadus, A. E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc Natl Acad Sci U S A, 93*(19), 10240-10245.

White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., et al. (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell, 6*(2), 159-170.

Whitfield, J. F., Isaacs, R. J., Chakravarthy, B., Maclean, S., Morley, P., Willick, G., et al. (2001). Stimulation of protein kinase C activity in cells expressing human parathyroid hormone receptors by C- and N-terminally truncated fragments of parathyroid hormone 1-34. *J Bone Miner Res, 16*(3), 441-447.

Wu, T. L., Vasavada, R. C., Yang, K., Massfelder, T., Ganz, M., Abbas, S. K., et al. (1996). Structural and physiologic characterization of the mid-region secretory species of parathyroid hormone-related protein. *J Biol Chem, 271*(40), 24371-24381.

Wysolmerski, J. J., & Broadus, A. E. (1994). Hypercalcemia of malignancy: the central role of parathyroid hormone-related protein. *Annu Rev Med, 45*, 189-200.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M., & Philbrick, W. M. (1994). Overexpression of parathyroid hormone-

related protein in the skin of transgenic mice interferes with hair follicle development. *Proc Natl Acad Sci U S A, 91*(3), 1133-1137.
Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E., & Philbrick, W. M. (1995). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development, 121*(11), 3539-3547.
Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., & Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development, 125*(7), 1285-1294.
Wysolmerski, J. J., & Stewart, A. F. (1998). The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. *Annu Rev Physiol, 60*, 431-460.
Yamada, T., Tsuda, M., Ohba, Y., Kawaguchi, H., Totsuka, Y., & Shindoh, M. (2008). PTHrP promotes malignancy of human oral cancer cell downstream of the EGFR signaling. *Biochem Biophys Res Commun, 368*(3), 575-581.
Yang, D., Guo, J., Divieti, P., & Bringhurst, F. R. (2006). Parathyroid hormone activates PKC-delta and regulates osteoblastic differentiation via a PLC-independent pathway. *Bone, 38*(4), 485-496.
Yasuda, T., Banville, D., Hendy, G. N., & Goltzman, D. (1989). Characterization of the human parathyroid hormone-like peptide gene. Functional and evolutionary aspects. *J Biol Chem, 264*(13), 7720-7725.
Yoneda, T. (2000). Cellular and molecular basis of preferential metastasis of breast cancer to bone. *J Orthop Sci, 5*(1), 75-81.
Zhang, X. H., Wang, Q., Gerald, W., Hudis, C. A., Norton, L., Smid, M., et al. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell, 16*(1), 67-78.
Zhao, M., Mueller, B. M., DiScipio, R. G., & Schraufstatter, I. U. (2008). Akt plays an important role in breast cancer cell chemotaxis to CXCL12. *Breast Cancer Res Treat, 110*(2), 211-222.
Zlotnik, A. (2004). Chemokines in neoplastic progression. *Semin Cancer Biol, 14*(3), 181-185.
Zlotnik, A. (2008). New insights on the role of CXCR4 in cancer metastasis. *J Pathol, 215*(3), 211-213.