PARATHYROID HORMONE-RELATED PEPTIDE:

A KEY FACTOR IN CELL ADHESION

By

JENNIFER ANNE ANDERSON

BSc. Hons (Aberdeen)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2006

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ACKNOWLEDGEMENTS

I would firstly like to thank my PhD supervisor, Professor Sue Watson, for her help and support during my PhD. I am also extremely grateful to Dr Anna Grabowska for her encouragement and guidance throughout these three years despite numerous other important commitments and other students knocking on her door.

I would also like to thank each of the members of the Academic Unit of Cancer Studies, past and present, in particular those who helped me with laboratory techniques: Wendy Kleie, Phil Clarke, Andrew McKenzie and Rajendra Kumari.

My thanks also go to two key CSU members - Jaime and Amanda for injecting both sanity and fun into the labs (coffee breaks wouldn't have been the same without you). And Joe, part of the PhD student gang, entertaining us with his rhyming slang. Peace out!

Special thanks go to Jane McClelland for her administrative support but more importantly the supply of indispensable (and often necessary) beer and chat at the Rose & Crown, amongst other reputable drinking establishments. Couldn't have done it without you!

I am immensely grateful to Google and Wikipedia, without which I wouldn't have made it this far.

Finally, I would like to thank all my friends and family for their emotional and financial support during my student years.

ABBREVIATIONS

[^{3}H]-thymidine	Tritiated thymidine
1,25$(OH)_2D_3$	Active metabolite of vitamin D
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CPM	Counts per minute
Ct	Threshold cycle
DAB	3,3'-diaminobenzidine
DAPI	4'-6-Diamidino-2-phenylindole
dNTP	Deoxynucleotide
DRB	5,6-dichlororibofuranosyl benzimidazole
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GI	Gastrointestinal
HHM	Humoral hypercalcemia of malignancy
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horseradish peroxidise
JC-1	5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide
LOH	Local osteolytic hypercalcemia
mRNA	Messenger RNA
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue

NLS	Nuclear localisation sequence
NO	Nitric oxide
NSCLC	Non-small-cell lung cancer
nt	Nucleotide
OAS	2',5'-oligoadenylate synthetase
ODC	Ornithine decarboxylase
ODNs	Oligodeoxynucleotides
PBS	Phosphate buffered saline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
PTH1R	PTH/PTHrP receptor
PTHrP	Parathyroid hormone-related protein
PVDF	Polyvinylidene fluoride
RISC	RNA-induced silencing complex
RNAi	RNA interference
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR
siRNA	Small interfering RNA
STAT	Signal transducers and activator of transcription
TBS	Tris buffered saline
TGFβ1	Transforming growth factor β1
UTR	Untranslated Region
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

ABSTRACT

Over-expression of parathyroid hormone-related protein (PTHrP) is commonly described in a number of different forms of cancer and it has been suggested that this over-expression leads to tissue-specific metastasis whereby primary tumours have a propensity to metastasise to one particular organ e.g. breast tumours metastasise to bone whereas gastrointestinal tumours favour the liver. The aim of my PhD was to examine the role PTHrP plays in cancer cell adhesion to the extracellular matrix (ECM), to explore a mechanism of action and to elucidate any tissue-specific differences to explain the apparent partiality during metastasis.

In order to do this a small interfering RNA was used to silence PTHrP gene expression and expression vectors containing cDNA for PTHrP were used to create several stable PTHrP over-expressing cell lines. Analysis of cell adhesion revealed that regulating PTHrP expression caused changes in adhesion to the ECM proteins collagen type I, fibronectin and laminin in breast cancer cell lines. However although cell adhesion of gastrointestinal cancer cell lines to collagen type I and fibronectin was similarly affected, adhesion to laminin was unchanged by variations in PTHrP expression.

The cell adhesion molecules integrins were subsequently investigated for their role in PTHrP mediated cell adhesion. Integrins are heterodimers composed of an α and β subunit and to date 24 different subunits have been identified. Each unique combination of subunits results in a different ligand specificity, which includes collagens, fibronectin and laminin. Analysis of integrin gene and protein expression in over-expressing cell lines and in cells where PTHrP had been silenced it was

possible to demonstrate a link between expression of PTHrP and a number of different integrin subunits. One of the more significant findings was the observation that the integrin subunit α_6 changed in parallel with PTHrP in breast but not gastrointestinal cancer cell lines. As laminin is a ligand for this subunit these results correlate with the results previously described regarding this ECM protein.

The effects of PTHrP appeared to be mediated at the transcriptional rather than the translational level so integrin transcriptional activity was investigated using a reporter vector containing the coding region for firefly luciferase. The integrin subunit α_5 had shown a link to PTHrP expression in both breast and gastrointestinal cell lines so the promoter region for this subunit was inserted into the reporter vector, which was then transiently transfected into PTHrP over-expressing cell lines. Subsequent examination of luciferase activity revealed a significant increase in promoter activity compared with control thus implicating PTHrP as a key factor in integrin gene transcription.

The results described here suggest that PTHrP increases cell adhesion by inducing integrin gene transcription and is able to regulate expression of different subunits in different tissues thereby encouraging tissue-specific metastasis.

Chapter 1:

Introduction

1.1 PARATHYROID HORMONE-RELATED PROTEIN

Malignancy associated hypercalcemia, which is characterised by a life threatening increase in serum calcium, can be subdivided into humoral hypercalcemia of malignancy (HHM) and local osteolytic hypercalcemia (LOH). LOH is the result of a local paracrine factor and is the product of bone invasion and destruction by tumour cells. HHM on the other hand is the product of a circulating hormone which induces osteoclastic bone resorption (Broadus, Goltzman et al. 1985).

Parathyroid hormone (PTH) is a circulating hormone and was originally thought to be the cause of HHM due to its ability to induce bone resorption; however radioimmunoassay tests on patient sera did not show evidence of an increase in PTH expression (Powell, Singer et al. 1973). The real culprit was identified in 1987 by a group at the University of Melbourne.

Using a cell line established from a hypercalcemic patient with a squamous cell carcinoma of the bronchus, Moseley *et al* purified an 18kDa protein from the cell medium with "PTH-like activity", which the authors defined as an ability to stimulate cAMP production. Due to its biological similarities with PTH it was named PTH-related protein (PTHrP). When they subsequently analysed the amino acid sequence they found a high degree of homology with the human PTH sequence, with 8 out of the first 13 residues being identical. Using PTH antagonists they were also able to inhibit the cAMP production induced by the conditioned media (Moseley, Kubota et al. 1987).

Around the same time, PTHrP was also isolated from breast and renal carcinomas (Burtis, Wu et al. 1987; Strewler, Stern et al. 1987) and in the studies that followed it was confirmed that PTHrP was not only responsible for HHM but also played a paracrine role in causing LOH.

HHM is an important health issue in patients with cancer as it develops suddenly and reduces a patient's survival rate; Coleman and Rubens reported that patients with breast carcinoma, bone metastases and hypercalcemia had a median survival time of three months survival compared with 24 months for patients with bone metastases alone. Similarly the development of hypercalcemia in colon cancer reduces patient median survival to only 35 days (Coleman and Rubens 1987; Lortholary, Cadeau et al. 1999).

HHM is most commonly found in breast cancer patients with bone metastases as the majority of these are osteolytic (Sloan and Anderson 2002). Although rare, HHM has been identified in other forms of cancer such as colon cancer, as described by Lortholary *et al* who reported two cases of HHM in colorectal carcinoma patients and also reviewed the literature and found only nine other reported cases, the first of which was described in 1963. In both of their reported cases Lorthorlary *et al* found an increase in serum PTHrP along with PTHrP immunoreactivity in the primary tumour however there were no detectable bone metastases. Likewise Sidler *et al* showed that the gene for PTHrP was amplified in colon cancer and coincided with the appearance of hypercalcemia (Sidler, Alpert et al. 1996; Lortholary, Cadeau et al. 1999).

Other reported cases of HHM associated with PTHrP include a germ cell tumour (Sorscher 2004), a pancreatic tumour (Miraliakbari, Asa et al. 1992) and cancer of the penis (Akashi, Fuse et al. 2002).

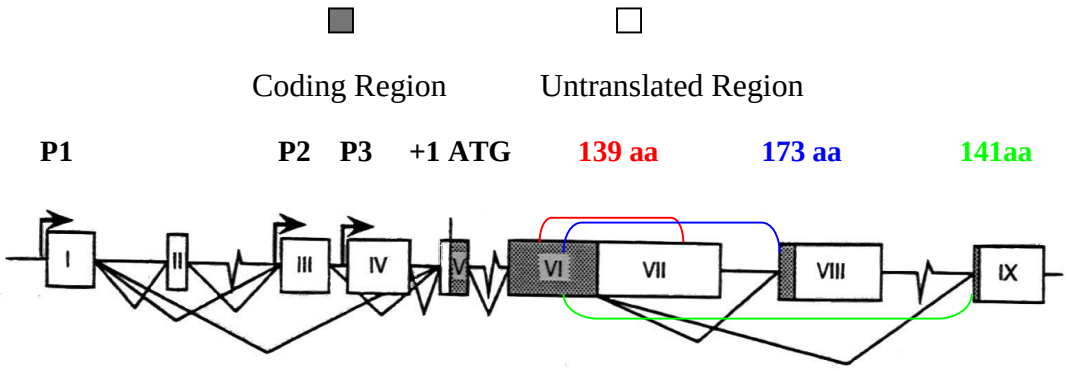
It can thus be seen that an increase in PTHrP expression brings about additional complications in cancer patients and as such should be taken into account when considering patient prognosis.

1.1.1 PTHrP Gene Structure

PTHrP was cloned by several groups shortly after it had been isolated (Suva, Winslow et al. 1987; Mangin, Webb et al. 1988). The PTHrP gene can be found on the short arm of chromosome 12 and consists of over 15 kilobases of genomic DNA (Yasuda T, Banville D et al. 1989); it is comprised of nine exons, includes three different promoters and alternative splicing allows the production of three different isoforms. Splicing of exon VI to VII produces an isoform 139 amino acids (aa) in length, splicing of exon VI to IX produces a 141aa isoform and VI to VIII a 173aa isoform. Exon V encodes the pre-pro region of PTHrP and consequently products of exons V and VI can be found in all three PTHrP transcripts (Wysolmerski and Broadus 1994). The structure of PTHrP can be seen in Figure 1.1.

PTHrP has three promoters which suggests a degree of transcriptional control; P1 is an upstream TATA promoter (5' to exon I), P2 is a GC-rich promoter (5' to exon III) while P3 is a downstream TATA promoter (5' to exon IV) (Southby J, O'Keeffe LM et al. 1995). It is believed that any of the three different isoforms can use any of these three promoters and to date there has been no evidence to suggest otherwise.

Figure 1.1 – Diagrammatic representation of PTHrP gene structure (Southby, O'Keeffe et al. 1995)



1.1.2 Regulation of PTHrP Gene Expression

PTHrP mRNA is unstable, with a half-life between 30 and 120 minutes. A number of factors have been shown to regulate PTHrP expression, including epidermal growth factor (EGF) (Heath, Southby et al. 1995) and transforming growth factor β1 (TGFβ1) (Heath, Southby et al. 1995; Sellers, Capen et al. 2002).

An increase in EGF receptor expression has been shown in a number of solid tumours, including breast, head-and-neck, non-small-cell lung (NSCLC), renal, ovarian and colon. This increase has been linked to an increase in cell growth and tumour invasion; in NSCLC, over-expression of the EGF receptor correlates with a high level of metastasis and tumour growth. As such it has been linked with poor prognosis (Herbst 2004).

Heath *et al* identified a role for EGF in regulating PTHrP expression using an immortalised keratinocyte cell line which constitutively produces PTHrP. EGF stimulation caused a dose-dependent increase in PTHrP protein and gene expression, which the authors went on to show was mediated via an increase in PTHrP transcription (Heath, Southby et al. 1995).

Heath *et al* also examined PTHrP mRNA stability using the transcriptional inhibitors actinomycin D and 5,6-dichlororibofuranosyl benzimidazole (DRB) to demonstrate that EGF causes a post-transcriptional increase in mRNA stability and proposed that these effects were mediated by post-translational modifications such as phosphorylation or intracellular translocation. In addition they examined expression of the three different PTHrP isoforms and showed that while EGF increased the stability of PTHrP (1-139) and (1-173), PTHrP (1-141) was unaffected.

Tovar Sepulveda and Falzon also used EGF to induce a dose-dependent increase in PTHrP mRNA and secretion rate in a prostate cancer cell line. They went on to demonstrate that the active metabolite of vitamin D ($1,25(OH)_2D_3$) is able to produce a dose-dependent decrease in PTHrP mRNA and secretion in prostate tumour cells but not in normal prostate epithelial cells. Further analysis revealed that both EGF and $1,25(OH)_2D_3$ were able to induce their responses at the transcriptional level (Tovar Sepulveda and Falzon 2002).

TGFβ1 has been described as a tumour suppressor in early stages of cancer however its over-expression has also been identified in a number of cancers including breast, colon, prostate, gastric, lung and liver. As with EGF, TGFβ1 has been linked with an increase in metastasis following studies demonstrating an increase in TGFβ1 expression compared with the primary tumour. TGFβ1 has thus been linked with poor prognosis (Glick 2004).

Sellers *et al* used TGFβ1 to increase the half-life of PTHrP in squamous carcinoma cells and using DRB demonstrated that, as with EGF, this increase in mRNA half-life appears to be mediated post-transcriptionally. (Sellers, Capen et al. 2002).

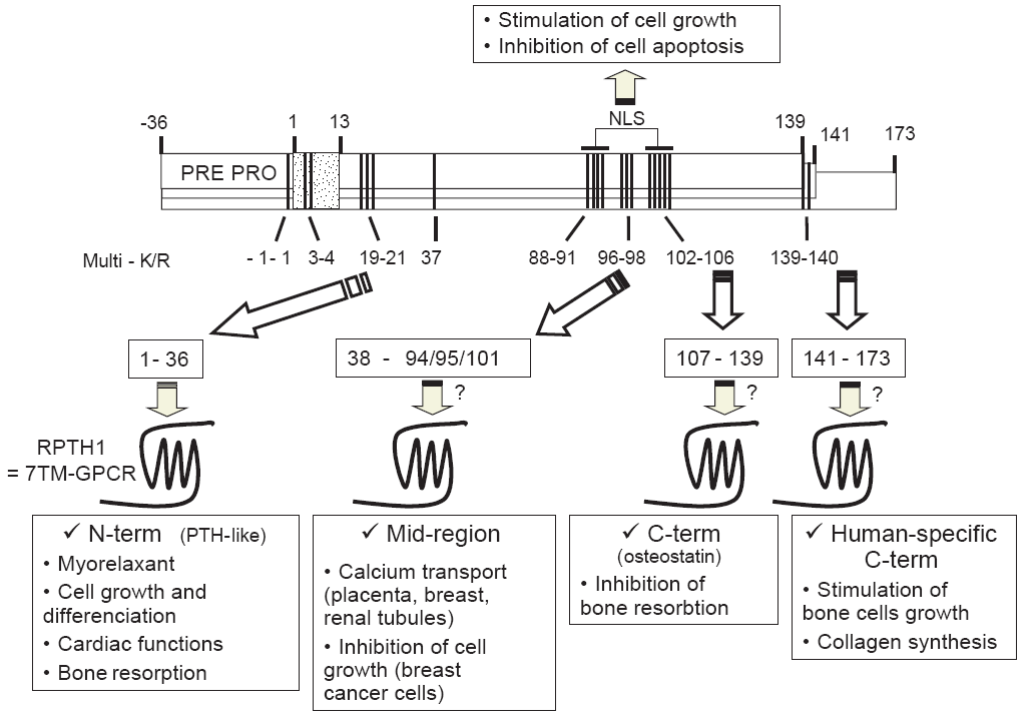
The same group expanded this study to include a human lung cancer cell line, a human keratinocyte cell line and a canine oral squamous carcinoma cell line and demonstrated a significant increase in PTHrP mRNA levels and PTHrP secretion following TGFβ1 treatment. When the individual isoforms were examined there was an increase in the half-life of PTHrP (1-141) but no change in the half-lives of (1-139) or (1-173) (Sellers, Luchin et al. 2004).

Heath *et al* demonstrated that EGF increased stability of PTHrP (1-139) but not (1-141) and Sellers *et al* showed that TGFβ1 had the opposite effect whereby it increased stability of PTHrP (1-141) but not (1-139). Although the coding regions of these two isoforms are very similar, differing by only two amino acids, the 3' untranslated regions (UTR) of their mRNAs are very different thus allowing different proteins to regulate their expression. Sellers *et al* (2004) carried out a U.V. cross-linking assay and found that different proteins bind to the two 3'-UTRs: the proteins bound to the 3'-UTR (1-141) had a mobility of 64, 80 and 100 kDa whereas those bound to (1-139) had a mobility of 45 and 55kDa. Although Sellers *et al* were unable to identify the proteins they hypothesised that they were involved in mRNA degradation and that TGFβ1 and EGF are able to regulate different 3'-UTR binding proteins, thereby affecting the stability of the different isoforms.

1.1.3 Post-Translational Products

Once PTHrP has been translated, its pre-pro sequence directs the mature protein to the endoplasmic reticulum where it undergoes endoproteolytic processing. Figure 1.2 shows that this post-translational processing results in the generation of a number of daughter peptides, which are believed to have a variety of functions in normal homeostasis (see Section 1.2) (Orloff, Reddy et al. 1994).

Figure 1.2 The post-translational products of PTHrP and the known cellular functions of the various daughter peptides (Sourbier and Massfelder 2006)



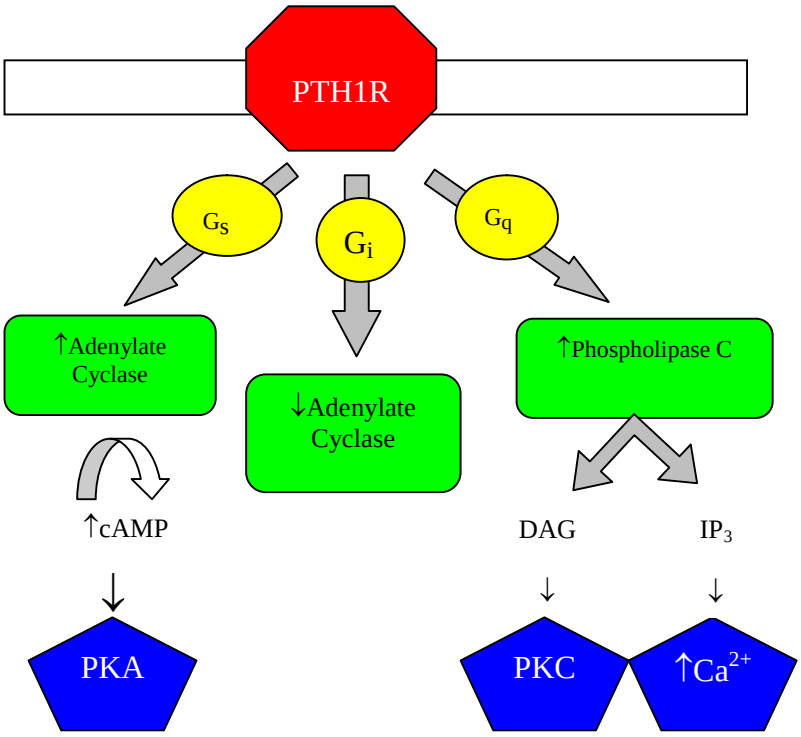
These daughter peptides can either enter transport vesicles (constitutive pathway) or secretory granules (regulated secretory pathway) depending on the cell type (Plawner, Philbrick et al. 1995). It has been shown that the secretory peptides are more predominant than the intact unprocessed peptide (Philbrick, Wysolmerski et al. 1996) and it is thought that each has a separate physiological function mediated through individual receptors; a distinct receptor for PTHrP (37-106) was proposed in

1990 (Ellis, Adam et al. 1990) but to date no such receptor has been isolated and at present only one receptor has been shown to respond to PTHrP.

1.1.4 A Receptor for PTHrP

The type I PTH/PTHrP receptor, or PTH1R, is a class II G-protein coupled receptor (Gardella and Juppner 2001). Following agonist binding there is a rapid desensitisation of PTH1R before internalisation of the agonist-receptor complex (Huang, Chen et al. 1995). As can be seen in Figure 1.3, PTH1R is coupled to G_s, G_q/G_{11} and G_i which allows it to increase cAMP, IP_3 and Ca^{2+} (Guo, Chung et al. 2002).

Figure 1.3 – Diagrammatic representation of the signalling pathways of PTH1R following ligand binding



PTH and PTHrP exhibit 70% homology within their first 13 amino acids and this allows them to share PTH1R, with only the first 34 amino acids of PTHrP being necessary for activation (Wysolmerski and Stewart 1998).

1.2 FUNCTIONS OF PTHrP IN NORMAL HOMEOSTASIS

Although initially discovered as a tumour product, PTHrP has subsequently been linked with a number of normal physiological functions in a variety of organ systems and these can be divided into three main roles: growth & development, calcium transport and homeostasis, and smooth muscle regulation.

1.2.1 Growth & Development

Studies by Kronenberg *et al* using knockout mice have shown that the PTHrP gene is necessary for foetal development. Their study showed that PTHrP (-/-) mice survive until birth but die soon after, which prompted Kronenberg *et al* to suggest that the mother is able to provide enough PTHrP for the foetus by way of the placenta in order to complete gestation, but unable to produce its own supply the offspring quickly dies (Kronenberg, Lanske et al. 1998).

Amling *et al* found that PTHrP is able to increase expression of the anti-apoptotic protein Bcl-2 in growth plate chondrocytes, which results in slowed differentiation thereby allowing bone to grow and elongate normally (Amling, Neff et al. 1997).

PTHrP has also been linked to mammary development and branching morphogenesis of the tubes comprising the mammary gland (Wysolmerski, Philbrick et al. 1998).

Furthermore, it has been found in the pregnant and lactating breast along with the milk which is subsequently generated (Lam, Thomas et al. 2000).

In addition, PTHrP has been shown to affect the development of skin, teeth, cartilage, extraembryonic endoderm, smooth muscle, the central nervous system and the placenta (Nissenson 2000).

1.2.2 Calcium Transport & Homeostasis

Expression of PTH1R is high in both the bone and kidney where it is utilised by PTH and PTHrP to maintain calcium homeostasis (Wysolmerski and Stewart 1998). In bone this is achieved through the stimulation of the cytokine RANKL, which in turn binds to its receptor RANK located on osteoclast progenitor cells. This results in the differentiation of these cells into mature osteoclasts, which then go on to cause bone resorption and calcium release (Mannstadt, Juppner et al. 1999). In the kidney PTH1R activation results in a decrease in calcium excretion in the distal tubule, an increase in phosphorus excretion and an increase in cAMP production and excretion (Martin, Allan et al. 1989).

As only the first 34 amino acids of PTHrP are necessary for activation of PTH1R the amino-terminal peptide is generally believed to be responsible for maintaining calcium homeostasis however it is thought that the mid-region peptide is responsible for stimulating trans-placental calcium transport. Using PTHrP and PTH1R knockout mice, Kovacs *et al* demonstrated that the PTHrP gene is necessary for maintaining calcium transport across the placenta and consequently maintaining calcium concentration in the foetus. Using exogenous PTHrP the authors were able

to reverse the impaired calcium transport using PTHrP (1-86) and (67-86). As PTHrP (1-34) was unable to affect calcium transport across the placenta it was subsequently suggested that the mid-region peptide is responsible for transplacental calcium transport. Also, as PTHrP (1-34) is necessary for activation of PTH1R, this study suggests that another receptor specific to the mid-region may be involved (Kovacs, Lanske et al. 1996).

While PTHrP (1-34) appears to stimulate osteoclast activation and bone resorption, the carboxyl-terminal of PTHrP appears to have the opposite effect. Fenton *et al* initially described the use of PTHrP (107-139) as an inhibitor of resorption in rat ostoclasts but went on to isolate this inhibitory activity to the (107-111) region and labelled it osteostatin (Fenton, Kemp et al. 1991).

1.2.3 Smooth Muscle and Endothelial Cells

Expression of the PTHrP gene has been shown in every smooth muscle cell and mechanical stretch stimulates PTHrP expression in the stomach, uterus and bladder where, by acting in a paracrine fashion, it can induce smooth muscle relaxation (Macica and Broadus 2003).

Kalinowski *et al* used PTHrP (1-34) to demonstrate that PTHrP was able to induce smooth muscle relaxation by stimulating nitric oxide (NO) production in endothelial cells. Using PTH (1-34) and PTHrP (1-34) antagonists, they were able to reduce NO release, which led the authors to propose that NO release was mediated via PTH1R activation. This theory was further supported when cAMP/protein kinase A

blockade reduced PTH (1-34) and PTHrP (1-34) stimulated NO release (Kalkinowski, Dobrucki et al. 2001).

PTHrP was identified as a neuroprotective vascular peptide when Funk *et al* showed for the first time that PTHrP is able to act within the central nervous system. The authors demonstrated an increase in PTHrP gene expression in the brain subsequent to focal ischemia. Prior to this increase there was an increase in TNF-α and IL-1β; two cytokines which are able to generate an increase in PTHrP expression during inflammation. The authors suggested that the increase in PTHrP expression resulted in an increase in cerebral blood flow by acting in a vasodilatory manner (Funk, Migliati et al. 2003).

PTHrP expression has also been shown in coronary endothelial cells where it appears to reduce coronary resistance. Not only have Grohe *et al* shown this to be the case but they were also able to show that this occurs in a sex-specific manner: they demonstrated that ventricular expression of PTHrP is higher in female rats than males, which results in a greater reduction in coronary resistance in the females. The authors proposed that this may explain the sex-specific differences observed in cardiovascular disease (Grohe, van Eickels et al. 2004).

1.3 A ROLE FOR PTHrP DURING CANCER

Since its initial discovery PTHrP has been associated with cancer. Following its initial identification as the key factor in HHM it is now frequently associated with increased metastasis and tumour cell proliferation. PTHrP has been linked with

numerous cancers however to date it is still most commonly associated with breast cancer.

1.3.1 Breast Cancer

Breast cancer is the most commonly diagnosed cancer among women and is the second leading cause of cancer death after lung cancer. It has previously been discussed that PTHrP plays a role in normal breast development and physiology (see Section 1.2); however it also appears to be involved in breast cancer metastasis to bone.

Southby *et al* examined the immunohistochemical localisation of PTHrP in 102 breast tumour samples and 60% of these samples stained positively for PTHrP (1-34). The authors also described stronger staining in the invasive element of the tumour compared with the intraductal component whilst there was no expression of PTHrP in normal epithelium. Furthermore, during follow up studies Southby *et al* found that five of the seven patients who had developed bone metastases exhibited positive PTHrP staining in the primary tumours. Additionally, all four patients who had developed nonosseous metastases showed no PTHrP staining in their primary tumours. In spite of this the authors observed that positive PTHrP staining did actually correlate with a favourable prognostic index (Southby, Kissin et al. 1990).

The same group expanded this study and considered not only bone metastases from breast cancer but metastases in the lung, skin, liver, brain and pericardium. 31 secondary tumours were analysed for PTHrP expression and in 13 bone metastases, 12 stained positively for PTHrP. There were a limited number of samples for the

other metastases, for example only four lung samples were obtained, two of which stained positively, and only one liver sample, which did not appear to express PTHrP. From this study it is not possible to make any general conclusions regarding the role of PTHrP in metastasis overall, however it is possible to conclude from this study that breast cancer does have a penchant to metastasise to bone and this appears to be linked with PTHrP expression (Powell, Southby et al. 1991). Furthermore, Southby and colleague's studies have led them to suggest that PTHrP could be used as a guide to predict which patients may develop bone metastases and in doing so highlight which patients may benefit from an anti-bone metastasis treatment.

Bundred *et al* also demonstrated a link between PTHrP and hypercalcaemia in breast cancer using an antibody raised against PTHrP (37-67) to examine PTHrP cytoplasmic staining in patients with breast cancer. In early breast cancer patients, who were normocalcaemic and exhibited no signs of metastasis, positive PTHrP staining was shown in 52% (55/106) of patients. In normocalcaemic patients who developed metastases primarily in the bone, 65% (47/72) exhibited positive PTHrP staining in the primary tumours. Despite being normocalcaemic at the outset of the study, 25 of the patients in this latter group developed hypercalcaemia; 88% (22/25) of these tumours stained positively for PTHrP whereas only 53% (25/47) of the remaining normocalcaemic patients did so ($p=0.004$). This study also examined plasma levels of PTHrP: the protein was detectable in only 9% (5/57) of patients who were diagnosed with early breast cancer and had no discernible metastases, whereas it was detected in 36% (10/28) of the patients who did have identifiable bone metastases. Further analysis showed that PTHrP was detected in 92% (12/13) of patients with hypercalcaemia and that the observed concentrations were

significantly greater than in those with early breast cancer ($p<0.01$) or who were normocalcaemic but had developed bone metastases ($p<0.001$). The results from this study clearly demonstrates a link between PTHrP, hypercalcaemia and metastases (Bundred, Ratcliffe et al. 1991).

However the premise that PTHrP aids tumour progression was challenged in a study by Henderson *et al* who examined PTHrP expression as well as patient prognosis and found that the presence of PTHrP actually improves survival in breast cancer patients. Using 367 samples from patients with operable invasive primary breast cancer, PTHrP was detected in 72% of samples. When they compared the 5-year survival rate of patients with PTHrP positive and negative tumours, there was an 87% survival rate in patients with PTHrP positive tumours compared with 73% in patients with PTHrP negative tumours ($p=0.002$). They also found that the presence of PTHrP in the primary tumour resulted in a reduced likelihood of the development of bone metastases after surgery with 27% of PTHrP negative patients developing bone metastases compared with only 12% of PTHrP positive patients ($p=0.001$). Similarly the PTHrP negative patients had an increased likelihood to develop lung metastases with 22% developing metastases compared with only 8% of PTHrP positive patients ($p<0.001$) (Henderson, Danks et al. 2001).

A number of studies have also examined PTH1R expression in breast cancer. For example, Linforth and colleagues examined co-expression of PTHrP and PTH1R and related it to patient survival in breast cancer patients. Using samples of malignant breast carcinoma tissue and bone metastases, receptor expression was examined using two different techniques. Using RT-PCR, 51% of early breast cancer tissue

and 84% of bone metastases were shown to express PTH1R. These results correlated well with those obtained by *in situ* hybridisation where 49% of breast cancer tissue and 80% of bone metastases exhibited PTH1R expression. When they examined PTHrP using immunohistochemistry they showed expression in 68% of breast carcinoma samples and 100% of bone metastases. Co-expression of the protein and receptor were found in 32% of breast carcinoma and 81% of bone metastases. When they looked at patient survival they showed that positive expression of either PTH1R and PTHrP was linked to a decrease in disease-free survival and that positive expression of PTH1R was linked with a significant decrease in overall survival. Examination of the 5-year mortality rates in patients with primary breast cancer who demonstrated either positive or negative PTH1R expression was 29% and 12%, respectively and this was a significant decrease in survival for the PTH1R positive patients ($p=0.003$). PTHrP expression alone did not appear to significantly affect overall survival however co-expression with PTH1R resulted in a 5-year mortality rate of 32%, which was greater than positive PTH1R expression alone. The 5-year mortality for patients who were both PTH1R and PTHrP negative was shown to be 6% (Linforth, Anderson et al. 2002).

Hoey *et al* also linked over-expression of the PTHrP receptor with breast cancer bone metastases. Their study found that PTH1R was evident in 58% of primary breast cancers compared with 85% of bone metastases. Their study included only 67 primary tumour samples and 13 bone metastases; as such their results were not statistically significant however they do suggest a link between receptor expression and bone metastases. The authors postulated that receptor over-expression might

provide a growth advantage by aiding autocrine PTHrP signalling (Hoey, Sanderson et al. 2003).

Most studies examining the role of PTHrP in breast cancer have examined expression of PTHrP (1-34) and the majority have demonstrated an increase in proliferation and metastasis. Luparello *et al* however examined the mid-region of PTHrP and demonstrated the ability of PTHrP (38-94)-amide to inhibit growth and invasion in several breast cancer cell lines by slowing cell proliferation and enhancing cell death. Luparello *et al* have also shown that PTHrP (67-86)-amide is able to inhibit breast cancer cell growth and invasion through matrigel as well as reducing tumorigenesis *in vivo*. These opposing effects may be the result of activation of separate receptors however until a mid-region receptor is cloned it will not be possible to verify this hypothesis (Luparello, Romanotto et al. 2001; Luparello, Sirchia et al. 2003).

An increase in PTHrP expression in breast cancer cells metastasising to bone explains the increase in bone resorption mediated by osteoclast activation which would lead to an increase in serum calcium and may possibly aid tumour cells in their ability to form metastases by allowing them to be substituted for bone; however it does not explain the apparent preferential ability of these cells to form the initial metastases.

1.3.2 Gastrointestinal (GI) Cancer

GI cancer encompasses cancer of the stomach, colon and pancreas. Colorectal cancer is the second most common cause of death from cancer in both males and

females, whilst pancreatic and gastric cancer are the sixth and seventh causes of death from cancer, respectively (http://info.cancerresearchuk.org/cancerstats/mortality/cancerdeaths/).

Shortly after its discovery, PTHrP expression was demonstrated in a wide variety of normal and neoplastic endocrine tissues, including endocrine tumours within the GI tract and pancreas (Asa, Henderson et al. 1990). Nishihara *et al* looked at PTHrP expression in human colorectal tumours and found elevated PTHrP mRNA and protein expression in the majority of patients. Similarly the intensity of PTHrP staining correlated with increasing grades of Dukes' classification; however neither PTHrP gene or protein expression was detected in either adenomas or adjacent non-neoplastic mucosae of the colon. (Nishihara, Ito et al. 1999).

Abdeen *et al* looked at 14 cases of gastric adenocarinoma and stained tumour specimens with antibodies directed towards both PTHrP (1-34) and (109-141). 13 of the 14 cases stained positively using an antibody directed against the carboxyl-terminal, where it was localised to the cytoplasm, whilst none of the cases stained positively using the antibody directed towards the amino-terminal. Significantly, PTHrP expression was not detected in ten control cases of normal gastric tissue using either antibody, which led the authors to suggest that PTHrP plays a role in growth regulation of gastric adenocarcinoma (Abdeen, Pandol et al. 1995).

In a larger study Alipov *et al* examined 92 cases of gastric carcinoma and using an antibody raised against PTHrP (38-64) showed positive staining in 77.2% of cases, which was again localised to the cytoplasm. This study also showed that cases

exhibiting deeper invasion along with poorly differentiated adenocarcinomas stained stronger for PTHrP expression than early invasive or well-differentiated adenocarcinomas. Furthermore, cases with lymph node metastasis showed stronger staining at both the primary site as well as in metastatic cells, compared with cases with no evidence of metastasis (Alipov, Ito et al. 1997).

The same group investigated a case of gastric cancer with multiple metastases including bone, kidney, liver, lung, spleen, peritioneum, retro-peritoneum and lymph nodes. Using an antibody raised against the (38-64) region of PTHrP, they found PTHrP present in the tumour cells of the metastases but not in the cells of the primary tumour thus providing further evidence for a role of PTHrP in GI tumour progression (Ito, Nakashima et al. 1997).

As mentioned earlier, PTHrP has also been found within the pancreas with expression often described in a number of cell lines derived from pancreatic tumours as well as in the tumours themselves (Drucker, Asa et al. 1989; Miraliakbari, Asa et al. 1992; Bouvet, Nardin et al. 2001). Furthermore it has been shown that exogenous PTHrP (1-34) is able to stimulate DNA synthesis in isolated rat pancreatic islets, which express PTHrP and PTH1R, whereas exogenous mid-region and carboxyl-terminal PTHrP had no effect on DNA synthesis (Villaneuva-Penacarrillo, Cancelas et al. 1999).

As with other cell types several studies have described the induction of PTHrP over-expression resulting in an increase in the resistance of pancreatic β cells to apoptosis (Cebrian, Garcia-Ocana et al. 2002; Hastings, Quintana et al. 2003).

1.3.3 Other Forms of Cancer

There are several examples of increased PTHrP expression in a variety of other cancers, where it is commonly discovered in association with hypercalcemia.

For example PTHrP over-expression is often demonstrated in prostate cancer cells when compared with normal cells and this over-expression has been linked to a propensity to metastasise to bone (Deftos 2000).

Iddon *et al* examined PTHrP expression in benign and malignant prostate tumours; PTHrP (1-34) expression was detected in 68% of benign prostate tumours compared with 95% of the malignant tumours ($p=0.04$). The authors also examined bone metastases and only 50% of the bone metastases from prostate cancer produced PTHrP (1-34) compared with 100% of bone metastases from other primary cancers. There was no apparent correlation between the Gleason grade and the intensity of staining and there appeared to be greater PTHrP expression in the primary tumours rather than in the metastases. These results suggest that PTHrP may not be linked to prostate tumour progression directly but rather facilitates metastases through some other means. However, of the metastases that did not stain for PTHrP (1-34), two out of seven did stain for PTHrP (37-67), which suggests that these results may in fact underestimate PTHrP expression in the metastases (Iddon, Bundred et al. 2000).

Another study examining the role of PTHrP in prostate cancer again used paired primary tumours and metastases. Not only was PTHrP gene expression shown in 13 out of the 14 primary tumours and in all 14 metastases but there was also an increase in PTHrP expression in the metastases. These results contradict the findings by

Iddon *et al* however as this study examined gene expression and Iddon *et al* protein expression, no direct comparison can be made (Bryden, Hoyland et al. 2002).

Studies by Asadi *et al* using a human prostate adenocarcinoma cell line showed that exogenous PTHrP (1-34) produced an increase in ornithine decarboxylase (ODC) mRNA expresson. ODC is the rate-limiting enzyme in polyamine biosynthesis and cell cycle progression and these results suggest that by increasing ODC PTHrP mediates its effects on cell growth by playing an indirect role in cell cycle progression (Asadi, Faraj et al. 2001).

Brandt *et al* examined PTHrP expression in each of the major lung cancer cell types, including cell lines derived from lung tumours and each one expressed and secreted PTHrP. Furthermore, Richard *et al* found a 131-fold increase in PTHrP gene expression in lung carcinoma compared with normal adjacent tissue (Brandt, Burton et al. 1991; Richard, Luchin et al. 2003).

Hastings and colleagues also examined the role of PTHrP in lung cancer and they published several reports in close succession. In one study they used exogenous PTHrP (1-34) and (67-86) to reveal a pro-apoptotic role in type II pneumocytes whilst in subsequent studies they have demonstrated that PTHrP (1-34) and (140-173) are able to protect lung cancer cells from apoptosis (Hastings, Araiza et al. 2003; Hastings, Quintana et al. 2003).

In the first study Hastings *et al* used alveolar type II epithelial cells isolated from healthy rats. Apoptosis was induced by ultraviolet exposure and treatment with

PTHrP (1-34) or (67-86) caused a further increase in apoptosis. In an ensuing study they used a squamous bronchial lung carcinoma cell line and again induced apoptosis by ultraviolet radiation. In this study the authors showed that pre-treatment with PTHrP (1-34) and (140-173) protected the lung cancer cells against apoptosis by causing a dose-dependent decrease in caspase-3, -8 and -9 activity as well as loss of cell mass and nuclear condensation. PTHrP (38-64), (67-86) and (107-139) meanwhile had no effect on caspase-3 activity. The results from these two studies appear to suggest that in normal cells PTHrP is pro-apoptotic whereas in tumour cells it switches to an anti-apoptotic factor.

The wide-spread actions of PTHrP are once again demonstrated by a report of increased PTHrP expression in penile cancer with bone metastases and hypercalcemia (Akashi, Fuse et al. 2002) and a reported case of a gallbladder carcinoma with liver metastasis where the patient not only exhibited increased serum PTHrP levels but also hypercalcemia (Ebinuma, Imaeda et al. 2002).

By considering all of these studies, which are summarised in Table 1.1, it appears that PTHrP is not only over-expressed in a variety of different tumours but that in doing so it is able to increase tumour cell proliferation and possibly plays a role in tumour progression. Despite the numerous studies investigating such a role, a mechanism of action has yet to be elucidated.

Table 1.1 Summary of PTHrPs links with tumour metastasis

Site of Primary Tumour	Site of Metastasis	Study
Breast	Bone	Southby, Kissin et al. 1990 Powell, Southby et al. 1991 Bundred, Ratcliffe et al. 1991 Linforth, Anderson et al. 2002
GI	Lymph Node	Alipov, Ito et al. 1997
	Bone, Kidney, Liver, Lung, Spleen, Peritoneum, Retro-peritoneum, Lymph Node	Ito, Nakashima et al. 1997
Prostate	Bone	Deftos 2000 Iddon, Bundred et al. 2000 Bryden, Hoyland et al. 2002
Penile	Bone	Akashi, Fuse et al. 2002
Gallbladder	Liver	Ebinuma, Imaeda et al. 2002

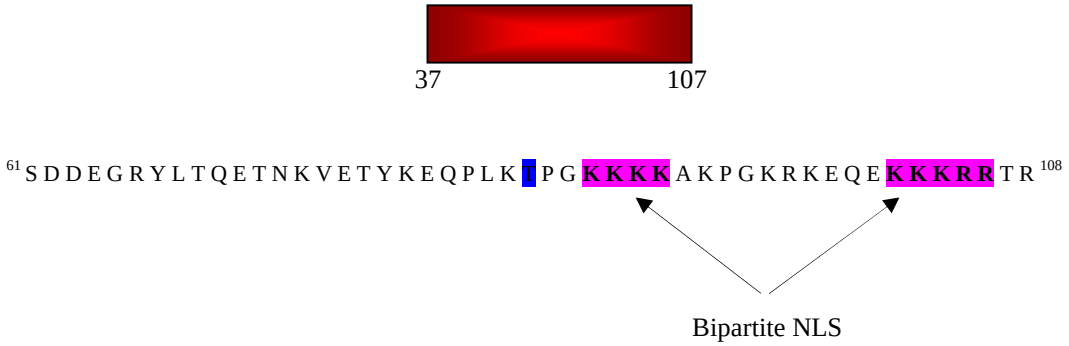
1.4 NUCLEAR ACTIONS OF PTHrP

Under normal conditions PTHrP does not enter the circulation but acts either in an autocrine, paracrine or intracine manner. This is demonstrated by the low serum level of PTHrP as well as the close association of the protein and receptor mRNA in breast and colon tumours (Carron, Fraser et al. 1997). The exact intracrine functions of PTHrP are unknown however they appear to be dependent on its presence within the cell nucleus.

1.4.1 Nuclear Transport

PTHrP is directed to the nucleus by a bipartite nuclear localisation sequence (NLS) within its mid-region. Figure 1.4 demonstrates the location of the two multibasic clusters at residues (88-91) and (102-106) (Nguyen and Karaplis 1998).

Figure 1.4 –Mid-region section of PTHrP, including the bipartite nuclear localisation sequence and the threonine which when phosphorylated prevents nuclear entry



Most proteins require a carrier in order to enter the nucleus and PTHrP is no exception; the NLS of PTHrP binds to the nucleo-cytoplasmic transport factor importin β. Importin β in turn docks to the nuclear pore complex, which makes a hole in the double membrane of the nucleus and facilitates entry of PTHrP-importin β. Following RanGTP binding, PTHrP dissociates from importin β and is free to act within the nucleus (Lam, Thomas et al. 2000).

Lam *et al* were able to localise PTHrP expression to the cytoplasm by deleting amino acids (67-94), the residues which bind to importin β. However although PTHrP (1-87) does not contain a functional NLS it has been identified within the nucleus. Furthermore, using fluorescently labelled PTHrP it has been shown that exogenous

PTHrP is also able to localise to the nucleus in a PTH1R dependent manner: Watson *et al* demonstrated nuclear localisation of PTH1R in a number of rat tissues including the liver, kidney, gut, uterus and ovaries which led them to propose the presence of a bipartite NLS in the carboxyl-terminal tail of PTH1R which could bind to importin α. It is known that ligand binding results in internalisation of the PTHrP/PTH1R complex and if the receptor does indeed contain an NLS this may offer a mechanism by which amino-terminal PTHrP can enter the nucleus independent of the mid-region. However if this is indeed the case, deletion of amino acids (67-94) shouldn't confine PTHrP to the cytoplasm. Further investigation will be necessary to establish the precise mechanism of PTHrP's nuclear translocation (Watson, Fraher et al. 2000; Lam, Thomas et al. 2002).

1.4.2 Actions of Nuclear PTHrP

Once in the nucleus it has been shown that PTHrP can affect the cell cycle and also inhibit apoptosis. For example, Tovar Sepulveda and Falzon used plasmids encoding for either intact PTHrP or PTHrP without the bipartite NLS to examine the actions of nuclear PTHrP. Over-expression of intact PTHrP in prostate cancer cells caused a two to three fold increase in proliferation compared with vector control containing cells, however where the NLS had been removed cell growth was slower compared with vector control. When the cell-cycle profile of the transfected cells was examined, the vector-control containing cells were mainly in the G_1 phase whilst over-expression of intact PTHrP resulted in an increase in the number of cells within the G_2+M phase, correlating with the apparent increase in cell growth. In cells over-expressing PTHrP minus the NLS there was an increase in the number of cells within the G_2+M phase compared with vector control, however this number was

significantly lower compared with cells where PTHrP was able to enter the nucleus and conflicts with their cell growth results (Tovar Sepulveda and Falzon 2002).

Lam *et al* also demonstrated that PTHrP localises to the nucleus in a cell-cycle dependent manner. Using a keratinocyte cell line, they demonstrated that PTHrP expression was greatest during the G_2+M phase of the cell cycle and showed that during mitosis PTHrP moves from the nucleolus to the cytoplasm. By phosphorylating PTHrP at Thr^{85} (Figure 1.4) the cyclin-dependent kinases cdc2 and cdk2 prevent nuclear entry and the mutation of Thr^{85} results in cytoplasmic accumulation. Lam and colleagues have shown that this phosphorylation activity begins during S phase and peaks during G_2+M, resulting in localisation of PTHrP to the cytoplasm during these phases. They went on to show the presence of PTHrP within the nucleus during G_1 and therefore suggested that once PTHrP has stimulated cell growth it is no longer required within the nucleus and is consequently exported to the cytoplasm (Lam, Olsen et al. 1997; Lam, House et al. 1999).

Henderson *et al* examined the effect of a functional NLS on the survival of chondrocytes during apoptotic conditions. Using plasmids encoding either intact PTHrP or PTHrP where the NLS had been deleted, the authors were able to demonstrate that the ability of PTHrP to translocate to the nucleus leads to an increase in the resistance of chondrocytic cells to serum deprivation. They also showed the presence of endogenous PTHrP in the nucleolus, an area of the nucleus where transcription and processing of ribosomal RNA occurs (Henderson, Amizuka et al. 1995).

In a similar study Aarts *et al* also showed that chondrocytes expressing PTHrP with an intact NLS were protected from serum deprivation. They showed that PTHrP (1-34) is insufficient to protect cells from apoptosis and consequently suggested that protection from apoptosis is unrelated to the cellular responses produced via PTH1R and may be mediated through a separate mechanism such as a mid-region receptor (Aarts, Davidson et al. 2001).

In an earlier study Aarts *et al* demonstrated that PTHrP can bind to poly(G) homopolymeric RNA, GC-rich double-stranded RNA and total cellular RNA located in the nucleus. Again using expression vectors encoding either full-length PTHrP or PTHrP without the NLS the authors were able to show that transient transfection of these vectors resulted in binding of PTHrP to cellular RNA and homopolymeric RNA only in the vector containing the NLS. Not only did they demonstrate that this interaction is of high relative affinity but they identified a core motif within PTHrP which is necessary for the binding: GxKKxxK. This motif is conserved among other RNA-binding proteins, adding further weight to the suggestion that PTHrP interacts with RNA (Aarts, Levy et al. 1999).

When Tovar Sepulveda *et al* expanded their earlier study to examine the effect of PTHrP on apoptosis they used breast cancer cells to demonstrate that the removal of the NLS leads to an increase in apoptosis in serum-deprived cells, which is consistent with Henderson *et al*'s study. The ratio of anti-apoptotic to pro-apoptotic proteins determines whether or not a cell will undergo apoptosis and Tovar Sepulveda *et al* were able to demonstrate that by entering the nucleus PTHrP increases the ratio of the anti-apoptotic proteins Bcl-2 and Bcl-x_L to the pro-

apoptotic protein Bax, thus leading to a decrease in apoptosis. Tovar Sepulveda *et al*'s results are consistent with Amling *et al*'s study which demonstrated that PTHrP is upstream of Bcl-2 in chondrocytes (Tovar Sepulveda, Shen et al. 2002).

The effect of nuclear PTHrP appears to be distinct from extracellular PTHrP. For example in vascular smooth muscle cells (VSMC) nuclear PTHrP promotes cell proliferation whereas extracellular PTHrP inhibits cell proliferation (Massfelder, Dann et al. 1997). These results led the authors to suggest that by translocating to the nucleus, PTHrP does not affect cell proliferation directly but rather decreases the amount of PTHrP available for secretion, thereby reducing the levels of extracellular PTHrP and diminishing its inhibitory effects.

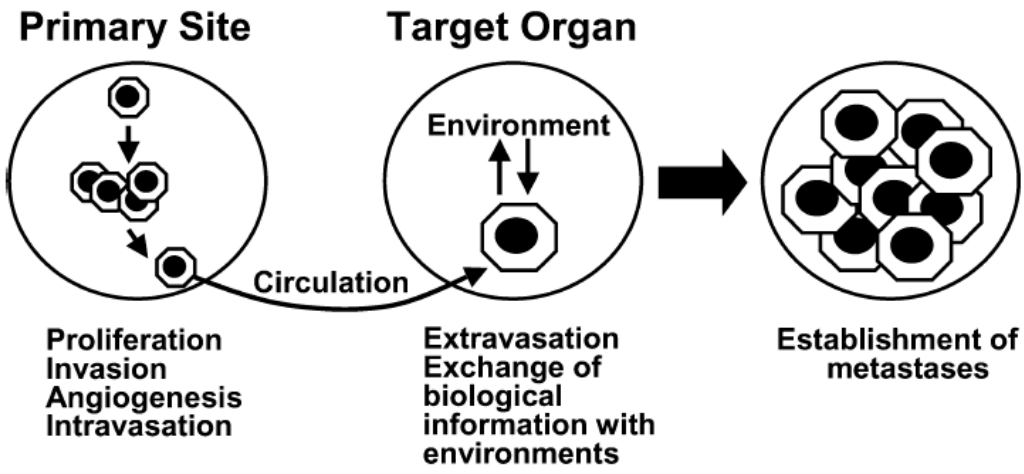
Each of these studies has shown a role for PTHrP as a mitogen or as an anti-apoptotic protein. However, PTHrP's NLS is homologous to the NLS of transcription factors such as *c-jun*, *c-fos* and p53, which has led to the suggestion that PTHrP may also be a transcription factor (Fiaschi-Taesch and Stewart 2003). It has also been hypothesised that intracrine PTHrP may be related to the ability of cells to adhere to the extracellular matrix.

1.5 TUMOUR PROGRESSION

1.5.1 Metastasis

Metastasis is a key element in tumour progression and as such it is a complex, highly regulated process which begins with a cancer cell dissociating from the primary tumour and entering the blood system, Figure 1.5.

Figure 1.5 Diagrammatic representation of metastasis including dissociation from the primary tumour, migration to a distant organ resulting in metastasis formation (Yoneda and Hiraga 2005)



However, once a tumour cell has entered the blood system, it is not simply a case of forming a secondary tumour in the nearest available organ; Paget's 'seed and soil' hypothesis suggests that a cancer cell (the 'seed') is only able to metastasise to an area (the 'soil') which has an advantageous microenvironment in which the cell may grow (Paget 1889).

The lung is the most common organ for metastasis, followed by the liver and bone (Mollabashy and Scarborough 2000). Melanoma and osteosarcoma tend to metastasise to the lung, while the liver is the most common site for colorectal cancer metastases. Breast cancer cells meanwhile metastasise predominantly to bone, as do tumour cells from the prostate, thyroid and kidney.

Bone metastasis is characterised by osteolysis, diffuse osteopenia and osteoblastic lesions (Mundy 1997). Bone metastases are a serious complication, not only due to the pain, nerve compression and ease of bone fracturing, but because the cancer can

no longer be cured and palliative therapy is the only option available. It is common for patients dying from lung and breast cancer to have bone metastases and as previously mentioned these are commonly associated with an increase in PTHrP expression.

1.5.2 The Extracellular Matrix

Tumour invasion into the extracellular matrix (ECM), including cell attachment to and penetration of the ECM, is a crucial step during metastasis. The ECM provides a structural framework around the cell and cellular interaction with the ECM also provides signals for cell growth and differentiation. There are many components of the ECM including collagens, fibronectin and laminin.

Collagenous proteins comprise the major component of the ECM. There are many types of collagen, however collagen type I is the most abundant type isolated from connective tissues such as skin, bone, tendon and liver. It is found in the interstitial matrix and provides these tissues with tensile strength, for example collagen type I makes up >95% of the bone matrix (Kiefer and Farach-Carson 2001).

Fibronectin is expressed in the basement membrane and plays a role in cell adhesion, embryonic cell migration and wound healing. Laminin is also expressed in the basement membrane and plays an important role in development, differentiation and migration. Both fibronectin and laminin are able to bind to cell surface receptors such as integrins and other extracellular molecules (Hay 1991).

In addition to growth and differentiation, binding to the ECM also aids cell migration. Changes in cell-cell adhesion and cell-matrix adhesion play an important role in the development of metastases with tumour cells using integrin-ECM interactions as a foothold during migration. Additionally several studies have shown that the ECM appears to stimulate tumour cell proliferation (Adams and Watt 1993; Ohtaka, Watanabe et al. 1996; Stupack and Cheresh 2002).

For example Kiefer and Farach-Carson examined the effect of collagen type I on proliferation of PC3 prostate carcinoma cells. After 24 hours of growth cells grown on collagen type I demonstrated a >50% increase in proliferation compared with cells grown in uncoated wells (Kiefer and Farach-Carson 2001).

Binding of cells to the ECM is mediated by cell surface receptors, the most abundant group being the transmembrane cell adhesion receptors, integrins.

1.5.3 Integrins

Integrins were first discovered in 1987 by Richard Hynes and have since been shown to have universal expression where they play a key role in cell-cell, cell-ECM and cell-soluble ligand interaction (Hynes 1987; Brakebusch, Bouvard et al. 2002).

These receptors are heterodimers composed of non-covalently linked α and β subunits. To date 18 α and eight β subunits have been discovered and these can form 24 different $\alpha\beta$ heterodimers with each different α and β subunit combination creating a different integrin-ligand interaction (Hynes 2002). Figure 1.6 shows the different combinations of the α and β subunits which have been identified to date.

Figure 1.6 Diagrammatic representation of the different α and β subunit interactions

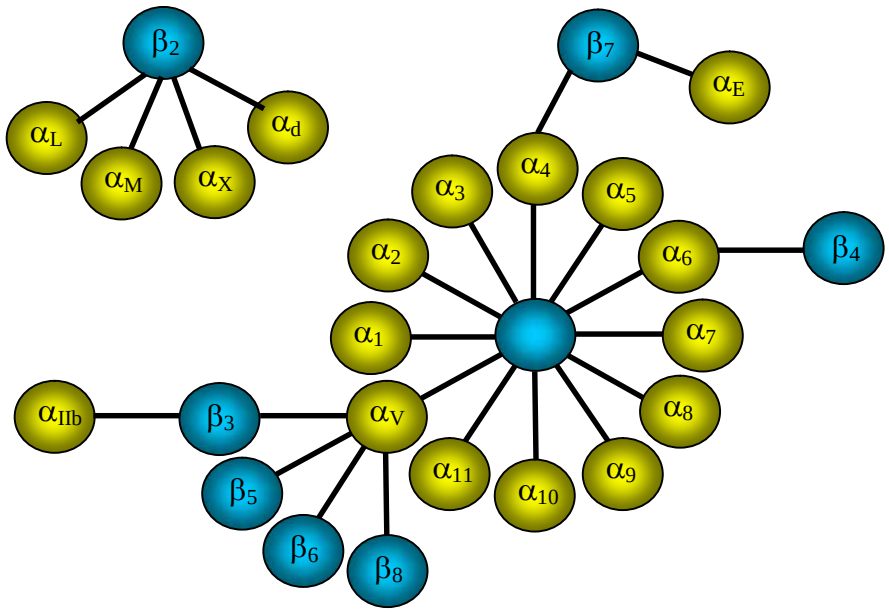


Table 1.2 also shows the different αβ heterodimers along with their integrin/ligand interactions and demonstrates that each ECM not only has multiple receptors but that each integrin receptor can bind to multiple ligands.

Table 1.2 Integrin-Ligand Interactions

Beta Subunit	Alpha Subunit	Ligand
β_1	α_1	Collagens, Laminin
	α_2	Collagens, Laminin
	α_3	Collagens, Laminin, Fibronectins
	α_5	Fibronectin
	α_7	Laminin
	α_8	Fibronectin
	α_{10}	Collagens
	α_{11}	Collagens
β_1 & β_7	α_4	Fibronectin
β_1 & β_4	α_6	Laminin
β_1, β_3 & β_6	α_V	Fibronectin
β_3	α_{IIb}	Collagens, Fibronectin
β_8	α_V	Collagens, Laminin, Fibronectin

Integrins have a large extracellular domain, a membrane spanning domain and a short cytoplasmic tail. The extracellular domain mediates cell-matrix and cell-cell interaction while the cytoplasmic tails interact with the cytoskeleton, consequently integrin signal transduction is bidirectional and provides a link between the cytoskeleton and ECM (Brakebusch, Bouvard et al. 2002).

Integrins are involved in multiple cellular functions including migration, cell cycle progression and programmed cell death. For example, during migration integrins interact with the actin cytoskeleton to stimulate cell contraction, integrin inactivation allows cell detachment from the substratum and integrins are also able to activate

extracellular matrix-degrading enzymes. It has been suggested that lower levels of integrin expression may protect against invasion and metastasis while a threshold level of integrin expression is required for cell invasion (Brakebusch, Bouvard et al. 2002).

Stallmach *et al* demonstrated a reduction in expression of a number of integrin subunits in colorectal carcinoma samples compared with normal, including α_2, α_3, α_5, α_6, β_1 and β_4. They also found that α_3 and α_5 expression was reduced in adenomas and absent in most colonic carcinoma suggesting a link between a reduction in integrin expression and tumour progression. In another study Stallmach *et al* showed α_5 expression in normal colonic epithelial cells but no expression in adenomas and carcinomas. Using HT29 clones which were positive and negative for this integrin, the authors were able to show that the ability of the positive cells to form tumours in nude mice was reduced compared with the negative cells. They proposed that expression of $\alpha_5\beta_1$ mediates the deposit of fibronectin at the epithelial-mesenchymal interface, thus providing a line of defence between the tumour cells and the host tissue and concluded that loss of $\alpha_5\beta_1$is part of tumour progression (Stallmach, von Lampe et al. 1992; Stallmach, Von Lampe et al. 1994).

However, Stallmach *et al*'s studies conflict with a number of other studies which have linked an increase in $\alpha_5\beta_1$ expression to cancer progression. For example Koretz examined integrin expression in a number of colon cancer cell lines and found α_5 expression in each of the five cell lines examined. Furthermore, Gong *et al* demonstrated a positive correlation between $\alpha_5\beta_1$ expression and the level of

invasiveness in a number of colon cancer cell lines (Koretz, Bruderlein et al. 1994; Gong, Want et al. 1997).

A number of studies have induced $\alpha_5\beta_1$ over-expression and linked expression of this receptor to cell death. For example, Lee and Juliano used human colon carcinoma cells to show that over-expression of $\alpha_5\beta_1$ results in an increased resistance to apoptosis induced by serum deprivation, aspirin, staurosporine and etoposide. Further analysis revealed that this effect was mediated via an increase in PKB/Akt activity (Lee and Juliano 2000).

Zhang *et al* also demonstrated a role for $\alpha_5\beta_1$ expression in cell survival. Using the Chinese hamster ovary cell line they induced over-expression of $\alpha_5\beta_1$ and $\alpha_V\beta_1$. When they subsequently examined cell growth on fibronectin in the absence of serum, fewer than 25% of cells over-expressing $\alpha_5\beta_1$ became apoptotic whereas nearly 100% of cells over-expressing $\alpha_V\beta_1$ displayed evidence of apoptosis. Both cell lines became apoptotic when grown on poly(lysine)-coated dishes or uncoated cell culture dishes. The authors concluded that the $\alpha_5\beta_1$ interaction with fibronectin protected the cells from apoptosis and went on to show an increase in the ratio of *Bcl-2/Bax* gene expression in $\alpha_5\beta_1$ over-expressing cells (Zhang, Vuori et al. 1995).

A number of integrins are also thought to aid breast cancer progression. For example the $\alpha_V\beta_3$ heterodimer has frequently been shown to play an important role during breast cancer metastasis to bone as it is highly expressed by both osteoclasts and the metastasising tumour cells. Expression of this integrin has also been linked

to the aggressiveness of breast cancer and as such has been used as a prognostic marker (Gasparini, Brooks et al. 1998). In addition, Pecheur *et al* demonstrated that breast cancer cells over-expressing $\alpha_V\beta_3$ exhibited an increase in the incidence and number of osteolytic lesions compared with control cells, with bone metastases visible at day 21 in all of the mice inoculated with $\alpha_V\beta_3$ cells, compared with 60-66% of control animals (Pecheur, Peyruchaud et al. 2002).

Expression of the integrin subunit α_6 has also been linked with breast cancer. For example, one study compared patient survival with α_6 expression in 119 patients where there was evidence of cell invasion into the basement membrane. These patients were classified into four groups based on whether they had low (grade I and II), intermediate (grade III) or high (grade IV) α_6 expressing tumours and examination of patient survival time revealed a link between increased α_6 expression and decreased patient survival time. All of the patients classified as having low α_6 expression survived whilst mortality increased to 11% in patients with intermediate expression and to 19% in patients with a high percentage of α_6 expressing cells. Furthermore, in 34 cases where distant metastases were observed 30 were shown to express high levels of α_6 (the other four were classified as having low α_6 expression), which led the authors to conclude that α_6 expression was linked to metastasis and patient survival. They also suggested that α_6 expression could be used as a prognostic marker (Friedrichs, Ruiz et al. 1995).

In a similar study Wewer *et al* examined the role of $\alpha_6\beta_1$ expression in tumour progression by eliminating expression of $\alpha_6\beta_1$ in a breast cancer cell line. The authors then used these cells to induce breast tumours in mice and found that the tumours formed by cells lacking $\alpha_6\beta_1$ demonstrated reduced growth and an increase in apoptosis compared with mice given $\alpha_6\beta_1$-expressing cells. Similarly these control cells formed lung metastases which the $\alpha_6\beta_1$-null cells were unable to do. The authors concluded that $\alpha_6\beta_1$ plays an important role in tumorigenesis and tumour cell survival in distant organs (Wewer, Shaw et al. 1997).

It is not only breast and colorectal cancer where integrin expression has been shown to be increased; Liu *et al* used a cDNA microarray to examine expression of 17 integrin genes and demonstrated an up-regulation of all 17 in hepatocellular carcinoma (HCC) compared with adjacent normal liver tissue. Similarly Masumoto *et al* investigated the relationship between β_1 integrin activity and HCC invasion and showed that cells with high β_1 integrin activity are more invasive than cells with lower activity (Liu, Jiang et al. 2002; Masumoto, Arao et al. 1999).

Secondary tumours increase the number of complications in cancer and are the leading cause of cancer related morbidity and mortality. The studies described here demonstrate the importance of integrins in tumour progression and as a result several groups have attempted to block integrin activity in an effort to inhibit tumour progression. For example an inhibitor of $\alpha_5\beta_1$ has been used in conjunction with continuous 5-FU infusion to reduce colorectal-liver metastases in mice (Stoeltzing, Liu et al. 2003). Reinmuth *et al* also examined colorectal metastasis in mice

however they used an antagonist of $\alpha_V\beta_3$ and not only reduced metastasis but also produced an increase in survival. Similarly another group used another antagonist of $\alpha_V\beta_3$ (S247) to suppress metastasis in a mouse model of breast cancer metastasis to bone. Following a pre-injection of S247, Harms *et al* demonstrated a significant reduction in the incidence and number of bone metastasis ($p=0.002$) following an intracardiac injection of MDA-MB-435 breast cancer cells. When they administered S247 after tumour cell inoculation they found only a small decrease in the incidence of bone metastases compared with vehicle control. This led the authors to suggest that $\alpha_V\beta_3$ was involved in the early steps of metastasis rather than the later stages i.e. adherence and colonisation rather than proliferation and osteolysis, respectively. (Reinmuth, Liu et al. 2003; Harms, Welch et al. 2004).

1.5.4 PTHrP and Cell Adhesion

Several studies have shown a relationship between PTHrP and integrin expression. For example, a study by Ye *et al* using human colon cancer cells over-expressing PTHrP demonstrated not only an increase in PTHrP secretion in these cells compared with vector-control, but also a two-fold increase in adhesion to collagen type I. However PTHrP over-expression had no effect on adhesion to either laminin or fibronectin. Ye *et al* examined the effect of exogenous PTHrP on adhesion and found that neither PTHrP (1-136) nor a PTH1R receptor antagonist affected cell adhesion to collagen type I, laminin or fibronectin (Ye, Seitz et al. 2001).

It has previously been discussed that certain tumours appear to have a predisposition to metastasise to certain organs and from these results Ye *et al* suggested that when entering the blood system, tumour cells over-expressing PTHrP may promote organ-

specific metastasis through their increased capacity to adhere to certain ECM components.

Another group at the University of Texas have shown a similar effect in a number of tumour derived cell lines. Using expression vectors containing PTHrP they showed that PTHrP over-expression in PC-3 prostate cancer cells causes an increase in adhesion to collagen type I, fibronectin and laminin (Shen and Falzon 2003). Subsequent studies showed an increase in adhesion to laminin in breast cancer cells over-expressing PTHrP (Shen, Qian et al. 2004), while in a colonic cell line they demonstrated an increase in adhesion to collagen type I, fibronectin and laminin (Shen and Falzon 2005).

When Shen and Falzon subsequently examined integrin expression in their over-expressing cell lines they found an increase in expression of the integrin subunits α_1, α_5, α_6 and β_4 in the prostate cancer cell line, an increase in the expression of α_6 and β_4 in the breast cancer cell line and an increase in α_2, α_5, β_1, β_4 and β_6 expression in the colonic cell line. They also used an expression vector containing PTHrP without the NLS to negate not only the increase in ECM adhesion but also the increase in integrin expression. Different subunits were increased in the three cell lines suggesting that PTHrP-induced up-regulation is not random but provides a more direct system of control which is tissue-specific.

It is known that PTHrP is over-expressed in a number of tumours and this has been linked to tumour progression. Integrin expression has also been described in association with tumour progression and Shen and Falzon's results point towards a

possible link between the two. A number of PTHrP's functions are similar to actions of certain integrins, for example an increase in cell growth and an increased resistance to apoptosis. It is therefore possible that PTHrP mediates some of its effects via an increase in integrin expression, an idea explored during the course of this thesis.

1.6 CURRENT PTHrP RELATED THERAPEUTICS

Shortly after PTHrP had been isolated and linked with hypercalcemia, Kukreja *et al* used an antibody directed against PTHrP to lower serum calcium in a mouse model of malignancy associated hypercalcemia. The anti-sera were directed against PTHrP (1-34) and (1-16) and both were able to reduce the biological activity of PTHrP (1-34) resulting in a decrease in serum calcium, urine calcium and urine cAMP (Kukreja, Shrevin et al. 1988).

This has been confirmed in several other studies where a PTHrP neutralising antibody has been used to treat various aspects of malignancy associated with PTHrP.

Following the suggestion that there was a link between PTHrP expression in breast cancer cells and bone metastasis, Guise *et al* used a PTHrP neutralising antibody in a mouse model of human breast cancer metastasis to bone. The present treatment for metastatic breast cancer involves the use of the osteoclast inhibitors, bisphosphonates. However their low absorption often necessitates high doses and they are often poorly tolerated resulting in significant GI side effects (Body 2002).

When Guise *et al* induced osteolytic lesions in mice they were unable to detect an increase in Ca^{2+} or plasma PTHrP levels. However they did detect an increase in bone marrow plasma PTHrP levels in mice with osteolytic lesions compared with control mice. When mice were subcutaneously treated with a PTHrP (1-34) neutralising antibody or IgG control seven days prior to tumour inoculation and treated twice a week during the course of the study, there was a significant reduction in both the number and area of metastatic lesions in mice receiving the antibody. There was also a significant reduction in the number of osteoclasts and while calcium concentration was unaltered, mice receiving IgG control rather than PTHrP antibody experienced significant weight loss (Guise, Yin et al. 1996).

Guise *et al*'s study not only indicated that antibodies directed against PTHrP could be used in the prevention of bone metastases but also suggested that PTHrP plays a role in weight loss in cancer patients. This hypothesis was given additional support by Iguchi *et al* who suggested that PTHrP was able to affect weight loss by acting on the hypothalamus and subsequently used a PTHrP neutralising antibody to bring about weight gain in mice suffering from cancer induced cachexia (Iguchi, Onuma et al. 2001).

Massfelder *et al* used a PTHrP neutralising antibody to examine the role of PTHrP in renal cell carcinoma. They demonstrated a decrease in cell proliferation with the antibody as well as a PTH1R antagonist and a parallel increase in apoptosis. When they then utilised a xenograft athymic mouse model they found that tumour-bearing mice treated with either the PTHrP (1-36) antibody or the PTH1R antagonist exhibited a significant decrease in tumour growth. With increased antibody dosage,

tumour regression was observed with the disappearance of 7/10 tumours and a 50-80% regression in three others. The antagonist was unable to bring about tumour regression however it was able to prevent tumour growth, with two out of ten tumours demonstrating a 90% and 60% regression. Due to the disappearance of the tumours the authors were unable to conduct histopathological analysis on antibody treated tumours however, when antagonist-treated tumours were subsequently examined there was an increase in apoptosis in antagonist-treated mice compared with control animals (Massfelder, Lang et al. 2004).

The successful use of PTHrP neutralising antibodies both *in vitro* and *in vivo* led the pharmaceutical company Chugai to develop an anti-PTHrP antibody called CAL for use in the treatment of metastatic breast cancer. This was successfully used to inhibit osteolytic bone metastases in mice and although Chugai began enrolment for a Phase I/II trial, at the time of writing development on this antibody has been suspended.

Immunotherapy is based upon neutralisation of the protein however PTHrP gene expression has also been targeted. After successfully using a PTHrP neutralising antibody, Teresa Guise went on to use nucleotide analogues of guanine to induce a 70% reduction in PTHrP promoter activity leading to a decrease in PTHrP mRNA concentration and PTHrP production. In mice bearing osteolytic bone lesions, the guanine analogues were able to significantly reduce the tumour lesion area from 3.97 $\pm$ 1.48mm^2 in untreated mice to 0.74 $\pm$ 0.14mm^2 in mice treated with 6-thioguanine ($p<0.05$). Similarly the number of osteoclasts per mm of tumour-bone interace was reduced from 15.62 $\pm$ 0.93 to 2.81 $\pm$ 1.74 ($p<0.05$). This led to an increase in

survival: all of the untreated mice had died by day 35 whereas 80% of the analogue treated mice were still alive ($p<0.05$). The analogues were also able to significantly decrease whole blood ionized calcium concentration and reduce mean serum PTHrP concentrations ($p<0.05$) (Gallwitz, Guise et al. 2002).

Other approaches have included the use of anti-sense oligodeoxynucleotides targeted against PTHrP to decrease translation of PTHrP mRNA in rat chondrocytes. Similarly Falzon and Du used anti-sense oligodeoxynucleotides to inhibit PTHrP production in a human breast cancer cell line and induced a decrease in cell proliferation (Tsukazaki, Ohtsuru et al. 1996; Falzon and Du 2000).

The active form of vitamin D ($1,25(OH)_2D_3$) has been shown to decrease PTHrP gene transcription as well as decreasing PTH1R expression in mouse osteoblasts (Goltzman, White et al. 2001). In a similar study Tovar Sepulveda and Falzon used $1,25(OH)_2D_3$ to decrease PTHrP expression in prostate cancer cells and suggested that it could be used in the prevention and treatment of prostate cancer (Tovar Sepulveda and Falzon 2002).

Francini *et al* also explored chemoprevention when they used PTHrP peptides to vaccinate mice against PTHrP and in doing so were able to stimulate an immune response against PTHrP (Francini, Kosmatopoulos et al. 2002).

Each of these studies describes systems whereby PTHrP could be targeted, but they also emphasise the number of cancer related complaints which could be treated by

doing so. These include hypercalcemia, tumour metastasis and cachexia. It can therefore be seen that PTHrP provides a key target in cancer therapy.

1.7 AIMS & HYPOTHESIS

PTHrP is commonly over-expressed in a number of cancers; it has been shown to be a growth and anti-apoptotic factor and there is evidence to suggest that it is also involved in cell adhesion. As PTHrP is also linked to tumour progression it is clear that it presents an ideal therapeutic target.

The principle aim of this work is to examine the role of PTHrP in cell adhesion. Specifically, it aims to investigate the mechanism by which this protein mediates cell adhesion, utilising a variety of methods including over-expression and RNAi of PTHrP.

Chapter 2:

Materials & Methods

2.1 CELL CULTURE

2.1.1 Cell Line Maintenance

The origin of the cell lines used in this study are shown in Table 2.1. They were cultured in Roswell Park Memorial Institute (RMPI) 1640 media (Sigma Aldrich, Dorset, UK) supplemented with 2mM glutamine (Sigma Aldrich) and 10% v/v heat inactivated foetal bovine serum (FBS, Sigma Aldrich). Genetically modified cell lines were cultured with the addition of 200μg/mL geneticin (Sigma Aldrich) in order to ensure transfection maintenance. Cells were housed in a humidified 5% CO_2 incubator at 37°C. Passage numbers were not recorded for the cell lines used during the course of this study.

2.1.2 Sub-culture

When harvesting cells for propagation or for assay purposes, growth media was removed and cells detached using 2mLs/75cm^2 of either 0.025% ethylenediaminetetracetic acid (EDTA) in phosphate buffered saline (PBS, pH 7.37, Oxoid, Basingstoke, UK) or 0.25% trypsin-EDTA (Sigma Aldrich). Cells were incubated for approximately five minutes or until cells detached, then pelleted by centrifugation at 1500rpm for five minutes using a Sanyo MSE Harrier 15/80 centrifuge.

A Neubauer haemocytometer was used in conjunction with trypan blue (Sigma Aldrich), which excludes live cells, in order to determine viable cell number. The haemocytometer contains a central platform with a ruled counting area 0.1mm under the coverslip. The counting area is divided into 25 squares with a total volume of

liquid encompassed by the primary square being $0.1mm^3$. A 0.2% stock of trypan blue was first diluted 3:1 with 4.25% saline solution (sodium chloride in distilled water, Sigma Aldrich) and then mixed 1:1 with cell suspension. Clear cells were deemed viable and blue cells non-viable. The number of viable cells contained within the squares of the hemocytometer were counted and the number of cells per mL calculated by multiplying the cell count by two (to allow for the 1:1 dilution) and $1x10^4$ (to allow for the volume underneath the coverslip).

Table 2.1 Cell lines used, including their tissue of origin and transfected variants.

Cell Line	Tissue of Origin	Transfected Variants
AGS[a]	Gastric adenocarcinoma	
C170HM2[a]	Colonic carcinoma	
COLO205[a]	Colonic adenocarcinoma	
HT29[b]	Colonic adenocarcinoma	PTHrP (1-139), PTHrP (1-139+NLS Mutation), Vector Control
MDAMB231[a]	Breast adenocarcinoma	PTHrP (1-141)[d], PTHrP (1-139), PTHrP (1-139+NLS Mutation), Vector Control
MGLVA1 *ascites*[c]	Gastric adenocarcinoma	
PAN1[a]	Pancreatic carcinoma	
PANC1[c]	Pancreatic carcinoma	

a - supplied by European Collection of Cell Cultures, Porton Down, Salisbury, UK
b - supplied by American Type Culture Collection, available from LGC Prochem, Middlesex, UK
c - derived within the Academic Unit of Cancer Studies, University of Nottingham, Nottingham, UK
d - provided by Dr Teresa Guise, Division of Endocrinology, University of Texas, USA

2.2 SMALL INTERFERING RNA

2.2.1 siRNA Design

Small interfering RNAs (siRNA) were used to induce post-transcriptional gene silencing of PTHrP and the integrin subunits α_5 and α_6. The PTHrP siRNAs were designed using Ambion's siRNA target finder (www.ambion.com/techlib/misc/siRNA_finder.html) while the integrin siRNAs were designed using a similar programme provided by Dharmacon (www.dharmacon.com/sidesign). While Dharmacon's design programme automatically eliminates siRNAs with high homology to other targets, Ambion's does not. Accordingly the potential PTHrP siRNAs were checked for homology with other genes using the web based tool BLAST (www.ncbi.nlm.nih.gov/BLAST) and three siRNAs targeted against different regions of the PTHrP gene subsequently chosen for investigation. The scrambled siRNAs were generated using another web based tool, RANDSEQ, which is available via workbench.sdsc.edu. Table 2.2 shows that target sequence of each siRNA investigated.

Table 2.2 siRNA Target Sequences

siRNA		Target Sequence
PTHrP	Target 4	AAGGGGAAGTCCATCCAAGAT
	Target 19	AAGACACCTGGGAAGAAAAAG
	Target 28	AAGAAAAAACGGCGAACTCGC
Scrambled PTHrP Target 19		AAGCACAAAGAGATGGAGAAC
Integrin α_5	α_5 A	AAGAATCTCAACAACTCGCAAAGCGAC
	α_5 B	AAGAATCTCAACAACTCGCAA
Scrambled Integrin α_5 A		AACCCTCGGACCTTAAGCAGAAACAAA
Integrin α_6	α_6 A	AAACATGGACCTTGATCGAAA
	α_6 A (27nt)	AAACATGGACCTTGATCGAAATTCCTA
	α_6 B	AACAGCAACCTTAAACTAGAA
	α6 C	AACAACAAGCAATCAAGATAA

2.2.2 Construction of siRNA

Sense and anti-sense oligonucleotide primers complementary to the siRNA target sequences were purchased from Eurogentec (Southampton, UK) and the siRNA constructed using the reagents provided with the *Silencer* siRNA Construction kit (Ambion, Huntingdon, UK).

This kit uses T7 RNA polymerase to transcribe the RNA; in order to do utilise this enzyme however it is necessary to first hybridise the oliognucleotide primers to T7 promoter primers. This is achieved by adding several nucleotides complementary to the T7 promoter (CCTGTCTC) onto the end of each oligonucleotide primer at the time of purchase.

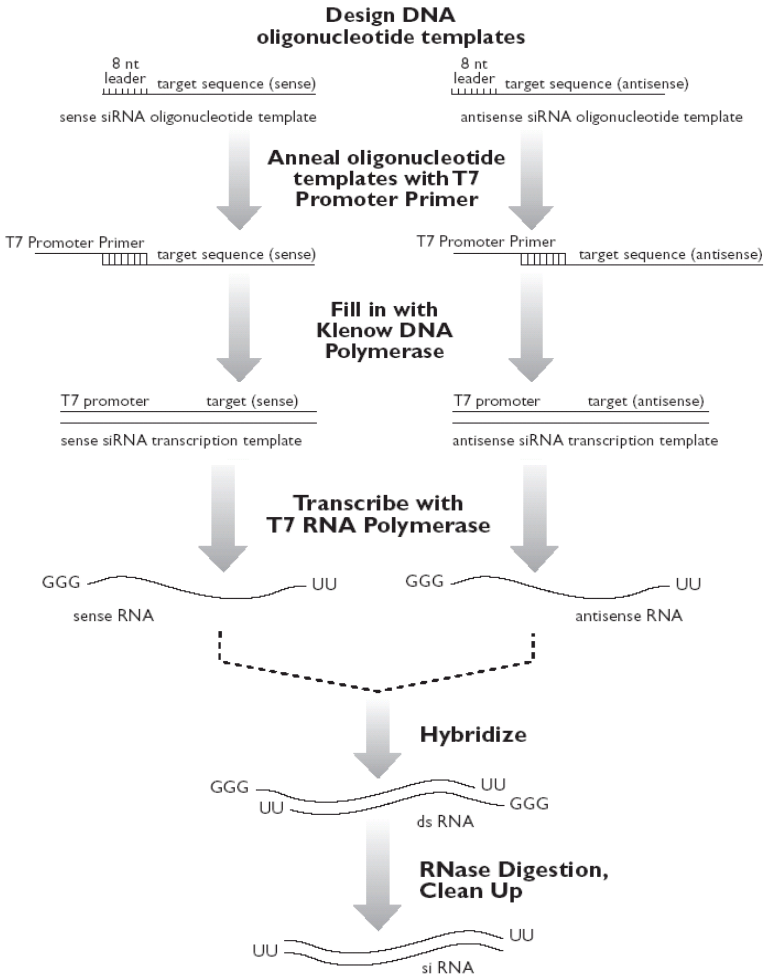
A five minute incubation of the template oligonucleotides with the T7 promoter primer and DNA hybridisation buffer at 70ºC encourages hybridisation of the primer

with the template. A further 30 minute incubation at 37ºC with Klenow reaction buffer, dNTP mix and Exo-Klenow enzyme promotes 3' extension of the hybridised oligonucleotide by the Klenow fragment of DNA polymerase.

The sense and anti-sense templates were then incubated at 37ºC with T7 RNA polymerase, T7 Reaction buffer and NTP mix to produce RNA transcripts. For fluorescent siRNA construction an NTP mix containing fluorescein-12-UTP (Roche, East Sussex, UK) was included in this reaction mix.

The sense and anti-sense transcripts were then combined and incubated at 37ºC overnight to allow hybridisation and the creation of double-stranded RNA. Incubation at 37ºC for a further two hours with a single-strand specific ribonuclease removed the leader sequence, while a deoxyribonuclease removed the DNA template. The siRNA were then purified by glass fibre filter binding in conjunction with an ethanol containing wash buffer to remove excess nucleotides, short oligomers, proteins, and salts. siRNAs were then eluted in nuclease-free water preheated to 75ºC. The resultant double-stranded 21-mer siRNAs were then ready for transfection into mammalian cells. A diagrammatic representation of siRNA construction can be seen in Figure 2.1.

Figure 2.1 siRNA construction using *Silencer* siRNA Construction kit (taken from instruction manual).

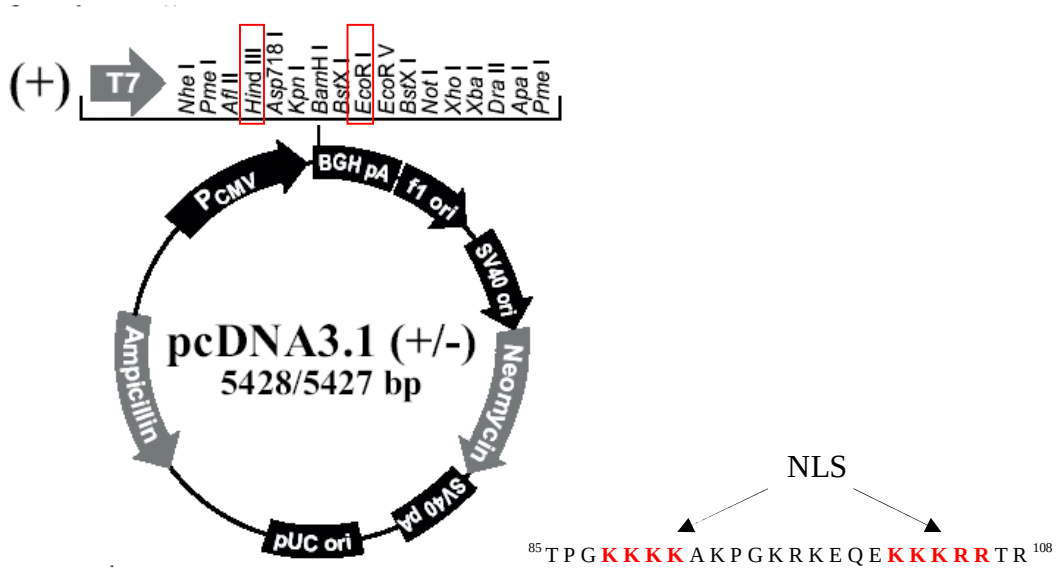


2.3 VECTOR CONSTRUCTION

2.3.1 PTHrP Plasmids

In order to examine the effects of PTHrP over-expression, mammalian expression vectors were used to create several stable cell lines. Three pcDNA3.1(+)vectors containing the coding regions for PTHrP were kindly donated by Professor Miriam Falzon at the University of Texas. The vectors contained either full-length PTHrP (-5 to +139) or PTHrP (-5 to +139) without the NLS. An empty vector control was also provided.

Figure 2.2 pcDNA3.1 vector with ligation sites for PTHrP (1-136) highlighted. The nuclear localisation sequence is also represented and highlights the deleted regions



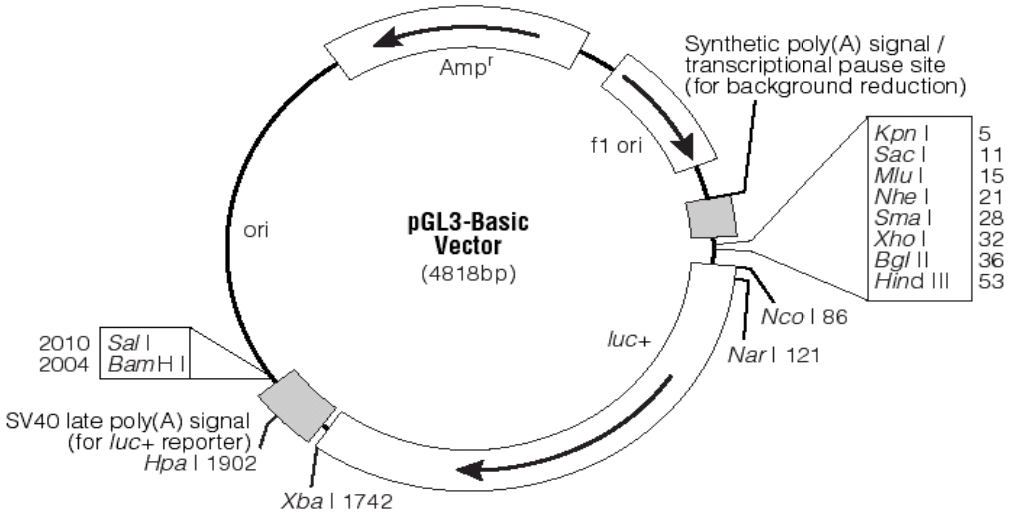
The cDNA encoding for PTHrP had been cloned into pcDNA3.1(+) by digesting the vector with the restriction enzymes *Eco*RI and *Hin*dIII (Figure 2.2). The deletion of the NLS had been carried out using a Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Palo Alto, CA), which resulted in the deletion of residues (88-91) and (102-106) and confirmed by DNA sequencing (Shen and Falzon 2003). A small volume of plasmid was initially obtained, so plasmid preparations were carried out to generate a greater working stock (see Sections 2.3.7 and 2.3.8).

2.3.2 Integrin Promoter Plasmids

The pGL3-Basic Vector (Promega, Southampton, UK) contains a coding region for firefly luciferase; by coupling this region to the coding region for a reporter gene it is possible to explore changes in promoter activity through changes in luciferase expression. For example by coupling the coding region for an integrin promoter it

was possible to examine the effects of PTHrP on the transcriptional activity of an integrin subunit.

Figure 2.3 pGL3-Basic vector circle map, containing coding region for firefly luciferase and with multiple cloning sites.



The sequence for each integrin promoter region was amplified by PCR as described in Section 2.7.5, using the primers noted in Table 2.3. Restriction enzyme sites were included to allow insertion of the promoter regions into the pGL3-Basic vector.

Table 2.3 Primers for amplifying integrin promoter sequences (restriction enzyme sequences are underlined).

Integrin Subunit	Primer Sequence 5' → 3'
α_2	**F** CGACGCGTTTCAGCAATGTGCTGGAAAT (*Mlu* I) **R** CGGGATCCCCCATCCTGGGTCTGACG (*Bam* HI)
α_5	**F** CGACGCGTGTTTACACCGATTAGGAGCTGAAGGT (*Mlu* I) **F** CGGGTACCGTTTACACCGATTAGGAGCTGAAGGT (*Kpn* I) **R** GAGAGATCTTCCTAAACCTCCCAGAGGCG (*Bgl* II)
α_6	**F** CGACGCGTCAGGAGCCTTCATGCCACCTACACA (*Mlu* I) **R** GAGAGATCTGAATGAGCCCGTTGTTCTCTGGAG (*Bgl* II) **R** GGCGACTTACTCGGGCAACAAGAG (from Lim, Lee et al. 2001)

2.3.3 Restriction Digest

Restriction digests of plasmids or amplified DNA were performed using restriction endonucleases. These enzymes each have a specific recognition sequence and cleave DNA at the sugar-phosphate backbone. Each enzyme was mixed with a specific enzyme buffer (New England Biolabs, Hertfordshire, UK) and RNase- and DNase-free water (Sigma Aldrich) to produce a 1:10 working concentration. The DNA and enzyme were incubated between four and 20 hours at 37°C and the subsequent digestion products mixed with 10x Blue Juice loading buffer (Invitrogen, Paisley, UK) and visualised using a 2% agarose gel containing 0.005% ethidium bromide (Sigma Aldrich), which intercalates between DNA base pairs then fluoresces when exposed to UV light. Buffers and incubation times can be seen in Table 2.4 and varied between the enzymes used and depending on whether these were used alone or in conjunction with another enzyme.

Table 2.4 Restriction enzymes used, including requisite buffers and incubation times

Restriction Enzyme	New England Biolab Buffer Used	Incubation Time (h)
Bam HI	2	2
Bgl II	3	20
Kpn I	1	2
Mlu I	3	20
Mlu I & *Bgl* II	3	20
Mlu I & *Bam* HI	3	20
Kpn I & *Bgl* II	2	20

A 100 base pair or 2-log DNA Molecular Weight Marker (New England Biolabs) was loaded onto each agarose gel and used for band size comparison. Digested products were visualised using GeneSnap software in conjunction with a Chemi Genius^2 BioImaging System (Syngene, Cambridge, UK).

2.3.4 Ethanol Precipitation of DNA

Ethanol precipitation was used to concentrate DNA and was performed as follows. Two volumes of 100% ethanol (Sigma Aldrich) along with 1/10th volume of 3M sodium acetate (Sigma Aldrich) were added to the DNA and incubated at -20°C for one hour. Following centrifugation[1], the DNA was washed twice with four volumes of 70% ethanol. After the final wash the supernatant was removed and the plasmid pellet allowed to air dry before being re-suspended in RNase- and DNase-free water (Sigma Aldrich).

2.3.5 Agarose Gel Purification

Following gel electrophoresis the desired DNA fragment was excised from the agarose gel using a clean scalpel and gel purified using reagents provided with the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK).

The agarose gel fragment was weighed and three volumes of Buffer QG used to solubilise the agarose gel at 50°C, where one volume is equivalent to 100mg of gel. In order to aid solubilisation the tube was vortexed every two to three minutes for approximately ten minutes until the gel fragment fully dissolved. In order to increase the yield of DNA, one gel volume of isopropanol was then mixed with the sample before it was transferred to a QIAquick column and centrifuged for one minute. The bound DNA was washed with a further 0.5mL of Buffer QG in order to remove all traces of agarose from the column before the DNA was washed with 0.75mL of Buffer PE. The column was dried by a further one-minute centrifugation before being placed in a clean tube and the DNA eluted using 30μL water. DNA

[1] Unless otherwise stated all centrifugations were carried out using a Hettich Mikro 20 bench top centrifuge

was quantified using a NanoDrop ND-1000 spectrophotometer (Labtech, East Sussex, UK).

2.3.6 Ligation of Vector and DNA Insert

The restriction endonucleases generated overhanging cohesive ends in the vector and DNA which allowed them to be ligated together. In order to join the vector and DNA together 100ng of pGL3-Basic vector was used alongside an appropriate amount of DNA to give a molar ratio of 1:3 (vector:insert) taking into account the base pair size of the vector and the DNA insert.

The vector and DNA insert were then ligated together at 4°C using T4 ligase (New England Biolabs), which creates a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. The vector, DNA insert and enzyme were also incubated with 10xbuffer and ATP to aid ligation (New England Biolabs). Following an overnight incubation the ligated vector and insert were ready for bacterial transformation.

2.3.7 Bacterial Transformation

Both the PTHrP and integrin containing plasmids were transformed into bacteria which are able to replicate the vector DNA and consequently generate additional plasmid stocks.

In a pre-cooled 15-mL falcon tube, 100μL of XL1-Blue MR Supercompetent Cells (Stratagene, La Jolla, CA USA) were mixed with 1.7μL β-mercaptoethanol, which

has been shown to increase the efficiency of the transformation. The sample was then incubated on ice for ten minutes, and mixed gently every two minutes.

Between 1 and 50ng of plasmid DNA was then mixed with the supercompetent cells and incubated for a further 30 minutes on ice. A tube containing supercompetent cells only was also included as a control. The DNA was then incorporated into the supercompetent cells via heat-shock; the tubes containing cells and DNA were immersed in a 42°C waterbath for 45 seconds before being cooled on ice for a further two minutes.

The mixture was diluted with 900µL of SOC medium (Sigma Aldrich), preheated to 42°C, and shaken for one hour at 37ºC to encourage bacterial growth. The transformed bacteria were then plated out on an LB agar plate containing 50µg/mL of the antibiotic ampicillin (Sigma Aldrich) and incubated overnight at 37ºC. [Each of the vectors used in this study contained an ampicillin resistance gene which enables selection of transformed bacteria.]

The agar plates were prepared by diluting the contents of one packet of FastMedia LB Agar Amp (Fermentas, Sunderland, UK), which is supplemented with 50µg/mL of ampicillin, with 200mL of distilled water. The mixture was then heated in a microwave for two to three minutes until the appearance of bubbles. The solution was then swirled gently and heated for a further 30-seconds or until the medium completely dissolved. The agar was left to cool before being aliquoted into petri dishes and allowed to set.

Multiple colonies were then selected for each plasmid and incubated in 2mL NZY media (0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, 1% NZ Amine, pH 7.5 (Sigma Aldrich)) again supplemented with 50µg/mL ampicillin and shaken at 37ºC overnight to promote bacterial growth. The plasmid DNA was then extracted from the overnight culture.

2.3.8 Plasmid DNA Extraction

Plasmid DNA was extracted using reagents supplied with the GenElute Plasmid Mini-prep kit (Sigma Aldrich), which is based on the alkaline lysis method.

The overnight culture was pelleted by centrifugation for one minute and the supernatant discarded. The cell pellet was re-suspended in 200µL Resuspension Solution containing RNase A, before the addition of 200µL of Lysis Solution. This alkali solution denatures the bacterial chromosomal DNA however, the circular plasmid DNA is covalently closed thus allowing it to remain double-stranded and intact. The sample was mixed by inversion and in order to ensure complete bacterial lysis the sample was incubated at room temperature for five minutes. 350µL of Neutralisation Solution was subsequently used to induce precipitation and the resultant bacterial debris pelleted by a ten-minute centrifugation. The supernatant (containing the plasmid DNA) was transferred to a Mini Spin Column and centrifuged for one minute. The column contains a silica-gel membrane which allows adsorption of the plasmid DNA in a high-salt buffer and elution in a low salt buffer. As RNA, cellular proteins and metabolites are not adsorbed by the membrane they are discarded in the flow-through.

The DNA was washed with 750μL of Wash Solution which contained ethanol, the column centrifuged and the flow-through discarded. The column was dried by another one-minute centrifugation before being transferred to a clean tube and the DNA eluted in 50μL of RNase and DNase free water. Eluted DNA was quantified using a NanoDrop ND-1000 spectrophotometer and either used immediately for cell transfection or stored at -20°C.

2.3.9 Sequencing of DNA Insert

Following PCR amplification of the integrin promoter regions (See sections 2.3.2) the sequence of the DNA was verified using the chain termination method, which was carried out by the School of Biomedical Sciences at the University of Nottingham.

The DNA being sequenced was used as a template in the synthesis of new DNA, which was initiated using the specific primers used for PCR amplification (See Table 2.3). The sequencing reaction consisted of an initial incubation at 94°C which induced the separation of the DNA into single strands. A further incubation at 50°C allowed the oligonucleotide primers to anneal to the template before the temperature was increased to 60°C in order to allow a DNA polymerase to extend the primers and produce a new copy of DNA.

Included in the reaction mix was a pool of single deoxynucleotide (dNTP) bases as well as fluorescently labelled dideoxynucleotide (ddNTP) bases. ddNTPs contain a hydrogen atom at the 3' carbon instead of the hydoxyl group found in dNTPs,

consequently when a ddNTPs is incorporated rather than a dNTP it brings about the termination of the growing DNA chain. Also as this incorporation occurs randomly, not only will chains of assorted lengths be produced but these will contain each of the four bases.

The resultant assorted truncated strands can then separated by size on a polyacrylamide gel. When the fragments migrate through the gel the fluorescently labelled ddNTPs are excited by a laser beam at the bottom, triggering the emission of light by the fluorescent molecules; this allows the complete DNA sequence to be reconstructed by matching the order of the bands with the ddNTP. Furthermore using different dye labels for the four ddNTPs allows the reaction to be performed in a single tube and analysed on a single gel.

5μL of gel purified PCR product was sent for analysis, which was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK).

2.4 TRANSFECTION PROTOCOLS

2.4.1 siRNA Transfection

Cells were either forward transfected using siPORT *Neofx* (Ambion) or reverse transfected using siPORT *Amine* (Ambion). siPORT *Neofx* is a lipid-based formulation while siPORT *Amine* is a blend of polyamines.

When forward transfected, cells were harvested and counted as described in Section 2.1.2 then re-suspended in growth medium at a concentration of $1x10^5$ cells/mL and incubated at 37°C until required. For each well that was transfected 2μL of siPORT

Neofx was combined with 23µL of Opti-MEM I reduced serum media (Invitrogen) and incubated at room temperature for ten minutes. siRNAs were diluted to their working concentration using Opti-MEM I and then combined with the previously diluted siPORT *Neofx*. The mixture was again incubated for ten minutes at room temperature to allow the formation of transfection complexes, which were then dispensed into the empty well of a 24-well plate. 450µL of cell suspension was then added to each well and incubated at 37°C, 5% CO_2 to promote transfection.

When cells were reverse transfected, it was necessary to plate $5x10^4$ cells/mL 24 hours prior to transfection in a 24-well plate so that cells achieved a level of confluence between 30-70% at the time of transfection. On the day of transfection 2µL of siPORT *Amine* was combined with 47µL of Opti-MEM I and incubated at room temperature for between ten and 30 minutes. siRNA were sufficiently diluted in RNase and DNase free water so that the addition of 1µL to the diluted siPORT *Amine* would result in the requisite working concentration. In order to allow formation of the siRNA/siPORT *Amine* complex, samples were incubated at room temperature for 15-20 minutes. The media was removed from the previously seeded cells and replaced with 200µL of fresh growth medium and the transfection reagents added drop-wise onto the cells. The cells were then incubated at 37°C, 5% CO_2 for approximately four to six hours before a further 1mL of growth medium media was added to the cells to prevent cytotoxicity.

2.4.2 Plasmid Transfection

Cells were harvested, counted and seeded in a 24-well plate 24 hours prior to transfection in order to achieve a level of confluence between 90-95% at the time of

transfection. For each well that was transfected 2µL of Lipofectamine 2000 (Invitrogen) was diluted in 50µL Opti-MEM I and incubated for five minutes at room temperature. Plasmid DNA was diluted to 500ng in 50µL Opti-MEM I before the DNA and Lipofectamine 2000 were combined and incubated for a further 20 minutes at room temperature to promote formation of transfection complexes. These complexes were then added to the previously seeded cells and incubated at 37°C, 5% CO_2.

In order to produce stable transfectants, transfected cells were passaged at 1:10 and re-seeded into a 6-well plate 24 hours later. The pcDNA3.1 vector contained a drug restriction marker which allowed selection of transfected cells following incubation of cells with 500µg/mL geneticin. Individual clones were selected in a 96-well plate before expansion and analysis of gene expression.

When only transient transfection was required, transfected cells were lysed and examined for luciferase expression one day after transfection, as described in Section 2.10.

2.5 CELL PROLIFERATION

2.5.1 PTHrP Stimulation Assay

Cells were harvested, counted and re-suspended in growth medium at a concentration of $5x10^3$ cells per well and 200µL of this cell suspension added to the inner 60 wells of an opaque 96-well plate (Corning, Surrey, UK). The remaining outer wells were filled with media only to prevent evaporation.

Serum has been shown to induce the release of PTHrP into the media, consequently following an overnight incubation, medium was aspirated from each well and replaced with 200µL of serum-free medium containing 100nM of PTHrP peptide and 1µCi per mL of tritiated thymidine ([^{3}H]-thymidine; Amersham Biosciences, Buckinghamshire, UK). The peptides employed were PTHrP (1-34), (67-86) (107-139), (140-173) and (67-101) (Bachem, St Helens, UK; Anaspec, Cambridge, UK), which correspond to amino-terminal, mid-region (without NLS), osteostatin, carboxyl-terminal and mid-region (with NLS) respectively. As a control, a (67-101) peptide was used which contained a five amino acid scrambled sequence linked to the PTHrP NLS sequence (Cambridge Research Biochemicals, Cleveland, UK).

Proliferation was assessed daily via thymidine incorporation, a nucleoside involved in the biosynthesis of DNA. Using [^{3}H]-thymidine it is possible to measure the incorporation of thymidine into DNA, with an increase in radioactivity correlating with an increase in cell proliferation.

At each time point, the medium containing [^{3}H]-thymidine was removed and the cells washed once with 100µL PBS before being lysed with 25µL of 1M sodium hydroxide. Plates were shaken using a Heidolph Titramax 1000 to aid cell lysis before the addition of 175µL of Opti-Phase 'HiSafe' 3 liquid scintillation cocktail (Perkin Elmer, Buckinghamshire, UK) to each well. Again plates were shaken to aid mixture of scintillant with cell lysates.

Well radioactivity was counted using a Trilux Microbeta Liquid Scintillation Counter for three minutes, with radioactivity reported in counts per minute (CPM). The use of an opaque 96-well plate prevented radioactive cross-talk between wells.

2.5.2 Antibody Neutralisation Assay

Two different antibodies (one raised against PTHrP (1-34) and the other against (38-64) were used to neutralise PTHrP and the effects on proliferation again assessed using [^{3}H]-thymidine incorporation. IgG1-κ murine ascites (Sigma Aldrich) was used as a control.

Cells were harvested and seeded into opaque 96-well plates as described previously. The following day, medium was removed and replaced with serum free medium containing 1μg/mL of antibody. At each time point, radioactivity was measured as described in Section 2.5.1.

2.5.3 Assay of siRNA Transfection and Proliferation

Cells were transiently transfected with either the PTHrP siRNA or the scrambled control siRNA, as described in Section 2.4.1. The following morning, the medium was replaced with fresh growth medium to maintain the wellbeing of the transfected cells and later the same day the cells were harvested, pelleted, re-suspended in growth medium and re-plated in an opaque 96-well plate; approximately one well of a 24-well plate provided enough cells for ten wells of a 96-well plate. The following day the medium were replaced with serum-free medium containing 1μCi per mL of [^{3}H]-thymidine and proliferation assessed daily.

2.5.4 Clonogenicity Assays

Clonagenicity assays were performed in order to compare the basal growth rate of cells transfected to over-express PTHrP. Cells were seeded in growth medium at a density of $5x10^3$ cells per well in opaque 96-well plates. The following day the medium was aspirated and replaced with serum-free medium containing 1µCi per mL of [^{3}H]-thymidine. The growth rate of different cell lines was compared by examining proliferation on a daily basis for up to 72 hours.

2.6 EXTRACELLULAR MATRIX ADHESION

2.6.1 ECM Preparation

The extracellular matrix (ECM) components rat-tail collagen type I (Sigma Aldrich), human fibronectin and mouse laminin (BD Bioscience, Oxford, UK) were used to investigate cell adhesion. Collagen type I was dissolved in 0.1M acetic acid (Sigma Aldrich) at a stock concentration of 1mg/mL and stored at -20°C. Fibronectin was dissolved in PBS and laminin in serum-free media, at a stock concentration of 100µg/mL. Fibronectin was stored at -20°C whilst laminin was stored at –80°C. Collagen type I and fibronectin were diluted to a working concentration of 5µg/mL in PBS; laminin was diluted in serum-free medium.

50µl of 5µg/mL ECM was added to the inner 60 wells of a clear 96-well plate (Corning) and incubated at room temperature for one hour, excess ECM was removed and wells washed in distilled water. Plates were air dried at room temperature then stored until required.

Prior to use adventitious binding sites were blocked with 1mg/mL bovine serum albumin (BSA; Sigma Aldrich) in PBS and left at room temperature for one hour. Excess BSA was then removed and wells again washed with distilled water.

2.6.2 Effect of Peptide Stimulation on Cell Adhesion

Cells were seeded in six-well plates at $1x10^5$/mL, or $2x10^5$/well, and grown overnight before the growth medium was removed and replaced with serum-free medium containing 100nM of PTHrP peptide. This seeding density produced a sub-confluent monolayer on the day of assay, 72 hours following PTHrP peptide stimulation.

On the day of assay, cells were harvested, counted and re-suspended in growth medium at a concentration of $5x10^5$/mL. 200µl of cell suspension was then added to an ECM coated plate and the outer wells filled with medium only. Cells were incubated for one hour at 37°C, after which time each well was washed twice with growth medium to remove any non-adherent cells and the number of viable, adherent cells determined using MTT uptake.

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Aldrich) is converted to the purple salt formazan by the mitochondrial enzyme succinate dehydrogenase and consequently only viable cells will be able to convert MTT (Mossman 1983).

Following the addition of 50μL of 1mg/mL MTT, cells were incubated for four hours at 37°C, 5% CO_2 to promote MTT uptake and conversion to formazan. Excess MTT was removed and the remaining formazan crystals, which are insoluble in aqueous solution, were solubilised with 75μL of dimethyl sulfoxide (Sigma Aldrich). The amount of solubilised formazan product was then photometrically quantified using an MRX microplate reader at a wavelength of 550nm (ThermoLab Systems, Chantilly, USA). An increase in the number of viable cells correlates with an increase in total metabolic activity and consequently a larger amount of formazan. Therefore an increase in absorbance can be taken as being proportional to an increase in the number of viable cells and used as a measure of proliferation.

2.6.3 Effect of PTHrP Neutralisation on Cell Adhesion

Cells were grown in six-well plates prior to assay. The day after cells were seeded, growth medium was removed and replaced with serum-free medium containing 1μg/mL of PTHrP neutralising antibody or IgG1-κ murine ascites control. Following a 72-hour incubation, cells were harvested, counted and re-suspended in growth medium. 200μL of cell suspension was added to an ECM coated plate to give a cell density of $1x10^4$ cells per well. Outer wells were filled with media only.

Following a one-hour incubation at 37°C, non-adherent cells were washed off and the number of viable, adherent cells determined by MTT uptake as described in Section 2.6.3.

2.6.4 Effect of Gene Silencing on Cell Adhesion

Cells were transfected with siRNA targeted against either PTHrP or the integrin subunits α_5 or α_6. At various time points cells were harvested and re-suspended in growth medium before being added to an ECM-coated plate. Transfected cells were incubated for one hour, non-adherent cells washed off and the number of viable cells remaining assessed by MTT uptake.

2.6.5 PTHrP Over-expression and Adhesion

In order to aid reproducibility, cells were seeded at a density sufficient to achieve a level of confluence of 80-90% on the day of assay. Cells were then harvested, counted and plated at a concentration of $1x10^4$ cells per well in growth medium. Cells were incubated for one hour at 37°C, washed twice and viable cell number determined using MTT uptake.

2.7 MOLECULAR BIOLOGY

2.7.1 RNA Isolation

RNA was isolated using either 1mL of RNA-Bee (Biogenesis, Poole, UK) to re-suspend a pellet of approximately $1x10^6$ cells or by using 500µL to lyse cells directly in a 24-well plate. RNA-Bee is a monophase solution containing phenol, which dissolves proteins and lipids, and guanidine thiocyanate, a protein denaturant. The addition of 200µL of chloroform (Sigma Aldrich), a vigorous shake for 10-30 seconds followed by a 30-minute centrifugation at 13,000rpm, 4°C resulted in the separation of the organic phase, containing DNA and proteins, and the aqueous

phase, containing the desired RNA. The RNA was then precipitated by mixing the aqueous layer with an equal volume of isopropranol (Sigma Aldrich) in a clean tube.

The sample was incubated at -20°C for at least 20 minutes before a 15-minute centrifugation at 13,000rpm, 4°C. Once the supernatant had been discarded the RNA pellet was washed with 1mL of 70% ethanol, followed by another five-minute centrifugation. This wash was then repeated and as much of the remaining liquid as possible removed. The tube was left open but covered in order to aid evaporation of any remaining ethanol. Once dry, the RNA pellet was re-suspended in 50µL of DNase and RNase free water and samples either stored at -80°C or used immediately for reverse transcription.

2.7.2 Reverse Transcription

A 10µL aliquot of RNA was mixed with 1.2µL of either random PdN6 hexamer primer mix (Amersham Biosciences) or water to provide a cDNA positive and negative sample, respectively. Both samples were incubated at 70°C, to aid the unfolding of the RNA and allow primer access. At the end of this incubation the samples were transferred onto ice to prevent re-folding of the RNA.

10µL of a reverse transcription reaction mix containing DTT, First-Strand Buffer, dNTP mix and SuperScript II RNase-H Reverse Transcriptase (Invitrogen) was then added to the cDNA positive sample; the negative sample contained the same mix with the exception of the reverse transcriptase.

Reverse transcription was carried out by heating the samples to 25°C for ten minutes, 42°C for one hour and finally 95°C for five minutes, which inactivated the enzyme. This reaction protocol allows the transcriptase to elongate the PdN6 primers previously annealed to the RNA template, thus generating cDNA.

Samples were then diluted 1:5 with water and stored at -20°C or used immediately for PCR.

2.7.3 Extraction of Genomic DNA

When amplifying the integrin promoter regions it was necessary to use genomic DNA as cDNA reverse transcribed from RNA does not contain this region. Gemomic DNA was thus obtained using the reagents provided with the DNeasy tissue kit (Qiagen).

A cell pellet containing $1x10^6$ PAN1 cells was re-suspended in 200µL of PBS before 20µL of proteinase K and 200µL of Buffer AL were added to the cell sample, vortexted and incubated at 70°C to induce cell lysis. 200µL of 100% ethanol was then added to the cell lysate which was again vortexted before being added to a silica-gel containing mini-spin column. In order to promote binding of the DNA to the membrane, the column was centrifuged at 8000rpm for one minute and the flow-through discarded. The bound DNA was then washed using 500µL of Buffer AW2 and centrifuged for three minutes at maximum centrifugal speed in order to dry the membrane. The spin column was then placed in a clean tube and 100µL of DNase and RNase free water used to elute the DNA, which was then either used immediately for PCR or stored at -20°C until required.

2.7.4 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 0.2mL thin wall tubes (Sigma Aldrich) using genomic DNA and a reaction mix containing the following: Reaction Buffer, Q solution, dNTP mix, previously optimised primer pair and HotStarTaq enzyme (Qiagen).

The samples were amplified using a PTC-200 Peltier Thermal Cycler (MJ Research, Hertfordshire, UK) using the following reaction protocol: 15 minutes at 94°C followed by 40 cycles of one minute at 94°C, 2.5 minutes at 55°C then 1.5 minutes at 72°C. This allowed denaturation and separation of the DNA strands, annealing of the oligonucleotide primers which were then extended by *Taq* DNA polymerase. By using two opposing primers copies of the target DNA sequence are able to accumulate at an exponential rate. A final ten minute incubation at 72°C allowed for completion of any unfinished transcripts.

PCR products were visualised following mixture with 10x Blue Juice loading buffer (Invitrogen) and separation using a 2% agarose gel (Sigma Aldrich) containing 0.005% ethidium bromide (Sigma Aldrich) for 45 minutes at 120V. A 100 base pair DNA Molecular Weight Marker (New England Biolabs) was used for band size comparison.

2.7.5 Real time-PCR (RT-PCR)

Using RT-PCR it was possible to quantify levels of a specific template cDNA in cell lysates and thereby examine gene expression levels. RT-PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems) using 5μL of diluted cDNA and 20μL of reaction mix which contained Reaction Buffer, SYBR green, $MgCl_2$, dNTP mix, previously optimised primer pair, UNG and HotGoldStar (Eurogentec).

Corresponding cDNA positive and negative samples were analysed using a 7500 Real-time PCR System (Applied Biosystems) using the following reaction protocol: 2 minutes at 50°C, ten minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and one minute at 60°C. As with PCR this reaction involved denaturation, annealing and extension. Again using two opposing primers copies of the target DNA sequence are able to accumulate at an exponential rate and using the fluorescent DNA binding dye, SYBR green, gene expression can be quantified.

SYBR green emits strong fluorescence upon binding to double-stranded DNA and can thus be used to quantify expression of the target gene by monitoring the levels of SYBR green fluoresence emitted at the end of each cycle; the greater the template at the beginning of the reaction the fewer the number of cycles it takes to reach a point at which the fluorescent signal is statistically significant above background, which is defined as the threshold cycle (Ct). Gene expression was not deemed not detectable if the Ct of the test gene was >35 when the Ct of HPRT was <25.

In order to allow for differences in template concentration, gene expression of the house-keeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was also measured and compared to expression of the test gene; this ΔC_t was calculated using $C_{t,\ target\ gene} - C_{t,\ HPRT}$. Relative quantification of gene expression was then calculated using the following equation:

$$\textbf{Relative gene expression} = 2^{-(\Delta Ct,\ test\ sample\ -\ \Delta Ct,\ control\ sample)}$$

This comparative C_t model, also known as the $2^{-\Delta\Delta Ct}$ model, was derived by Livak and Schmittgen. In order for the equation to be valid the amplification efficiencies of the target and house-keeping gene must be approximately equal. To examine whether this is the case, it is necessary to serially dilute a cDNA template and examine the ΔC_t for the target and reference genes. If the amplification efficiencies are equal then a plot of cDNA dilution versus ΔC_t is <0.1 (Livak and Schmittgen 2001).

The oligonucleotide primers used during PCR can be seen in Table 2.4. These were purchased from Eurogentec and prior to use each primer pair was optimised to prevent primer-dimer formation and the amplification efficiency for each primer pair compared with that of the housekeeping gene, HPRT.

Table 2.5 Oligonucleotide primer pairs used during PCR

Target Gene	Primer Sequence 5' → 3'	Working Concentration (µM)
HPRT	**F** GACCAGTCAACAGGGGACAT **R** CGACCTTGACCATCTTTGGA	2.5
PTHrP	**F** GCTCGGTGGAGGGTCTCA **R** TTGTCATGGAGGAGCTGATGTT	2
Integrin α_1	**F** GGAGAAAACGTGACCCATGA **R** TGGATGACCTTCTTCAGTCG	5
Integrin α_2	**F** AACATCCCAGACATCCCAAT **R** CATCATGTGATTCACCGTCAG	5
Integrin α_3 (Guidry, Bradley et al. 2003)	**F** ACGAAGTCAGCAATGGCAAG **R** CAGCCACAGCTCGATTTGG	3.33
Integrin α_5 (Oki, Matsuo et al. 2004)	**F** GGCAGCTATGGCGTCCCACTGTGG **R** GGCATCAGAGGTGGCTGGAGGCTT	5
Integrin α_6	**F** TCAATTGCTGGAAACATGGA **R** GGCGGAGGTCAATTCTGTTA	3.33
Integrin β_1 (Oki, Matsuo et al. 2004)	**F** GTGGTTGCTGGAATTGTTCTTATT **R** TTTTCCCTCATACTTCGGATTGAC	5
Integrin β_3 (Schneider, Whitson et al. 1999)	**F** CTACCTGATGGACCTCTCCCTG **R** GTACTGCATCAAAGCCCCCCTC	3.33
Integrin β_4 (Utermark, Kaempchen et al. 2003)	**F** ATAGAGTCCCAGGATGGAGGA **R** GTGGTGGAGATGCTGCTGTA	5

Where there is no reference given primers were designed using the web based programme Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), which was developed by Steve Rozen and Helen Skaletsky. To avoid amplification of

contaminating genomic DNA the primers were designed to span exon-exon junctions in the cDNA sequence. Other criteria adhered to during design included limiting the melting temperature to 50-60°C, using oligonucleotides between 15-30 bases and with a G/C content between 30-80% (Rozen and Skaletsky 2000).

In addition, OAS, STAT and HPRT gene expression was examined using ABI Taqman primer sets (Applied Biosystems) labelled with the fluorescent reporter dye FAM, which negated the need for SYBR green.

2.8 PROTEIN BIOLOGY

2.8.1 Western Blotting

It is possible to detect a specific protein in a lysed cell sample using immunoblotting (more commonly referred to as western blotting) in conjunction with specific primary antibodies. The methods described here are based on the original protocol developed by Towbin *et al* and involves the separation of charged molecules through a polyacrylamide matrix via the application of an electrical field before the electrophoretic transfer of these proteins from the polyacrylamide gel to a nitrocellulose or PVDF membrane (Towbin, Staehelin et al. 1979; Burnette 1981).

2.8.1.1 Cell Lysate Preparation

In order to obtain both nuclear and cytoplasmic fractions ~$5x10^6$ cells were lysed first with a a lysis buffer containing 20mM Tris-HCl at pH 7.6, 0.5% Triton X-100, 250mM NaCl, 3mM EDTA, 3mM EGTA, 1mM PMSF, 1mM DTT (Sigma Aldrich) and 10μL/mL Protease Inhibitor Cocktail III (Calbiochem, Nottingham, UK).

Following a ten-minute centrifugation at 13,000rpm, 4°C, the supernatant was removed, stored separately and taken to represent the cytosolic fraction of the cell.

The cell pellet was then further lysed using RIPA buffer containing 20mM Tris buffer, 137mM NaCl, 10% Glycerol, 1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 2mM EDTA, 1mM PMSF, 1mM DTT (Sigma Aldrich) and 10μL/mL Protease Inhibitor Cocktail III. This was then taken to represent the nuclear fraction of the cell.

To aid loading of the samples, 4x NuPAGE sample buffer (Invitrogen) was mixed with the cell lysates and the samples then heated at 99°C for two minutes. When heated, SDS coats the protein resulting in a uniform negative charge, whilst DTT causes the disruption of the non-covalent inter- and intra-molecular associations i.e. the protein no longer has a secondary or tertiary structure. Samples were either used immediately for gel electrophoresis or stored at -80°C.

2.8.1.2 Gel Electrophoresis

Proteins were separated by electrophoresis using a polyacrylamide gel; depending on the size of protein being investigated either a pre-cast 12% NuPAGE or 8-16% Tris-glycine gel (Invitrogen) was used. Due to the uniform negative charge, the proteins were separated according to molecular weight rather than charge. NuPAGE gels have a neutral pH, rather than the highly alkaline pH used with Tris-glycine gels, which increases the stability of not only the proteins but also the acrylamide matrix. NuPAGE gels also allow a wider range of separation and are able to separate proteins through both low and high molecular weight ranges. NuPAGE gels were

used to examine PTHrP protein expression, which has a low molecular weight, whereas PTH1R expression was examined using Tris-glycine gels, which allow analysis of a mid-range of molecular weights.

The polyacrylamide gels were inserted into an XCELL *SureLock* Mini-Cell (Invitrogen). The inner portion of the cell was completely filled with running buffer and the outer portion filled with enough running buffer (NuPAGE MES SDS Running Buffer or Novex Tris-Glycine SDS Running Buffer; Invitrogen) to cover the buffer inlet into the gel. The sample concentration was determined using a NanoDrop ND-1000 spectrophotometer and approximately 20µg of each sample was loaded into individual wells of the gel and a constant voltage of 200V applied to NuPAGE gels for 35 minutes whereas 125V was applied to Tris-glycine gels for 90 minutes.

2.8.1.3 Blotting

Proteins separated using NuPAGE and Tris-glycine gels were transferred to a 0.2µm nitrocellulose membrane or a 0.2µm polyvinylidene fluoride (PVDF) membrane (Invitrogen), respectively to allow for antibody detection of the protein of interest.

A sandwich was assembled in an XCell Blotting Module (Invitrogen) using blotting pads saturated with transfer buffer (NuPAGE Transfer Buffer or Novex Tris-Glycine Transfer Buffer, Invitrogen), the polyacrylamide gel and an appropriate membrane. The blotting apparatus included a cathode plate and an anode; with the gel positioned towards the cathode plate and the membrane towards the anode plate the proteins

transfer from the gel to the membrane following the application of an electrical current.

The membranes were equilibrated in transfer buffer for 10-15 minutes prior to sandwich assembly. As the PVDF membrane is hydrophobic it was also necessary to soak this membrane in methanol (VWR, Poole, UK) for 30 seconds prior to equilibration.

A constant voltage of 30V was applied to the blotting module containing NuPAGE gels for 60 minutes and Tris-glycine gels for 90 minutes.

2.8.1.4 Antibody incubation

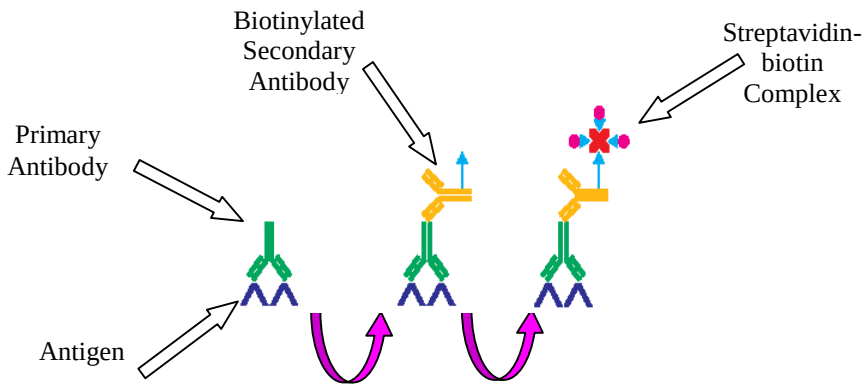
Prior to antibody incubation, non-specific binding sites on the membrane were blocked using 5% dried milk in Tris buffered saline (TBS: pH 7.6, 20mM Tris-base, 127mM sodium chloride, Sigma Aldrich). To detect the target protein each primary antibody was diluted as required in TBS and incubated with the membrane overnight at 4°C. Information relating to the antibodies used in this study is given in Table 2.6.

The excess antibody was removed by washing the membranes with TBS+0.1% Tween-20 (Sigma Aldrich) five times, with each wash lasting approximately five minutes. The primary antibody-antigen complex was then detected by incubating the membrane with a biotin conjugated secondary antibody directed against the species in which the primary antibody was raised (see Table 2.6). Again excess antibody was removed using TBS+0.1% Tween-20.

2.8.1.5 Visualisation

In order to visualise the protein of interest, a streptavidin-biotin-peroxidase complex (DAKO, Cambridgeshire, UK) was used. Figure 2.4 demonstrates the binding of the complex to the biotin conjugated secondary antibody which provides a substrate for the chemiluminescent solution ECL Advance (Amersham Biosciences). Luminescence was visualised using GeneSnap software in conjunction with a Chemi Genius^2 BioImaging System.

Figure 2.4 Illustration of the biotinylated secondary antibody binding to the primary antibody, which is followed by binding of the streptavidin-biotin complex to the secondary antibody.



An alternative method utilised 3,3'-diaminobenzidine (DAB), a horseradish peroxidase (HRP) substrate. Hydrogen peroxide causes oxidative polymerization of DAB by HRP which results in a brown insoluble precipitate at the site of the bound antibody. The developing solution contained 100mL TBS, 0.05g DAB, 0.07g imidazole and 150µL hydrogen peroxide (Sigma Aldrich). Membranes were incubated with developing solution for approximately ten minutes or until the appearance of the brown oxidation product. Peroxidase activity was quenched with running water and membranes left to dry.

A SeeBlue Plus2 Pre-Stained Standard or MagicMark™ XP Western Protein Standard (Invitrogen) was loaded into one well of each polyacrylamide gel and used for band size comparison.

2.8.1.6 Densitometry

The intensity of the bands present on the membranes is proportional to the amount of bound secondary antibody and hence the amount of target protein present in the sample. Band intensities were calculated using the Chemi Genius^2 BioImaging System and compared intra-gel.

2.8.2 Immunocytochemistry

Cells were harvested and counted as previously described and $5x10^4$ cells seeded in tissue culture-treated 8-well chamberslides (VWR) and incubated overnight. The following day, cells were fixed for five minutes with 4% electron microscope grade, methanol free paraformaldehyde (Science Services, Munich, Germany). The excess aldehyde was quenched using 10mM diethylamine (Sigma Aldrich). Following a five-minute incubation, cells were washed twice with PBS before permeabilisation with 0.1% Triton X-100 (Sigma Aldrich) and a further two PBS washes. The cells were incubated with primary antibody at room temperature for one hour (see Table 2.6).

The cells were washed twice in PBS before application of fluorescent secondary antibody directed against the species in which the primary antibody was raised (see Table 2.6) for one hour at room temperature.

Cells were then washed twice in PBS and slides cover-slipped using Antifade with DAPI mounting media (Molecular Probes at Invitrogen). DAPI (4'-6-Diamidino-2-phenylindole) forms a fluorescent complex with DNA and thereby allows counterstaining of the nucleus. Slides were sealed using clear nail varnish and fluorescence was visualised using a Zeiss LSM510uv META combi confocal microscope and 364nm ex/ 470-500BP, 488nm ex/505-530nm and 543nm ex/585-615 emission filters were used to examine DAPI, FITC and TRITC respectively.

2.8.3 Flow Cytometry

Both intracellular and extracellular protein expression can be examined using flow cytometry. For extracellular staining a cell pellet containing $2x10^5$ cells was washed twice in 1mL PBA (PBS containing 0.5% BSA and 0.1% sodium azide, Sigma Aldrich). The presence of the BSA prevents non-specific antibody binding while sodium azide inhibits antibody uptake during live cell staining.

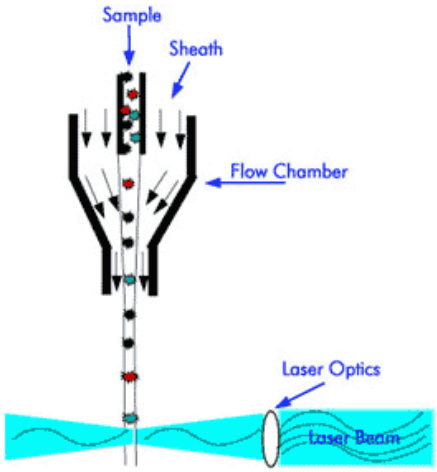
For intracellular staining it was necessary to first fix cells in 4% formalin (formaldehyde diluted in PBS, Sigma Aldrich) at room temperature for 15 minutes. Fixed cells were then washed once with 1mL saponin solution (PBA containing 0.1% saponin, Calbiochem, and 0.9% glucose, Sigma Aldrich) in order to permeabilise the cell membrane and allow antibody entry. This was followed by a further two PBA washes.

When conducting both extracellular and intracellular staining, cells were incubated first with 2µL of primary antibody (see Table 2.6), washed twice with 1mL PBA, then incubated with 1µL of fluorescently labelled secondary antibody (Table 2.6)

and again washed twice with 1mL PBA. Cells were then fixed with 1% formalin before analysis using a Beckman Coulter FACS analysis machine. Both antibody incubations were carried out in the dark for 30 minutes at 4°C.

Within the FACS analysis machine the cells are hydro-dynamically forced into a stream of PBS before the fluorescently labelled cells are then excited one at a time using a laser beam. The light emitted is then detected by a photomultiplier tube and the level of fluorescence of each cell recorded (Figure 2.5). The resulting data was analysed using WinMDI software (The Scripps Institute, La Jolla, CA, USA).

Figure 2.5 Diagrammatic representation of a flow cytometry flow chamber (taken from biology.berkeley.edu/crl/flow_cytometry_basic.html).



Each of the antibodies used to examine protein expression in this study, both primary and secondary, can be seen in Table 2.6, which also includes the optimal concentration or dilution used, where relevant.

Table 2.6 Antibodies used in the various methods of examining protein expression, including supplier and working concentration

Primary Antibody	Technique[§]	Supplier	Concentration (μg/mL) or dilution used
PTHrP (1-34)	WB/IHC/FC	Aphton[a]	3
PTHrP (38-64)	WB, IHC, FC	Oncogene[b]	3
PTHrP (polyclonal)	WB, IHC	Aphton	3
PTH1R	WB, IHC	Oncogene	1
α_2	FC	BD Bioscience	12.5
α_5	FC	BD Bioscience	12.5
α_6	FC	BD Bioscience	12.5
β_1	FC	BD Bioscience	12.5
β_3	FC	BD Bioscience	12.5
β_4	FC	BD Bioscience	12.5
β-actin	WB	Abcam[c]	1:5000
Secondary Antibody	**Technique**	**Supplier**	**Dilution**
Biotin labelled rabbit anti-mouse	WB	DAKO	1:1000
Biotin labelled goat anti-rabbit	WB	DAKO	1:1000
AlexaFlour 488 labelled goat anti-rabbit	IHC	Molecular Probes[d]	1:200
AlexaFlour 594 labelled rabbit anti-mouse	IHC	Molecular Probes	1:200
FITC labelled rabbit anti-mouse	FC	DAKO	-
PE labelled rabbit anti-mouse	FC	DAKO	-

[§]WB=western blotting, IHC=immunohistochemistry, FC=flow cytometry

a - Aphton Corporation, Woodlands, CA, USA; b - Oncogene, Nottingham, UK;
c - Abcam, Cambridge, UK; d – Molecular Probes at Invitrogen, Paisley, UK

2.9 MITOCHONDRIAL MEMBRANE POTENTIAL ASSAY

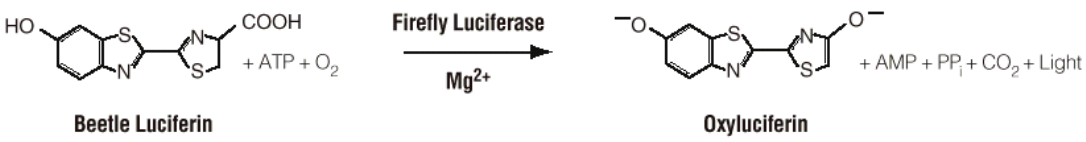
JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide, Molecular Probes) is a cationic dye which exhibits potential-dependent accumulation in mitochondria. A shift in fluorescence emission from red (~590nm) to green (~525nm) represents mitochondrial membrane depolarisation, a characteristic of apoptosis.

Cells were prepared as previously described and seeded in black 96-well plates (Corning). On the day of assay growth medium was removed from each well, replaced with growth medium containing 5µM JC-1 and incubated with cells for one hour at 37°C, 5% CO_2. Cells were washed three times with 100µL Hanks Buffered Saline Solution (Sigma Aldrich) before fluorescent emission was examined using a FlexStation II^{384} (Molecular Devices, Wokingham, UK).

2.10 LUCIFERASE SINGLE REPORTER ASSAY

Firefly luciferase is an enzyme which catalyses mon-oxygenation of luciferin in the presence of Mg^{2+}, ATP and molecular oxygen to generate both oxyluciferin and luminescence, Figure 2.6.

Figure 2.6 Catalysis of luciferin to oxyluciferin (taken from Promega Technical Manual No. 051)



Cells were transfected with the pGL3-Basic plasmid, which contains the coding region for firefly luciferase (see Section 2.3.2). 24 hours after transfection, cells were lysed at room temperature using 100µL of Lysis Buffer (Promega). Lysates were transferred to a 1.5mL microcentrifuge tube and cell debris pelleted by centrifugation. Steady-Glo Assay Reagent was prepared by mixing lyophilised assay substrate with Steady-Glo luciferase assay buffer (Promega) and equal volumes of this reagent mixed with cell lysates in a black solid 96-well plate (Corning). Following a five-minute incubation at room temperature, luminescence was analysed using a MicroLumi XS Microplate Luminometer (Harta, Guildford, UK).

2.11 STATISTICAL ANALYSIS

Parametric data (following a normal distribution) were analysed by Students unpaired t-test (comparison of two means) or one-way analysis of variance (ANOVA, comparison of more than two means). All statistical analysis was performed using Prism statistical software package (GraphPad Software, San Diego, USA), with $p<0.05$ considered to be significant.

Chapter 3:

Characterisation of PTHrP

Expression and Activity

3.1 INTRODUCTION

The mature PTHrP protein undergoes post-translational processing resulting in several daughter peptides (Orloff, Reddy et al. 1994). These have often been used to manipulate cell growth but conflicting reports have emerged and exogenous PTHrP has been shown to be both pro- and anti-mitogenic.

For example Massfelder *et al* added a variety of PTHrP peptides to vascular smooth muscle cells and observed a dose-dependent decrease in proliferation with amino-terminal containing peptides. Mid-region and carboxyl-terminal fragments had no effect on proliferation (Massfelder, Dann et al. 1997).

Several studies by Luparello and colleagues have shown that exogenous PTHrP also causes a decrease in breast cancer cell proliferation. For example using (38-94)-amide they slowed MDAMB231 cell proliferation and accelerated cell death. In another study PTHrP (1-34), (67-86) and (107-139) were shown to inhibit 8701-BC proliferation (Luparello, Burtis et al. 1995; Luparello, Romanotto et al. 2001).

Conversely the addition of exogenous PTHrP (1-34) has been shown to stimulate proliferation in a number of cell lines including those derived from prostate (Asadi, Faraj et al. 2001) and pancreatic tumours (Villaneuva-Penacarrillo, Cancelas et al. 1999). However when Cataisson *et al* used exogenous PTHrP (1-34) and [^{3}H]-thymidine incorporation to examine proliferation in a breast cancer cell line their initial studies failed to demonstrate any effect on breast cancer cell proliferation. The authors attributed this to high levels of endogenous PTHrP so subsequently used a PTHrP (1-34) neutralising antibody to demonstrate a dose-dependent decrease in

[^{3}H]-thymidine incorporation. The authors also examined the role of the PTHrP receptor PTH1R, and through the use of an antagonist were again able to demonstrate a dose-dependent decrease in proliferation. These results led the authors to suggest that the autocrine effects of endogenous PTHrP are mediated via PTH1R. Miyaji *et al* also used a monoclonal antibody directed against the (1-34) region of PTHrP to induce a dose-dependent increase in apoptosis in chondrosarcoma cells (Cataisson, Lieberherr et al. 2000; Miyaji, Nakase et al. 2003).

It has been suggested that PTHrP is involved in the propensity of primary tumour cells to metastasise to one particular organ. For instance, bone is the primary location for breast cancer metastases (Powell, Southby et al. 1991), whereas GI tumours predominantly metastasise to the liver (Fidler 1990; Belluco, Mammano et al. 2005). As yet, the mechanism by which PTHrP promotes this specificity is unknown however it has been suggested that it is linked to PTHrP's ability to alter cell adhesion (Ye, Seitz et al. 2001).

A large portion of the research conducted on PTHrP has related to breast cancer; it was therefore decided to give more emphasis to the role of PTHrP in GI cancer. However as part of this project a breast cancer cell line stably transfected to over-express PTHrP was obtained. By comparing this cell line with the corresponding wild-type cells it was believed that this would provide a positive control for changes in PTHrP expression and activity. Using this positive control cell line in conjunction with several GI cell lines it was also hoped that it would be possible to investigate potential tissue-specific differences in cell adhesion.

As described above, there is a degree of inconsistency in the results obtained using exogenous PTHrP peptides. It may be possible to attribute this disparity to the different cell lines used or perhaps to variations in experimental approach. It was therefore necessary to screen a panel of tumour-derived cell lines, characterise their activity and explore ways in which it would be possible to manipulate PTHrP.

3.2 AIMS

- To characterise PTHrP gene and protein expression in a panel of tumour-derived cell lines.
- To further examine the preliminary finding that there is a link between PTHrP expression and cell adhesion and to consider possible mechanisms of action.
- To explore the effects of exogenous PTHrP and neutralising antibodies on proliferation and adhesion.

3.3 METHODOLOGY

3.3.1 RT-PCR Analysis of PTHrP Gene Expression

As described in Section 2.7, total RNA was extracted from approximately $1x10^6$ sub-confluent cells using RNA-Bee and cDNA generated by reverse transcription. PTHrP expression was assessed using RT-PCR in conjunction with primers designed to detect all forms of PTHrP. Gene expression was deemed not detectable if the Ct of the test gene was >35 when the Ct of HPRT was <25. All RT-PCR assays were

carried out in triplicate and results shown are expressed relative to the cell line MDAMB231 and are average values from three replicate assays.

3.3.2 Analysis of PTHrP and PTH1R Protein Expression

PTHrP and PTH1R protein expression was detected by western blotting (Section 2.8.1) and immunocytochemistry (Section 2.8.2). For the former method, cells were lysed, the sample concentration determined using a NanoDrop ND-1000 spectrophotometer and approximately 20µg of each sample loaded into individual wells of the gel. Samples were then separated by electrophoresis before transfer to either a nitrocellulose (PTHrP) or PVDF (PTH1R) membrane. Using the appropriate monoclonal primary and biotin conjugated secondary antibody, PTHrP and PTH1R expression were detected via chromogenic DAB visualisation.

Cells analysed by immunocytochemistry were first seeded into 8-well chamber slides, fixed and stained for PTHrP and PTH1R using specific polyclonal and monoclonal antibodies, respectively. Using a fluorescent labelled secondary antibody (AlexaFlour 488 conjugated goat anti-rabbit or AlexaFlour 594 conjugated rabbit anti-mouse) it was possible to directly visualise PTHrP and PTH1R expression and co-localisation.

PTHrP protein expression was also examined by flow cytometry. The cells were first fixed and the cell membrane permeabilised in order to allow staining of intracellular PTHrP. Cells were then incubated with an anti-PTHrP monoclonal antibody followed by a FITC conjugated rabbit anti-mouse secondary antibody. Fluorescence was measured using a CoulterAltra flow cytometer and results

analysed using WinMDI software (Section 2.8.3). The results shown are the average values from three separate assays.

3.3.3 ECM Adhesion Assay

As outlined in Section 2.6, clear 96-well tissue culture plates were coated with 5μg/mL of either collagen type I, fibronectin or laminin. [During optimisation studies adhesion to increasing concentrations of ECM protein (0.1-100μg/mL) was examined and 5μg/mL shown to be optimal. Incubation time was also investigated, with adhesion found to be maximal after one hour (data not shown).]

Cells were harvested, added to pre-coated 96-well plates and incubated for one hour at 37°C, 5% CO_2. Non-adherent cells were removed and cell-ECM adhesion assessed using MTT uptake. To examine the effect of exogenous peptides or neutralising antibodies on adhesion, cells were seeded in a 24-well plate and after 24 hours growth medium removed and replaced with serum free medium containing either 100nM of the relevant PTHrP peptide or 1μg/mL of neutralising antibody. Either a scrambled PTHrP peptide or IgG1-κ murine ascites was used as the relevant control. Following a further 48 hour incubation, cells were harvested and seeded into 96-well plates coated with the appropriate ECM. The concentration of PTHrP peptide used was based on previous in-house studies whereas the antibody concentration was chosen following preliminary dose-response studies (data not shown).

MTT assays were carried out in quintuplicate and results shown are the average values from three replicate assays.

3.3.4 Effect of Peptide Stimulation or Neutralising Antibodies on Proliferation

Proliferation assays were carried out to assess the effect of either PTHrP antibodies or exogenous PTHrP peptides on cell proliferation. Cells were seeded into opaque 96-well tissue culture plates and the following day, growth medium replaced with serum free medium containing 1μCi/mL [^{3}H]-thymidine along with either 100nM PTHrP peptide or 1μg/mL neutralising antibody; again scrambled PTHrP peptide or IgG1-κ murine ascites were used as controls. Radioactivity was evaluated on successive days, with an increase in radioactivity proportional to an increase in cell number (Section 2.5.1 and 2.5.2). Proliferation assays were carried out in quintuplicate and the results shown are the average values from three replicate assays.

3.3.5 Analysis of Mitochondrial Membrane Permeability

Using the cationic dye JC-1, it was possible to examine the effect of PTHrP antibody incubation on mitochondrial membrane depolarisation, an early stage of apoptosis. Cells were seeded into black 96-well tissue culture plates and the following day the growth medium was replaced with serum free medium containing either 1μg/mL of PTHrP neutralising antibody or IgG1-κ murine ascites. After a further 48 hours, this medium was replaced with JC-1 containing growth medium and incubated for one hour. Excess JC-1 was washed off with Hanks Buffered Saline Solution, and the fluorescent emission measured, with an increase in the ratio of green (~525nm) to

red (~590nm) indicating an increase in apoptosis (Section 2.9). Each JC-1 assay was performed in triplicate and the results shown are the average values from three separate assays.

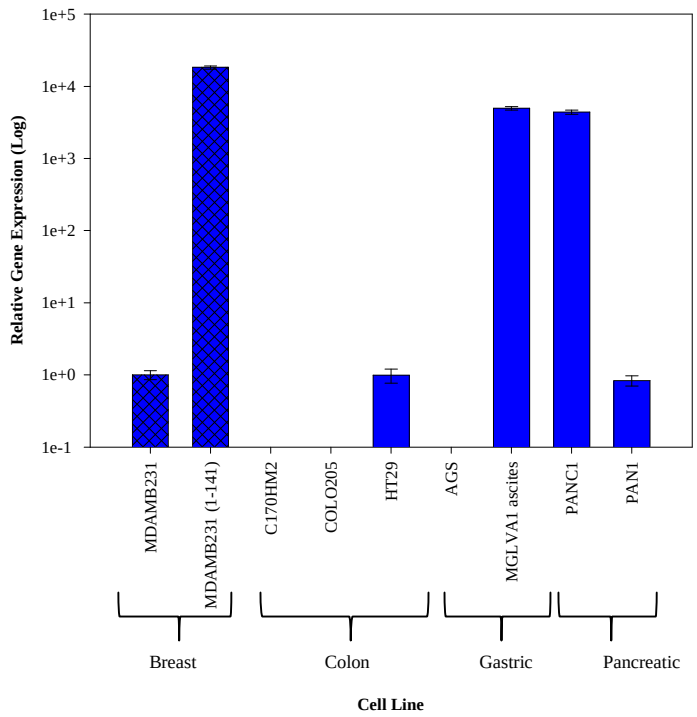
3.4 RESULTS

3.4.1 PTHrP Expression in a Panel of Tumour-Derived Cell Lines

3.4.1.1 Analysis of PTHrP gene expression

PTHrP gene expression was determined in a range of tumour-derived human cell lines. These cell lines were established from colorectal (C170HM2, COLO205, HT29), pancreatic (PAN1, PANC1) and gastric (AGS, MGLVA1 *ascites*) tissue. Included in the panel were the breast cancer cell lines MDAMB231, and MDAMB231 cells stably transfected with PTHrP cDNA encoding for amino acids (1-141) were used as the positive control cell line (hereafter referred to as MDAMB231 (1-141)).

Figure 3.1 PTHrP gene expression in a range of tumour-derived cell lines.



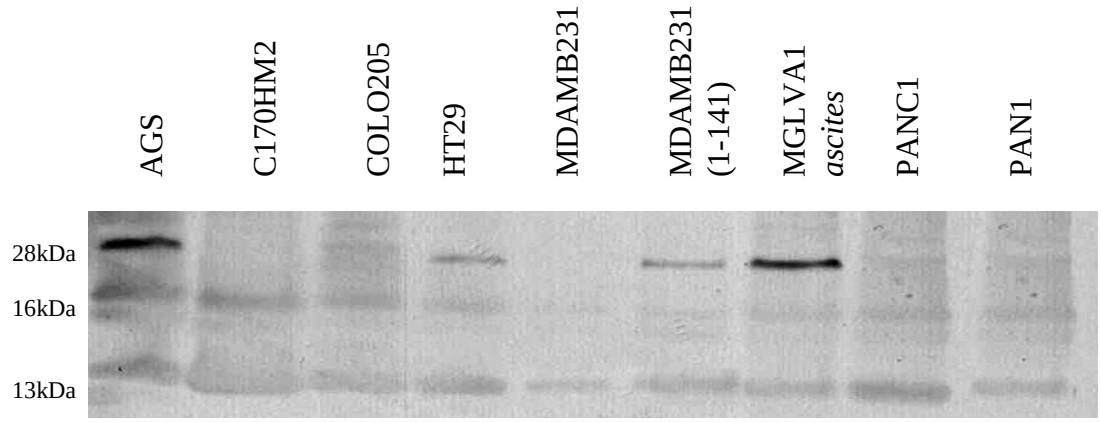
It can be seen in Figure 3.1 that of the nine cells analysed, PTHrP gene expression was detected in six. It was identified in one of the three colonic cell lines, one of the two gastric cell lines, both of the pancreatic cell lines and both of the breast cell lines examined. Where PTHrP was detected, the expression levels varied, with the highest expression identified in the gastric cell line MGLVA1 *ascites*, the pancreatic cell line PANC1 and the PTHrP over-expressing cell line MDAMB231 (1-141).

3.4.1.2 Analysis of PTHrP protein expression

Translational regulation and post-translational processing may result in differences between gene and protein levels. Therefore, PTHrP protein expression was examined by western blotting using the PTHrP monoclonal antibody raised against the amino-terminal of PTHrP. Three forms of PTHrP were detected; a representative

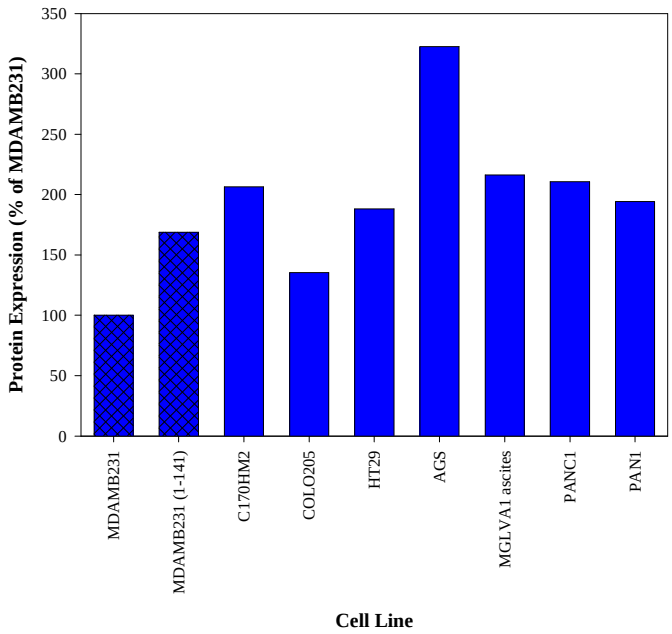
blot can be seen in Figure 3.2. The weight of the 13 and 16kDa bands correspond to amino-terminal proteins with different levels of post-translational processing. The identity of the bands visible at approximately 28kDa in the AGS, HT29, MDAMB231 (1-141) and MGLVA1 *ascites* cell lines are unknown, however the size suggests that this band represents the entire PTHrP protein including the pre-pro region.

Figure 3.2 PTHrP protein expression in a panel of tumour-derived cell lines using a specific PTHrP antibody and visualised using a biotin conjugated secondary antibody in conjunction with DAB.



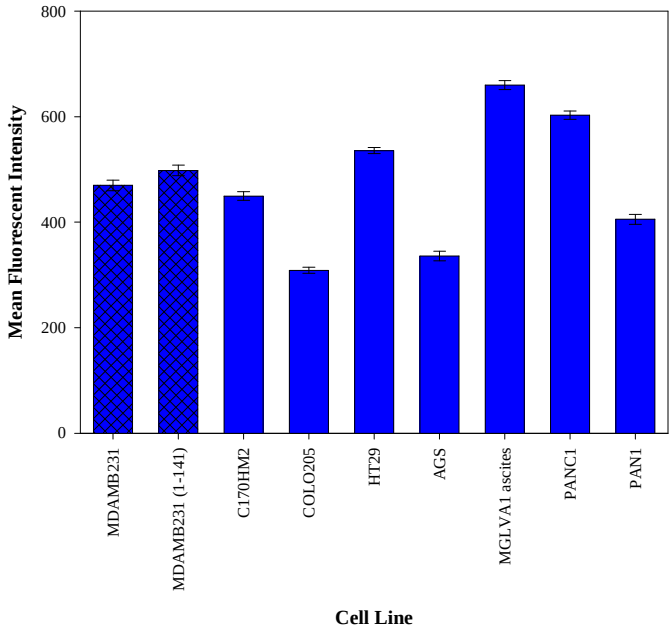
All three bands were quantified by densitometry analysis, added together and total PTHrP expressed relative to the MDAMB231 cell line (Figure 3.3). Although the results partly correlate with the gene expression data shown in Figure 3.1 there are some obvious differences; specifically it appears that the highest amount of protein expression was found in the AGS cell line whereas gene expression was not detected at all in these cells.

Figure 3.3 Densitometry analysis of PTHrP western blot



Flow cytometry analysis was subsequently used to corroborate the results obtained by western blotting (Figure 3.4).

Figure 3.4 PTHrP protein expression a panel of tumour-derived cell lines, measured by flow cytometry in conjunction with a specific PTHrP antibody.



PTHrP expression was again found in all nine cell lines, which was consistent with the results obtained with western blotting; however, the relative protein expression varied with the highest amount of protein expression found in MGLVA1 *ascites* rather than AGS. This high expression of PTHrP in MGLVA1 *ascites* and PANC1 cells, and low expression in AGS and COLO205 cells, is more consistent with the gene expression results shown in Figure 3.1. As a result, the level of PTHrP expression determined by flow cytometry was used to compare PTHrP expression with the results obtained in subsequent assays. The hierarchy of increasing PTHrP expression in the GI cell lines was therefore COLO205, AGS, PAN1, C170HM2, PANC1 and MGLVA1 *ascites*.

3.4.2 PTHrP Receptor Expression in a Panel of Tumour-derived Cell Lines

As the effects of PTHrP (1-34) are mediated via PTH1R, it was important to first check the presence of this receptor prior to peptide stimulation.

Western blotting with the PTH1R antibody displayed expression of an 80kDa receptor monomer and a 160kDa receptor dimer. As with PTHrP protein expression all nine cell lines exhibited presence of PTH1R (Figure 3.5). Densitometry analysis was carried out on PTH1R monomer expression (Table 3.1), which revealed the highest expression in the cell lines previously shown to express high amounts of PTHrP i.e. MDAMB231(1-141), MGLVA1 *ascites* and PANC1 which may be indicative of the autocrine/paracrine mechanism by which PTHrP functions as high protein expression may lead to increased receptor expression or *vice versa*.

Figure 3.5 PTH1R expression in a panel of tumour-derived cell lines, using a specific PTH1R antibody and visualised via a biotin-labelled secondary antibody in conjunction with DAB

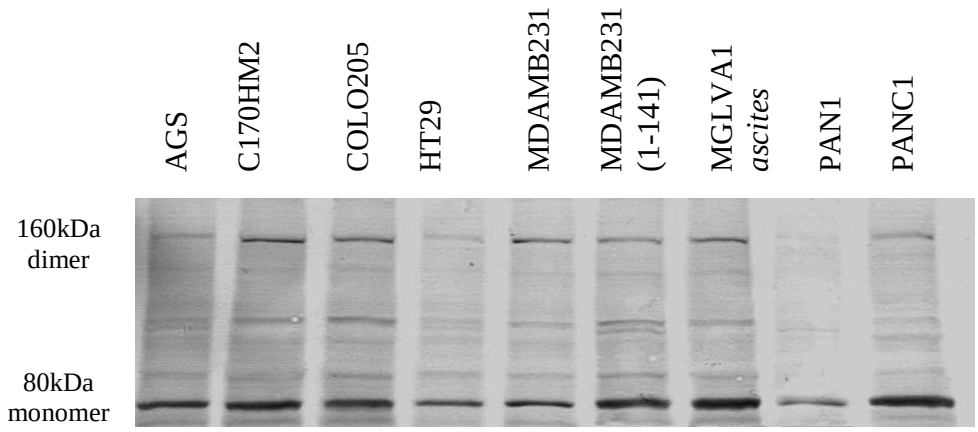


Table 3.1 Densitometry analysis of PTH1R monomer expression

Cell Line	Densitometry analysis (% of MDAMB231)
AGS	105
C170HM2	129
COLO205	114
HT29	87
MDAMB231	100
MDAMB231(1-141)	164
MGLVA1 a*scites*	188
PAN	78
PANC1	203

3.4.3 PTHrP and PTH1R localisation

Four cell lines were used to investigate co-localisation of PTHrP and its receptor. These cell lines represented low (COLO205 and MDAMB231) and high (MGLVA1 *ascites* and MDAMB231(1-141)) PTHrP and PTH1R expression.

It has previously been reported that PTHrP translocates to the nucleus so the cellular localisation of both PTHrP and PTH1R were examined using fluorescent

visualisation with dual staining in order to examine co-localisation of the protein and the receptor.

Figure 3.6 shows the presence of PTH1R in all four cell lines. PTHrP protein expression was visible in three of the four cell lines. Although PTHrP was detected in COLO205 cells by immunocytochemistry it was too low to visualise by fluorescent microscopy suggesting that this technique is less sensitive than the other two methods; however, these results do correspond with the gene expression data. By overlaying the different fluorescent signals it was possible to identify co-localisation of the protein and the receptor, indicated by a yellow signal. Distinct co-localisation was observed, which although primarily located within the cytoplasm was visible within the nucleus.

Figure 3.6 Immunocytochemical detection of PTH1R (green) and PTHrP (red) in four tumour-derived cell lines. Expression was detected using a polyclonal PTHrP antibody, monoclonal PTH1R antibody and fluorescently labelled secondary antibodies. The nucleus was visualised using DAPI.

a) COLO205 **b) MGLVA1 *ascites***

c) MDAMB231 **d) MDAMB231 (1-141)**

3.4.4 Adhesion of a Panel of Tumour-derived Cell Lines to the Extracellular Matrix

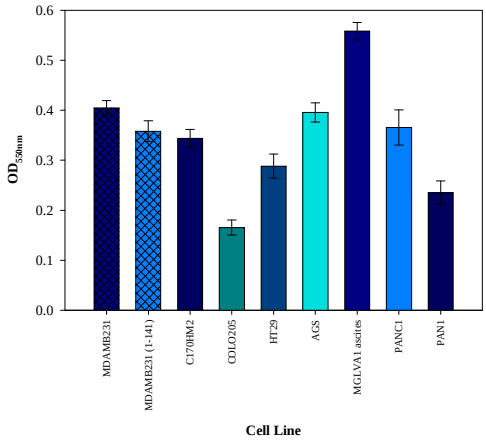
Having characterised PTHrP gene and protein expression, adhesion to collagen type I, fibronectin and laminin was explored in an effort to highlight a link between PTHrP expression and adhesion.

By comparing PTHrP expression in these cell lines to their adhesion to the three ECM components, Figure 3.7 demonstrates an apparent association between PTHrP expression and adhesion to both collagen type I and fibronectin. However, although it can be seen that overall an increase in PTHrP expression is associated with an increase in adhesion, AGS cell adhesion to collagen type I is higher than would be expected; similarly C170HM2 adhesion to fibronectin is lower than expected.

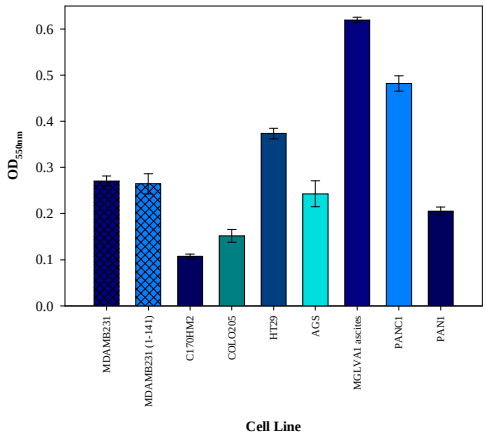
GI cell adhesion to laminin was generally poor and there was no obvious link with PTHrP expression. The breast cancer cell lines did show relatively strong adherence to laminin. However, while there was an increase in adherence of the over-expressing cell line MDAMB231 (1-141) compared with the wild-type, this increase was relatively small and not statistically significant. Similarly there was no apparent increase in adhesion of MDAMB231 (1-141) cells to collagen type I or fibronectin, compared with the wild-type.

Figure 3.7 Adhesion of a panel of tumour-derived cell lines to the extracellular matrix components collagen type I, fibronectin and laminin.

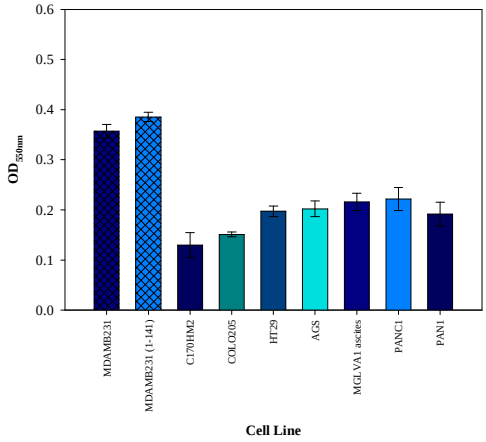
a) Collagen type I



b) Fibronectin



c) Laminin



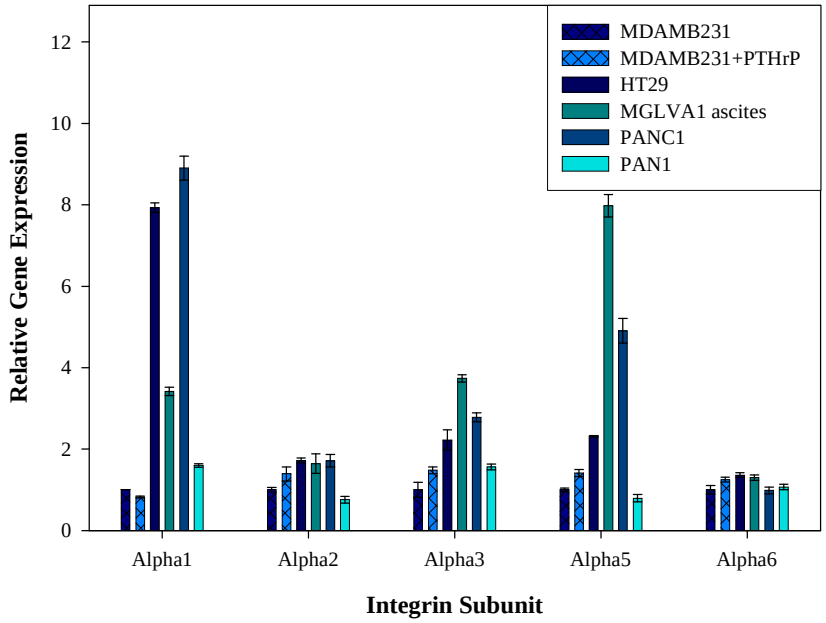
3.4.5 Analysis of Integrin Expression in a Panel of Tumour-derived Cell Lines

The cell adhesion molecules integrins are important mediators of cell-ECM interaction. Gene and protein expression was therefore examined in an effort to elucidate the apparent link between PTHrP expression and cell adhesion. Only the six cell lines which had demonstrated positive PTHrP expression at both the gene and protein level were analysed.

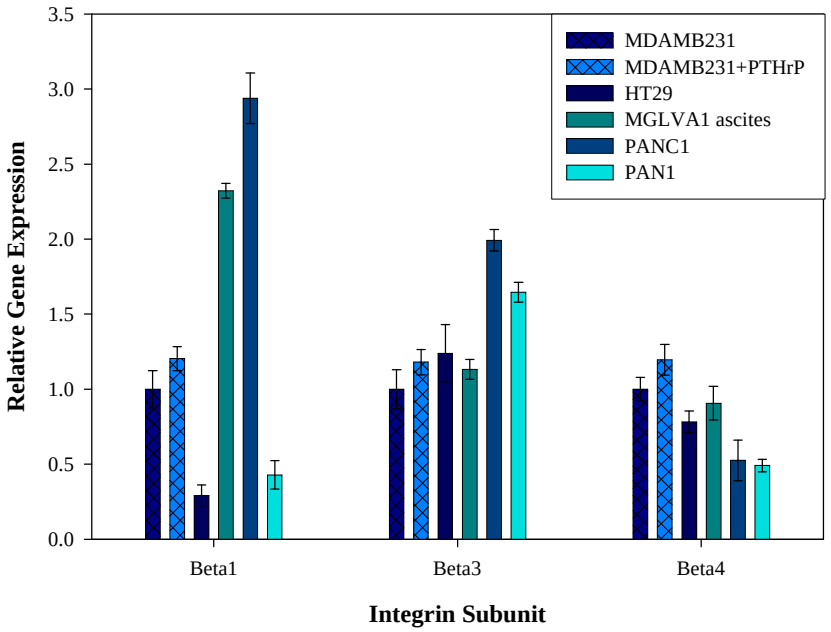
When integrin gene expression is considered in the GI cell lines there also appears to be an association between PTHrP and expression of the integrin subunits α_1, α_3, α_5 and β_1 (Figure 3.8). When expression off the integrin subunits are similarly compared with PTHrP expression in the two breast cancer cell lines, only a small increase in integrin expression is detected and this increase was not statistically significant. However this is consistent with the results obtained when adhesion of these cell lines was analysed.

Figure 3.8 Integrin gene expression in panel of tumour-derived cell lines.

a) α Subunit



b) β Subunit

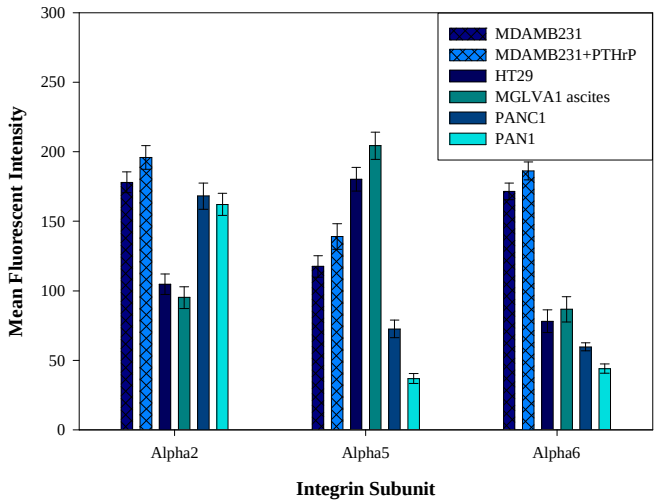


The integrin subunits α_2, α_5, α_6 β_1, β_3 and β_4 were chosen for analysis of protein expression. As with the integrin gene expression data, an increase in PTHrP

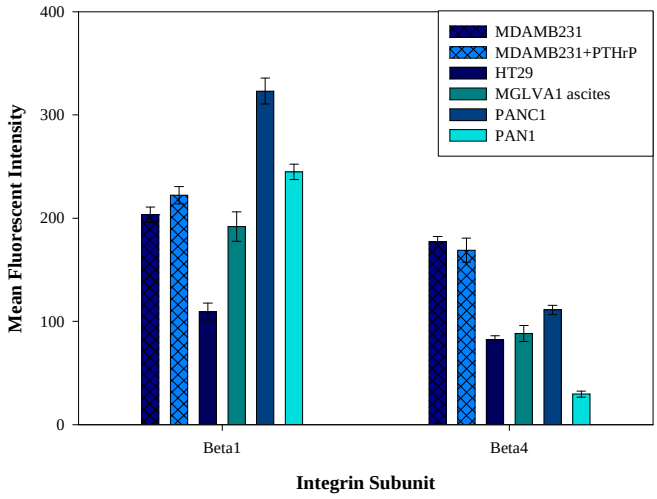
expression is associated with an increase expression of α_5 and β_1 expression in the GI cell lines and α_5, α_6 β_1 and β_4 expression in the breast cancer cell lines (Figure 3.9). As before the increase in integrin expression between the two breast cancer cell lines was relatively small and not statistically significant. β_3 expression was not detected in any of the cell lines examined, consequently the data is not shown.

Figure 3.9 Integrin protein expression in a panel of tumour-derived cell lines.

a) α Subunit



b) β Subunit



3.4.6 Effect of PTHrP Manipulation on Cell Proliferation

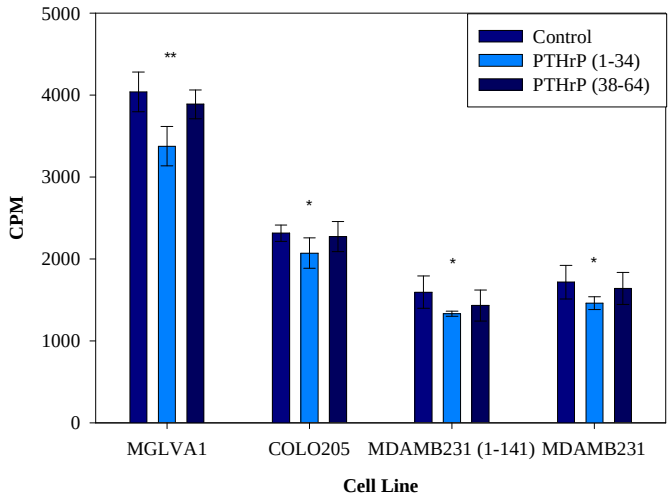
As mentioned earlier, various exogenous peptides have been used in an attempt to elucidate the role of PTHrP in cell growth, with varying results. It was therefore decided to explore the effects of neutralising antibodies and exogenous PTHrP daughter peptides on cell proliferation.

3.4.6.1 Effect of neutralising antibodies on cell proliferation

Two antibodies were used to neutralise the effects of PTHrP. One was used to neutralise PTHrP (1-34) and the other PTHrP (38-64). Having carried out a titration of both antibodies, the maximal effect was observed using 1µg/mL after 72 hours (data not shown).

The PTHrP (1-34) antibody produced a modest, although significant decrease in proliferation compared with control; however, the PTHrP (38-64) antibody had no effect (Figure 3.10, One-way ANOVA * $p<0.05$, ** $p<0.001$). The greatest decreases were observed in the cell lines expressing the most PTHrP: there was a ~16.5% decrease in proliferation in MGLVA1 *ascites* and MDAMB231 (1-141) cells treated with the PTHrP (1-34) antibody compared with control. The MDAMB231 and COLO205 cell lines demonstrated a decrease in proliferation of only 14.9% and 10.4%, respectively.

Figure 3.10 Effect of PTHrP antibodies and proliferation, after 72 hours.

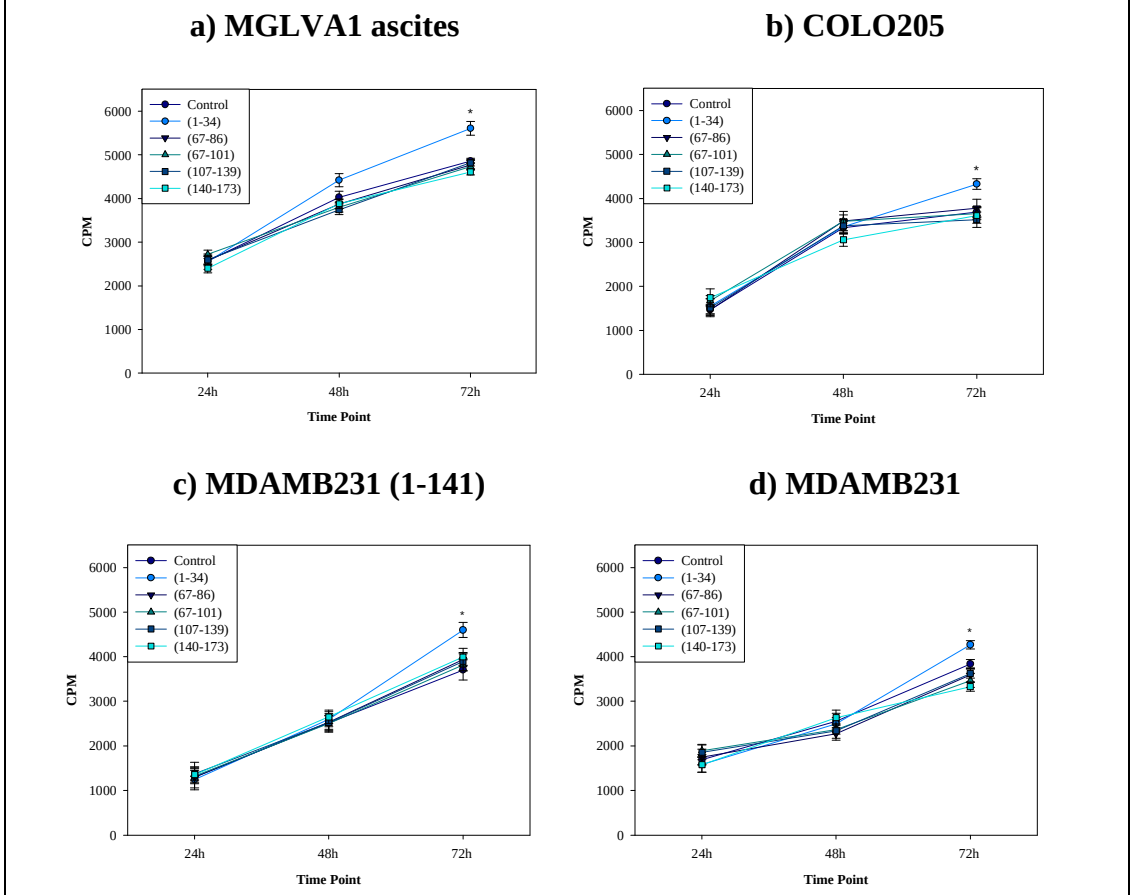


3.4.6.2 Effect of exogenous PTHrP peptides on cell proliferation

Various PTHrP daughter peptides were used to examine the effect on cell proliferation over 72 hours. These included PTHrP (1-34), (67-86), (67-101) (107-139) and (140-173), which correspond to amino-terminal, mid-region without NLS, mid-region including NLS, osteostatin and carboxyl-terminal, respectively.

After 72 hours there was a significant increase in proliferation in cells treated with PTHrP (1-34) (One-way ANOVA, $p<0.05$). None of the other peptides were able to significantly affect proliferation in any of the four cell lines examined (Figure 3.11).

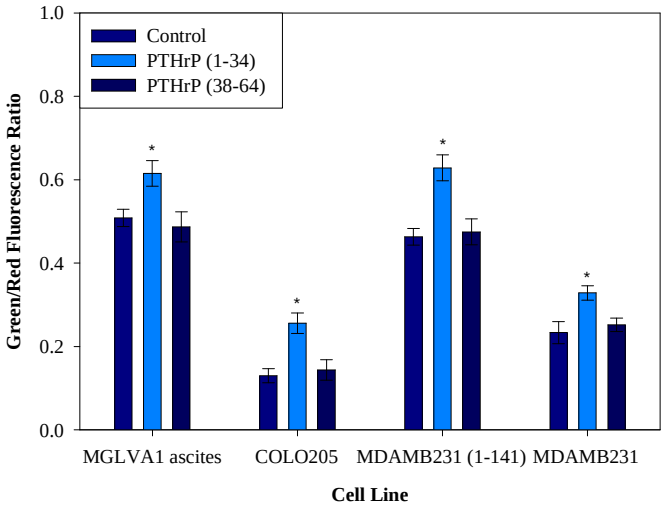
Figure 3.11 Effect of peptide addition on proliferation.



3.4.7 Assay of Anti-apoptotic Effects of PTHrP

The effect on apoptosis was subsequently examined using 1µg/mL antibody in conjunction with the JC-1 assay. Again only the PTHrP (1-34) neutralising antibody produced a response, with a significant increase in apoptosis compared with control (Figure 3.12, One-way ANOVA, * $p<0.0001$).

Figure 3.12 Effect of antibody addition on apoptosis, measured using JC-1 assay.



3.4.8 Effect of PTHrP Manipulation on Cell Adhesion

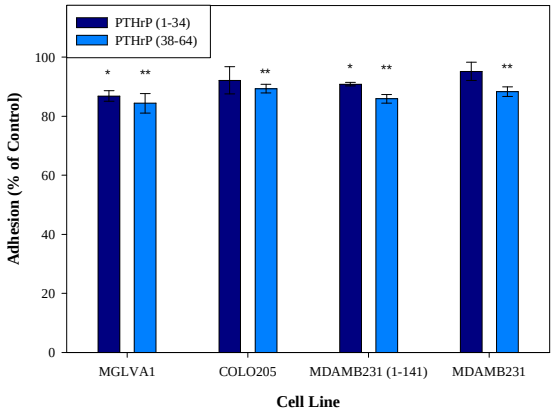
Both the exogenous peptides and neutralising antibodies were assayed for their impact on cell adhesion.

3.4.8.1 PTHrP neutralisation and adhesion

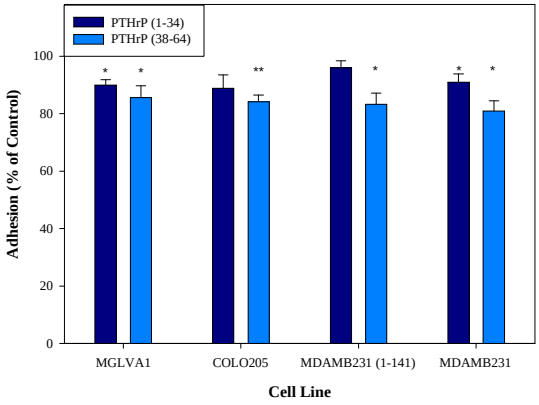
The PTHrP (1-34) neutralising antibody had previously been used to induce a significant decrease in cell proliferation and was subsequently used to further examine the role of PTHrP in cell adhesion. Despite not causing a significant reduction in proliferation, the effect of the (38-64) antibody was also examined in order to determine if mid-region PTHrP played a role in adhesion.

Figure 3.13 Effect of pre-incubation with PTHrP antibodies on ECM adhesion as determined by MTT uptake. Results shown are expressed as percentage of control.

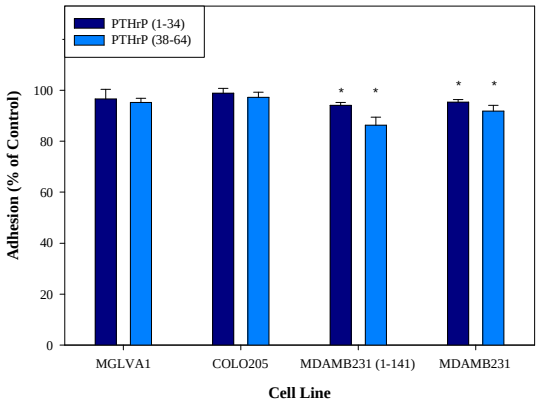
a) Collagen Type I



b) Fibronectin



c) Laminin



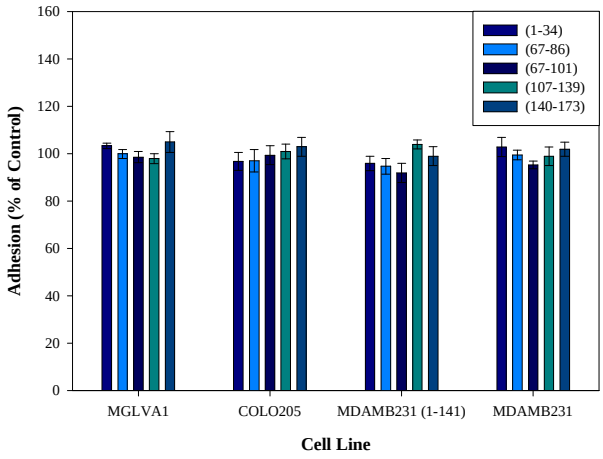
Again the antibodies produced modest results however Figure 3.13 demonstrates that the PTHrP (38-64) antibody caused a significant reduction in adhesion to collagen type I and fibronectin in all four cell lines examined. It was also able to reduce adhesion of both breast cancer cell lines to laminin. The (1-34) antibody also caused a significant reduction in breast cancer cell adhesion to laminin as well as a significant reduction in MGLVA1 *ascites* adhesion to collagen type I and fibronectin. This antibody also reduced MDAMB231 (1-141) adhesion to collagen type I and MDAMB231 adhesion to laminin (One-way ANOVA, * $p<0.05$, ** $p<0.005$).

3.4.8.2 Effect of exogenous peptides on cell adhesion

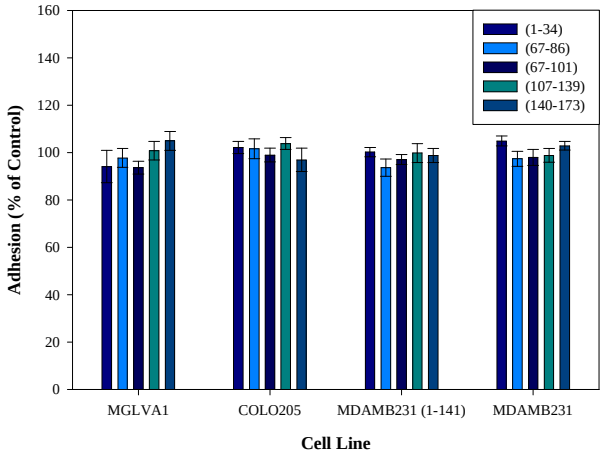
Analysis of adhesion following antibody addition indicated that amino- and mid-terminal PTHrP was involved. When the effect of exogenous peptide addition on adhesion was examined, there was no effect on adhesion to collagen type I or fibronectin (Figure 3.14); effects on adhesion to laminin were not investigated.

Figure 3.14 Effect of peptide addition on ECM adhesion as determined by MTT uptake. Results shown are expressed as percentage of control.

a) Collagen Type I



b) Fibronectin



3.5 DISCUSSION

Western blotting and flow cytometry were both used to examine protein expression. Both assays demonstrated that although low gene expression does not always allow detection of PTHrP, the protein is expressed by each of the cell lines used in this study. Each of the methods has its drawbacks for instance as western blotting

separates proteins by size, proteins with different levels of processing are detected thereby making it more specific but also making it more difficult to fully quantify the total level of PTHrP protein expression. Flow cytometry analysis on the other hand appears to demonstrate lower sensitivity compared with western blotting, although it did correlate well with the gene expression and cell adhesion data. Immunocytochemistry also allowed the examination of PTHrP protein expression and although it was not possible to quantify the data, the results did correspond with the gene expression data. Following densitometry analysis it can be seen that the results obtained by western blotting also correlated with the gene expression results, with the exception of AGS and C170HM2, which showed relatively high protein expression. Although protein levels were quantified so that equal amounts of protein would be loaded, a loading control such as β-actin was not used. Therefore there may have been differences in the amount of protein actually loaded onto the gel so repeating the western blot protein characterisation using a loading control would help to clarify the protein expression in each of these cell lines.

By comparing PTHrP expression (as determined by flow cytometry) and adhesion to the ECM proteins collagen type I, fibronectin and laminin there was an indication of a relationship between PTHrP and adhesion with an increase in PTHrP expression parallel to an increase in cell adhesion to collagen type I and fibronectin. Adhesion of the GI cell lines to laminin was generally poor and there was no indication of a relationship between PTHrP expression and cell adhesion to this ECM protein.

Although it was possible to examine PTHrP expression in a panel of cell lines and use this to investigate its relationship with cell adhesion, it was also considered

important that a control cell line be included whereby changes in PTHrP expression could be directly correlated to downstream effects, independent of possible differences between cell lines. The breast cancer cell line MDAMB231 (1-141), which contained the coding region for PTHrP (1-141), was used. Despite observing a 10,000 fold increase in PTHrP gene expression, when adhesion of this cell line to the ECM proteins was examined and compared with the corresponding wild-type cell line although there was a small increase in adhesion to laminin when PTHrP was over-expressed this was not statistically significant and there was no increase in adhesion to either collagen type I or fibronectin. Taken in isolation, this result suggested that PTHrP did not play a role in ECM adhesion. However, taking into consideration the results obtained from the panel of GI cell lines, which did show a positive correlation between PTHrP expression and cell-ECM adhesion, reservations concerning this cell line's role as a positive control arose.

A number of studies had suggested a role for PTHrP in cell growth, with exogenous PTHrP daughter peptides shown to be mitogenic (Villaneuva-Penacarrillo, Cancelas et al. 1999; Asadi, Faraj et al. 2001), anti-mitogenic (Luparello, Romanotto et al. 2001) and anti-apoptotic (Aarts, Davidson et al. 2001; Tovar Sepulveda, Shen et al. 2002). However, due to some apparent inconsistency in the published date it was unknown how the panel of tumour cell lines used here would respond to peptide stimulation.

Only PTHrP (1-34) was able to induce any change in cell proliferation, inducing a significant increase in proliferation in all four cell lines examined. When antibodies were used to neutralise endogenous PTHrP there was a significant decrease in

proliferation in all four cell lines with the antibody directed towards PTHrP (1-34) whereas the antibody directed towards PTHrP (38-64) had no effect on cell proliferation. These results suggest that PTHrP (1-34) stimulates proliferation and it can be hypothesised that this is mediated via PTH1R.

When cell-ECM adhesion was examined, none of the exogenous peptides induced any significant effect; however, neutralisation of endogenous PTHrP resulted in a decrease in cell adhesion. So proliferation appears to be mediated by both exogenous and endogenous PTHrP suggesting an autocrine and/or paracrine mechanism. Adhesion, on the other hand, appears to be mediated solely by an intracrine mechanism.

The three ECM proteins used here are ligands for different integrin heterodimers and the combination of the subunits determines the specificity. When integrin expression was examined there were differences in endogenous integrin subunit expression between the GI cell lines. Furthermore, several of these subunits showed a connection to PTHrP expression with a correlation between integrin and PTHrP expression. For example, higher PTHrP expression was associated with higher expression of the integrins α_1, α_3, α_5 and β_1. The increase in α_5 and β_1 gene expression was also seen at the protein level. There were no changes in expression of α_6 and β_4 and as $\alpha_6\beta_4$ is a receptor for laminin this relates to the adhesion data. Similarly as $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ are receptors for collagen type I, fibronectin or both these results also tie in with the adhesion data for the GI cell lines.

Although there was slightly higher expression of α_3, α_5, β_1 and β_4 in the PTHrP over-expressing breast cancer cell line compared with the wild-type cell line this was not statistically significant, therefore correlating to the lack of difference in adhesion observed with these two cell lines.

The aim of these initial studies was to characterise PTHrP expression and to then explore a role for this protein in cancer cell adhesion. The results described here suggest a role for PTHrP in proliferation, apoptosis and adhesion however they also show that the exogenous peptides do not mimic the effects of endogenous PTHrP and that endogenous PTHrP is related to adhesion, the main focus of this study. Furthermore, owing to the limited effects of the neutralising antibodies as well as the costs involved, it was felt important to find an alternative means of regulating PTHrP expression.

Although, using the panel of tumour-derived cell lines, it was possible to identify a trend whereby PTHrP was linked to adhesion and integrin expression it was difficult to come to any definitive conclusions as the endogenous levels of integrin expression varied between tissues. For example, the two pancreatic cell lines exhibited lower levels of integrin expression than the other GI cell lines. In addition, although it was possible to compare endogenous integrin and PTHrP expression it was unknown how manipulating PTHrP expression would affect integrin expression and unclear as to the manner in which the two were linked. In order to further examine the mechanism of PTHrP's action on integrin expression an alternative means of regulating PTHrP expression was sought.

Chapter 4:

Manipulating the Actions of

PTHrP

4.1 INTRODUCTION

The results described in Chapter 3 suggested a link between PTHrP expression and adhesion, which was mediated via the cell adhesion molecules, integrins. However although there was evidence of an association, the results were by no means definitive.

The use of two different antibodies directed towards the amino-terminal and mid-region of PTHrP provided a successful means of reducing the PTHrP-mediated effects on cell adhesion and proliferation. However, not only was this a costly way of blocking the actions of PTHrP but the resultant effects were relatively minor. Alternative methods were therefore considered in an effort to find a more effective means of negating the effects of PTHrP.

Following the sequencing of the human genome, silencing gene expression has been an effective and valuable tool in exploring the functions of previously uncharacterised genes. Anti-sense oligodeoxynucleotides (As-ODNs) have frequently been used in this capacity following their initial description in 1977 (Paterson, Roberts et al. 1977) and have to date been the method of choice for regulating gene expression both *in vivo* and *in vitro*.

Anti-sense technology has previously been successfully used to inhibit PTHrP. For example, furin cDNA, a proprotein convertase within the secretory pathway, has been used in an anti-sense orientation to inhibit processing of PTHrP in rat leydig tumour cells (Liu, Amizuka et al. 1995). Tsukazaki *et al* used anti-sense oligonucleotides targeted against PTHrP directly and were able to decrease

translation of PTHrP mRNA which resulted in inhibition of DNA synthesis (Tsukazaki, Ohtsuru et al. 1996).

In spite of their wide-spread use, As-ODNs have a number of weaknesses which have made their use problematic. For example, selecting a sequence within the mRNA target is not an easy task as mRNA associated proteins and the tertiary structure of mRNA often physically block binding of the As-ODN. A hit and miss approach is commonly used which results in a low success rate. Delivery is another problem area, as a result of degradation by endonucleases and exonucleases, and passive diffusion across the cell membrane cannot occur as As-ODNs are polyanionic. This necessitates As-ODN stabilisation through modifications of phosphodiester bridges and sugar moieties. It has been demonstrated that As-ODN uptake is time, energy, temperature and concentration dependent with Gewirtz and colleagues showing that at lower concentrations a "receptor-like mechanism" is responsible for uptake whereas other studies have shown that at higher concentrations this switches to endocytosis (Beltinger, Saragovi et al. 1995). Once an effective As-ODN has been found and successfully introduced into a cell, it is often necessary to use high concentrations in order to induce a prolonged inhibition of mRNA translation. So it can be seen that the use of As-ODNs can be both time-consuming and expensive (Gewirtz, Sokol et al. 1998).

The discovery of RNA interference however has made gene silencing a much simpler task and has revolutionised this area of research as it has quickly emerged as a powerful tool for suppressing gene expression. As with most discoveries, RNA interference was first stumbled upon by a group of researchers who were attempting

to deepen the purple colour of petunias by over-expressing a key enzyme in plant coloration (Napoli, Lemieux et al. 1990). What they found instead was that the introduction of the cDNA for this enzyme actually caused 42% of the flowers to turn either totally white or to contain white areas. Further study showed that the introduction of this gene reduced expression of the homologous gene, a phenomenon the authors described as "co-suppression" but one that they were unable to explain.

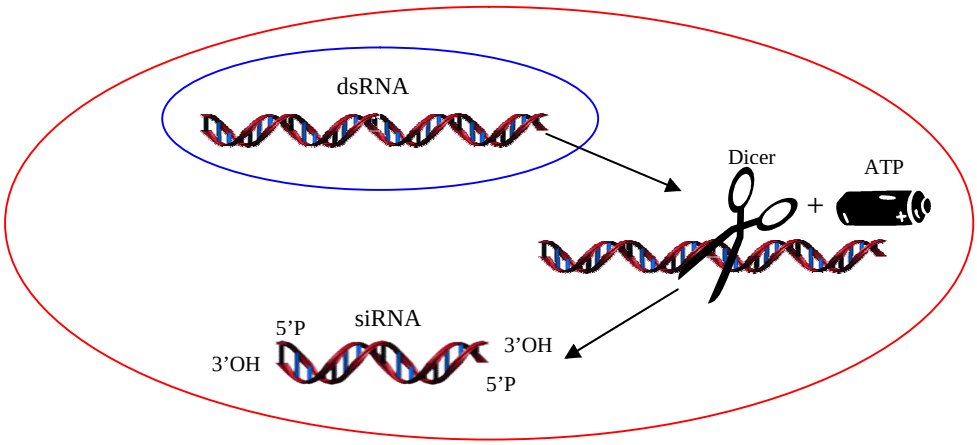
Napoli *et al*'s idea of "co-suppression" was further developed when the researchers Guo and Kemphues used anti-sense RNA to inhibit *par-1* gene expression in *Caenorhabditis elegans* (*C.elegans*). They used the corresponding sense strand as a control and to their surprise the sense strand was able to induce a similar reduction in gene expression compared with the anti-sense strand (Guo and Kemphues 1995).

Fire *et al* expanded on this finding by demonstrating that the use of double-stranded RNA (dsRNA) to silence gene expression in *C.elegans* was more effective than using either the sense or anti-sense strand alone and were the first to label this event RNA interference (RNAi). Their research also showed that only a few molecules of dsRNA were required to induce a significant decrease in gene expression, that dsRNA targeted against introns or promoter sequences were ineffective at reducing gene expression, and that the gene silencing was also inherited by the offspring of the targeted *C.elegans* (Fire, Xu et al. 1998). For their efforts Dr Andrew Fire and Dr Craig Mello were awarded the 2006 Nobel Prize for medicine.

4.1.1 RNAi Mechanism of Action

Double stranded RNA are generated in the nucleus and following transport to the cytoplasm are cleaved by Dicer (a highly conserved RNase-III-like dsRNA-specific ribonuclease) in an ATP dependent, processive manner to produce dsRNA 21-23 nucleotides (nt) in length. These dsRNA are known as small interfering RNA (siRNA) and their processing, which is known as the initiator step, is illustrated in Figure 4.1 (Timmons 2002; Lieberman, Song et al. 2003).

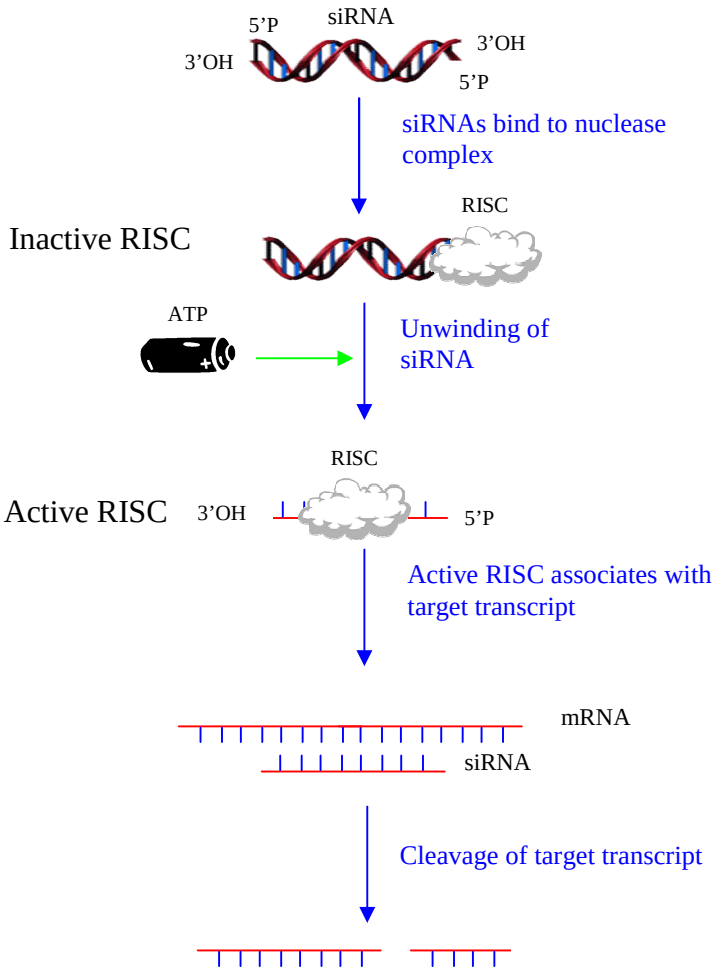
Figure 4.1 –Initiator step of siRNA production, whereby dsRNA moves from the nucleus to the cytoplasm where it is cleaved by Dicer to produce small interfering RNA



The siRNA then undergo further processing to produce an siRNA with a free 5'-monophosphate and a 3'–hydroxyl group, which are necessary for incorporation into the RNA-induced silencing complex (RISC) (Lipardi, Wei et al. 2001; Lieberman, Song et al. 2003; Lieberman, Song et al. 2003). The presence of the two-base 3' overhang has also been shown to increase the potency of the siRNA (Elbashir, Harborth et al. 2001).

The introduction of chemically synthesised 21nt siRNA results in the bypass of this initiator step and the siRNA are able to enter directly into RISC. When the siRNA goes on to induce gene silencing this is known as the effector step, which can be seen in Figure 4.2.

Figure 4.2 –Effector step of siRNA action



During this step, the duplex siRNA is unwound by an ATP-dependent helicase. One strand is then incorporated into RISC, a multi-protein complex that includes an effector nuclease. The siRNA strand then binds to the homologous target RNA and RISC is then able to guide RNA cleavage. It is thought that RISC includes an

endonuclease which is responsible for cleaving the RNA in the middle of the homologous sequence, ~12 nucleotides from the 3' terminus of the siRNA (Lieberman, Song et al. 2003).

Unlike As-ODNs, it is relatively easy to design an effective siRNA and various guidelines have been developed based on previously verified effective siRNA. Criteria taken into account when designing an siRNA include G/C content, thermodynamic stability, an avoidance of internal repeats and the preferential use of specific bases at known positions on the sense strand (i.e. positions 3, 10, 13 and 19). However, it has also been shown that some siRNA which did not satisfy these criteria were still functional siRNA; as such there are no absolute rules for siRNA design. Nevertheless by using their general rules Reynolds *et al* were able to increase their success rate at selecting an siRNA able to induce more than 80% knockdown by 3.5 fold (Reynolds, Leake et al. 2004).

Not only are siRNAs now commonly used within the laboratory to induce gene silencing but there are a number of siRNA currently undergoing clinical trials. For example Cand5 has been developed to silence VEGF expression in macular degeneration (Tolention, Brucker et al. 2004). Patient enrolment for a Phase II trial was completed in early 2006 and so far the trial has produced positive results. Initial results have shown it to be safe and there is evidence of dose-dependent efficacy (www.acuitypharma.com).

Although siRNA have been shown to be an effective tool in silencing gene expression, and are a valuable tool for examining the role of novel genes, they do

have a number of disadvantages which require consideration. For example, a recent study demonstrated that not all siRNA which have been shown to silence gene expression also go on to silence protein expression. Whilst developing a method whereby they could examine changes in protein expression in novel genes silenced by siRNA, Wu *et al* examined the effects of a siRNA on both gene and protein expression. Their initial study examined four genes and used three different siRNA for each gene. When this was expanded to 30 genes they showed that in three cases even though there was a decrease in mRNA levels there was no decrease in protein expression. As such, it is ill-advised to make conclusions regarding the effects of gene silencing without knowing that the corresponding protein has also been down-regulated (Wu, Hodges et al. 2004).

Another factor which must be taken into account when using siRNA is the observation that the introduction of dsRNA into mammalian cells can result in the induction of an antiviral pathway. Interferons are cytokines which are involved in the first line of defence during viral infection and as dsRNA are commonly associated with viruses their introduction to the cell appears to inadvertently trigger these cytokines. The innate interferon response appears to be mediated by the protein kinase PKR, which phosphorylates several substrates including the translation initiation factor eIF2α. Once phosphorylated, eIF2α initiates a cascade leading to inhibition of protein synthesis (Stark, Kerr et al. 1998). It has been shown that dsRNA >30nt in length are also able to bind and activate 2',5'-oligoadenylate synthetase which in turn activates RNaseL, a general ribonuclease that degrades cellular RNAs and shuts down protein translation (Wang and Carmichael 2004).

PKR activation is dependent on the length and concentration of dsRNA with the minimum length required for PKR activation being 33bp. Elbashir *et al* demonstrated that the use of 21bp siRNA circumvents induction of the interferon pathway (Elbashir, Harborth et al. 2001).

As with ODNs, delivery of siRNA can also be problematic, however due to the potency of siRNAs it is not necessary to deliver as much in order to be effective. Indeed, when Bertrand *et al* compared As-ODNs and an siRNA, both directed towards the coding region of GFP, they were able to show that the siRNA not only induced a greater decrease in GFP expression but that this decrease lasted longer than that induced by the ODN (Bertrand, Pottier et al. 2002).

Due to the numerous advantages of RNAi it was decided to use this technique as a means to down-regulate PTHrP during the course of this study.

The breast cancer cell line MDAMB231 transfected with the cDNA encoding for PTHrP (1-141) was intended to be used as a positive control for PTHrP over-expression. However, there was no increase in adhesion compared with the wild-type cell line and as a result it was also decided to either obtain or construct PTHrP expression vectors in order to produce some alternative over-expressing cell lines.

Xiaoli Shen and Miriam Falzon have constructed several PTHrP over-expressing cell lines and by deleting the nuclear localisation sequence have shown some noteworthy intracrine effects. Upon application, Professor Miriam Falzon kindly agreed to donate her PTHrP-containing vectors.

It was hoped that both the siRNA and expression vectors would provide useful tools for investigating the actions of PTHrP.

4.2 AIMS

- To develop an effective mechanism of silencing PTHrP expression.
- To use the PTHrP-containing expression vectors to stably transfect a GI and breast mammalian cell line.
- To explore the effects of PTHrP on cell growth.

4.3 METHODS

4.3.1 siRNA Construction and Transfection

As described in Section 2.2.1, Ambion's siRNA Design Centre was used to identify over 30 possible targets within the PTHrP nucleotide gene sequence. These siRNAs were checked for homology with other genes using the web based tool BLAST and three siRNAs subsequently chosen. The sense and anti-sense oligonucleotides of these targets were purchased and the siRNA constructed using the *Silencer* siRNA Construction kit. Fluorescent siRNA were constructed using an NTP mix containing fluorescein-12-UTP.

An siRNA specific to the house-keeping gene GAPDH was used as a control whilst assessing the activities of the three PTHrP siRNA. Once the most effective of the

three had been identified, a scrambled version was constructed for use as an alternative positive control.

Cells were harvested and seeded into 24-well plates one day prior to transfection. The following day the cells were transiently transfected with the appropriate preformed siRNA/siPORT *Amine* complex (see Section 2.4.1). During optimisation studies different volumes of siPORT *Amine* were tested (1, 2 and 4µL); 2µL was found to induce the greatest decrease in gene expression and this volume was subsequently used during all future siRNA transfections.

4.3.2 Analysis of Gene Expression

In order to analyse gene expression, RNA was extracted using RNA-Bee and reverse transcribed to produce cDNA. This cDNA was then used to analyse gene expression of PTHrP, OAS and STAT by RT-PCR, as described in Section 2.7. All RT-PCR assays were performed in triplicate. Unless otherwise stated results shown are expressed relative to the scrambled PTHrP siRNA or vector control, where appropriate, and are the average values from three replicate assays.

4.3.3 Analysis of PTHrP Protein Expression

Following siRNA transfection, PTHrP protein expression was measured using both flow cytometry and western blotting. For flow cytometry, cells were harvested, fixed and stained with a primary antibody raised against PTHrP (1-34), followed by a PE-conjugated secondary antibody. Fluorescence was measured using a CoulterAltra flow cytometer and results analysed using WinMDI software (Section 2.8.3).

Samples for western blotting were prepared by cell lysis, the proteins separated by electrophoresis before transfer to a nitrocellulose membrane and the detection of PTHrP protein using a primary PTHrP (1-34) antibody in conjunction with a biotin-labelled secondary antibody. β-actin was used as a loading control to ensure that equal amounts of protein were loaded per lane. Protein expression was visualised using ECL in conjunction with a Chemi Genius^2 BioImaging System (Section 2.8.1).

4.3.4 Transfection Protocol

The pcDNA3.1 vectors containing a geneticin selection marker as well as cDNA encoding for PTHrP were transfected into MDAMB231 and HT29 cells using Lipofectamine 2000. Three vectors were used; one contained cDNA encoding for amino acids (-5 to +139), one contained PTHrP encoding for cDNA with the nuclear localisation sequence (residues 88-91 and 102-106) deleted, and an empty vector was used as a negative control (Section 2.4.2).

Transfected cells were selected using 500µg/mL geneticin and resistant cells selected in 96-well tissue plates and expanded into T75cm^2 tissue culture flasks. Over-expression of PTHrP was assessed using RT-PCR and two clones from each cell line chosen for further analysis.

4.3.5 PTHrP Silencing and Proliferation

The effect of PTHrP silencing was examined using [^{3}H]-thymidine incorporation as a measure of cell proliferation, as described in Section 2.5.3. Transfected cells were re-plated into an opaque 96-well plate and incubated with 1µCi/mL [^{3}H]-thymidine.

Radioactivity was counted daily, with an increase being proportional to an increase in DNA synthesis and therefore related to an assessment of proliferation.

4.3.6 Clonagenicity Assays

Cells transfected with either the pcDNA3.1 vector containing PTHrP cDNA (with and without the NLS) or the empty vector control were seeded into opaque 96-well plates and incubated with 1µCi/mL [^{3}H]-thymidine. Radioactivity was counted daily, where an increase in radioactivity was indicative of an increase in proliferation (Section 2.5.4). Assays were carried out in quintuplicate and all results shown are the average values of three replicate assays.

4.3.7 Mitochondrial Membrane Potential Assay

As described in Section 2.9, apoptosis was examined using the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria. Cells transfected with either the PTHrP siRNA or scrambled control were seeded into black 96-well plates and grown for 72 hours before a one hour incubation with 5µM JC-1 dye diluted in 10% FBS growth medium. Excess JC-1 was then removed and fluorescent emission examined with a shift in fluorescence emission from red (~590nm) to green (~525nm) was indicative of a lack of mitochondrial membrane potential and, therefore, apoptosis.

4.4 RESULTS

4.4.1 PTHrP Small Interfering RNA

4.4.1.1 PTHrP gene expression following siRNA transfection

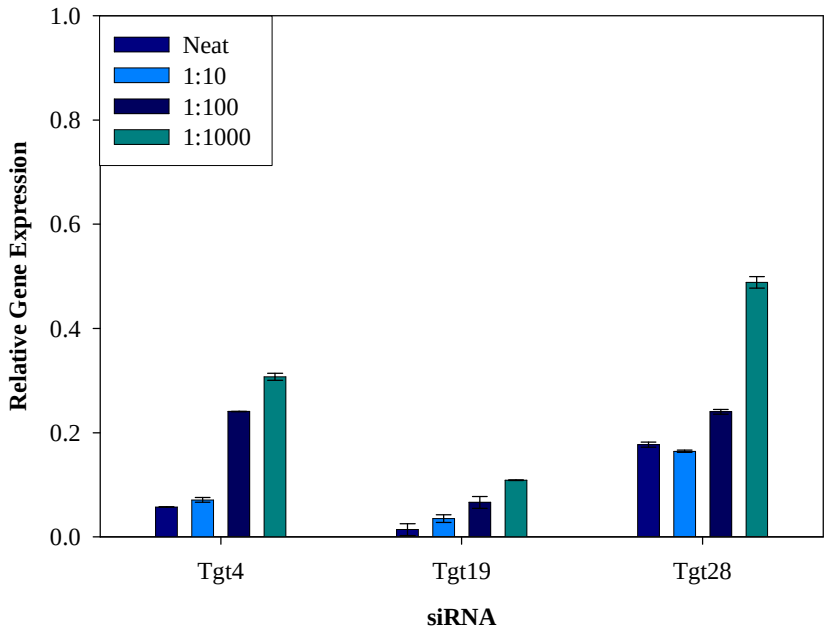
Three siRNAs were chosen for construction and transiently transfected into MGLVA1 *ascites.* This cell line was chosen as it had previously demonstrated a high level of PTHrP expression, both at the gene and protein level. All three siRNAs target sequences within exon VI, which is present within all three PTHrP isoforms.

Each siRNA was serially diluted. In a final transfection volume of 250µL the working concentration of neat siRNA (20µM) was equivalent to 80nM, with successive dilutions equivalent to 8nM, 0.8nM and 0.08nM.

The level of PTHrP gene expression was measured 24 hours following transfection and Figure 4.3 demonstrates that all three siRNAs were able to significantly reduce PTHrP gene expression (Students t-test, $p<0.0001$). The siRNA designated Target 19 proved to be the most effective at down regulating PTHrP, followed by Target 4 and Target 28. Even at its lowest concentration, Target 19 produced a greater than 80% decrease in PTHrP gene expression and was consequently chosen for further study.

For the remaining experiments the chosen siRNA was used at a working concentration of 8nM as it was felt that this would bring about the most effective and economical gene silencing. Upon selection of the chosen siRNA, a scrambled version was constructed to replace the control GAPDH siRNA.

Figure 4.3 PTHrP gene expression following siRNA transfection in MGLVA1 *ascites*. Results shown are expressed relative to GAPDH siRNA and are the mean of two separate transfection assays.



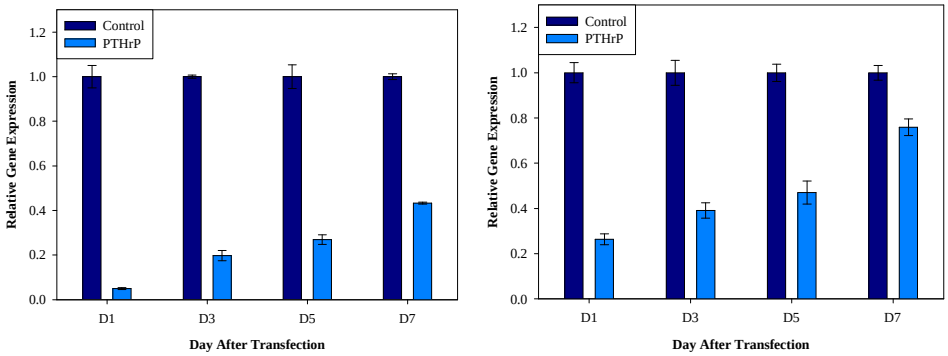
4.4.1.2 The temporal effect of PTHrP silencing

Twenty-four hours after the PTHrP siRNA had been transiently transfected into MGLVA1 *ascites* there was a >90% decrease in PTHrP gene expression. The temporal effect of siRNA transfection was then examined and the study expanded to include a further three cell lines. The wild-type and over-expressing MDAMB231 cell lines used previously were included; however, as PTHrP gene expression was undetectable in COLO205 cells, this cell line was replaced by another GI cell line, HT29, which had previously demonstrated PTHrP gene expression similar to that of MDAMB231.

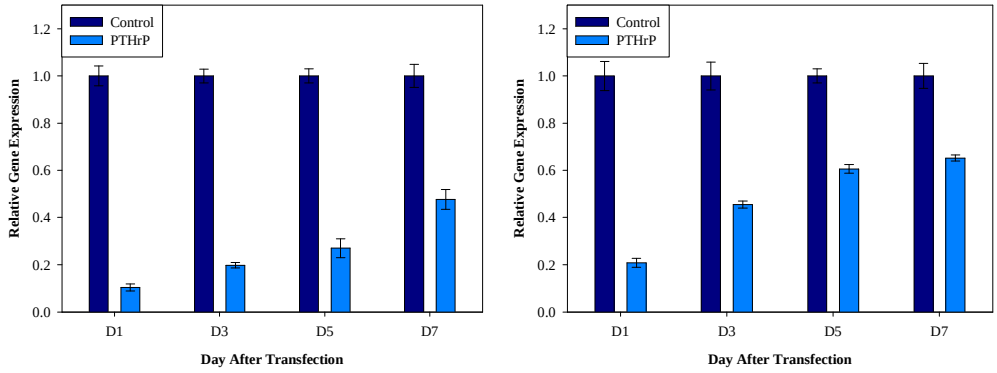
Gene down-regulation was maximal at day one in all four cell lines when there was a significant reduction in PTHrP gene expression of 80-90% compared with control. On subsequent days PTHrP gene expression slowly increased which is consistent with the transient nature of siRNA (Figure 4.4; Students t-test, $p<0.0001$).

Figure 4.4 Duration of PTHrP gene silencing in four tumour-derived cell lines.

a) MGLVA1 *ascites* **b)HT29**



c) MDAMB231 (1-141) **d) MDAMB231**



4.4.1.3 Protein down-regulation

Protein expression following siRNA transfection was examined in order to confirm that gene silencing translated into a decrease in protein expression.

Protein expression was first examined using flow cytometry. Used in conjunction with a fluorescent siRNA this technique allowed the examination of PTHrP protein expression as well as providing an indication of transfection efficiency.

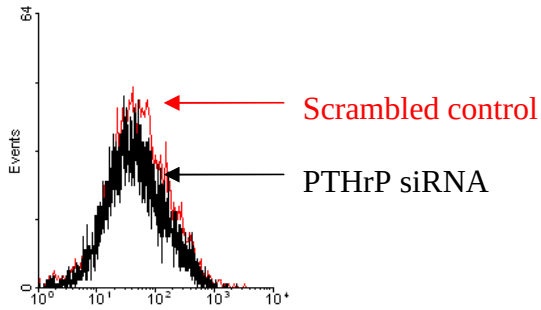
Fluorescent siRNA were constructed (see Section 4.3.1) in order to compare the level of transfection obtained with the control and PTHrP siRNA. MGLVA1 *ascites* were transfected with either fluorescent control or PTHrP siRNA and the fluorescence emitted from the siRNA detected by flow cytometry. The overlay of the histograms obtained from these cells can be seen in Figure 4.5a and there is no apparent difference between the two siRNAs. Furthermore, the mean fluorescent intensities obtained from the two siRNAs were similar. These results indicate that the transfection efficiency of both siRNAs are comparable and therefore any changes in protein expression can be attributed to PTHrP gene silencing rather than differences in siRNA uptake.

When PTHrP protein expression was examined by flow cytometry following siRNA transfection a decrease in protein expression was observed. This decrease is illustrated by the shift in fluorescence between the control and PTHrP siRNA transfected cells (Figure 4.5b). Figure 4.5b also demonstrates that two cell populations were sometimes detected in PTHrP siRNA transfected cells. Population one appears to represent cells in which the siRNA was taken up and PTHrP silenced: population two contains cells where the siRNA was also taken up but was ineffective. These two populations were not always detected as the two peaks often merged into one.

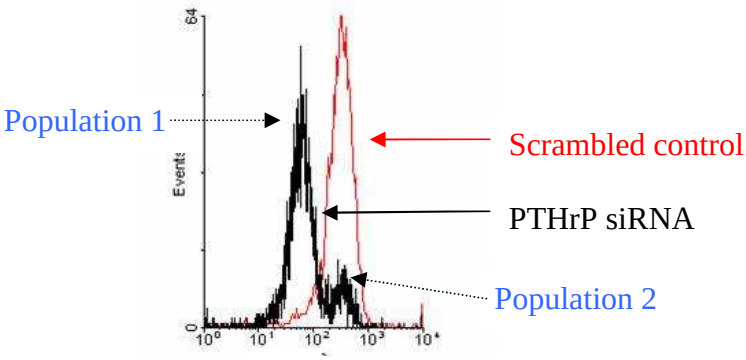
When the temporal effect of siRNA transfection on protein expression was examined, the decrease in expression was maximal five days after transfection (Figure 4.5c). However, despite achieving a decrease in gene expression of more than 90% a reduction in protein expression of only ~30% was detected.

Figure 4.5 Flow cytometry analysis of siRNA transfected cells

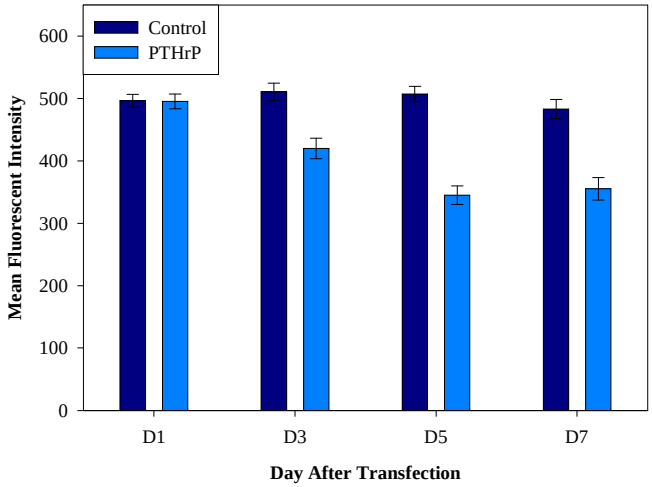
a) Fluorescence emitted from fluorescent control or PTHrP siRNA transfected MGLVA1 *ascites*



b) Fluorescence emitted from rabbit anti-mouse FITC secondary antibody in MGLVA1 *ascites* transfected with either scrambled control or PTHrP siRNA

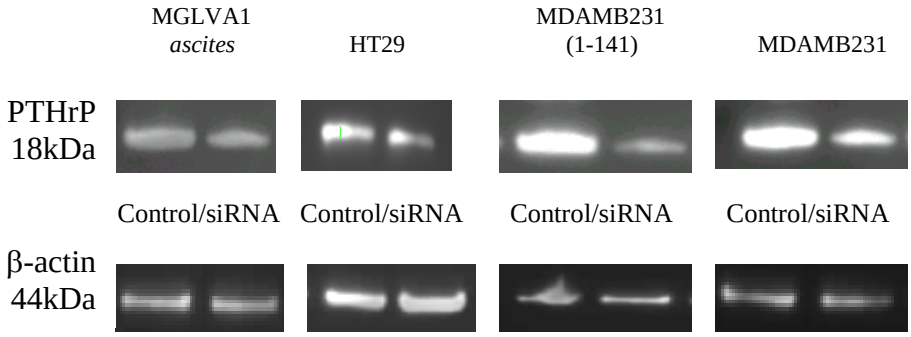


c) Temporal effect of siRNA transfection on protein expression in MGLVA1 *ascites* (mean results from three separate assays)



When protein expression was examined in MGLVA1 *ascites* following siRNA transfection using western blotting, the greatest decrease in protein expression was observed five days after transfection, which correlated with the results obtained by flow cytometry. However, a 45% decrease in PTHrP protein expression was observed whereas only a 32% decrease was observed using flow cytometry. Western blotting was therefore subsequently used to examine protein expression in the other three cell lines. Again the greatest decrease in protein expression occurred five days after transfection in these cell lines (Figure 4.6).

Figure 4.6 PTHrP and β-actin protein expression five days following transfection with either the PTHrP siRNA or the scrambled control.



The results from densitometry analysis are summarised in Table 4.1 and show a decrease in protein expression between 40 and 70% compared with the scrambled control siRNA. β-actin was used as a loading control and Figure 4.6 shows similar protein levels in both control and PTHrP siRNA samples, thereby demonstrating that the differences in protein expression observed can be directly attributed to PTHrP silencing.

Table 4.1 Densitometry analysis of western blots shown in Figure 4.6

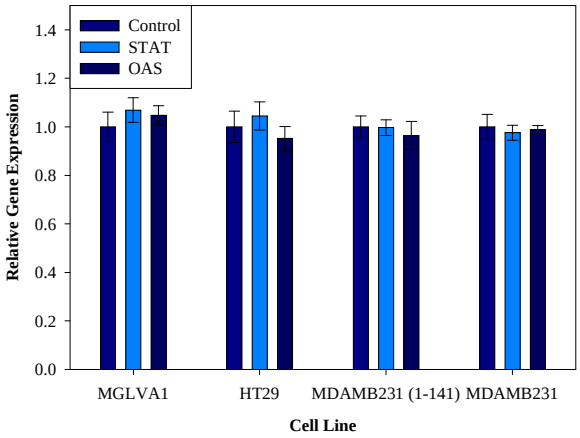
Cell Line	Protein Expression (% of scrambled control)
MGLVA1 *ascites*	55.85
HT29	56.2
MDAMB231 (1-141)	32.17
MDAMB231	59.03

4.4.1.4 Induction of the innate interferon response

Several groups have shown that siRNA are able to induce the innate interferon response and thereby cause the cessation of protein translation. Interferon expression was therefore examined in order to demonstrate that the siRNAs used in this study did not induce such a response.

Using primers specific for the interferon subunits STAT and OAS all four cell lines were examined for interferon expression. There was no apparent increase in expression of either STAT or OAS in any of the four cell lines examined following siRNA transfection which indicates that the innate interferon response was not induced and therefore the decrease in protein expression observed was the result of the specific actions of the PTHrP siRNA (Figure 4.7). Furthermore, as β-actin expression did not change following siRNA transfection (Figure 4.6) this also demonstrates that protein expression wasn't affected.

Figure 4.7 Effect of PTHrP siRNA transfection on interferon induction, determined using primers specific for the interferon genes STAT and OAS. Results shown are expressed relative to scrambled control siRNA and are the mean values from two replicate assays.

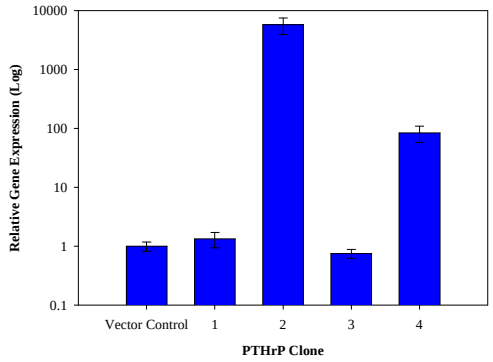


4.4.2 Construction of PTHrP Over-expressing Cell Lines

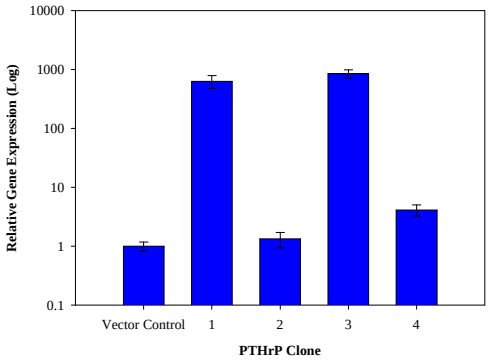
The breast cancer cell line MDAMB231 and the colonic cancer cell line HT29 were transfected with either the empty expression vector or those containing cDNA encoding for PTHrP. Figure 4.8 shows PTHrP expression in several of the selected geneticin resistant clones. Varying levels of PTHrP expression were observed in the transfected cells and two positive clones were selected for each plasmid. The HT29 cells over-expressing full-length PTHrP were designated PTHrP (2) and PTHrP (4). Cells expressing PTHrP but lacking the NLS were designated NLS (1) and NLS (3). Similarly the MDAMB231 cells were designated PTHrP (1), PTHrP (2), NLS (1) and NLS (3).

Figure 4.8 PTHrP expression in cells transfected with pcDNA3.1 vectors containing cDNA encoding for PTHrP (with and without the NLS). Results shown are expressed relative to cells containing the vector only and are the mean values of two separate RT-PCR assays.

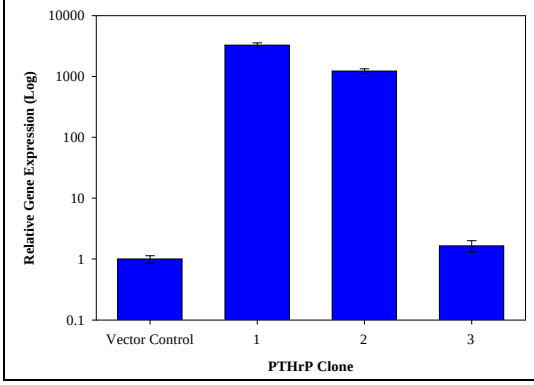
a) HT29+PTHrP



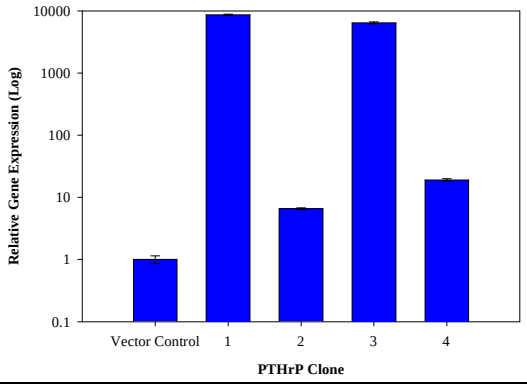
b) HT29+PTHrP NLS Deleted



c) MDAMB231+PTHrP



d) MDAMB231+PTHrP NLS Deleted



4.4.3 PTHrP and Cell Proliferation

4.4.3.1 Effect of PTHrP silencing on proliferation

Using the siRNA targeted against PTHrP, protein expression had successfully been reduced therefore, resulting effects on proliferation were examined.

Figure 4.9 shows that there was a significant reduction in proliferation in each of the four cell lines examined, which was maximal seven days following transfection (Students t-test, * $p<0.05$, ** $p<0.005$). Table 4.2 shows the percentage decrease in proliferation on day seven and demonstrates that MDAMB231 (1-141) was more sensitive to gene silencing than the corresponding wild-type, although this was not a significant difference. Similarly MGLVA1 *ascites* was more sensitive than the other GI cell line HT29. The greater decrease in proliferation thus correlates with the higher endogenous PTHrP expression found in MGLVA1 *ascites* and MDAMB231 (1-141) and is consistent with the greater decrease in gene and protein expression previously demonstrated (See Figures 4.4 and 4.6).

Figure 4.9 Effect of transfection of either PTHrP siRNA or scrambled control on proliferation.

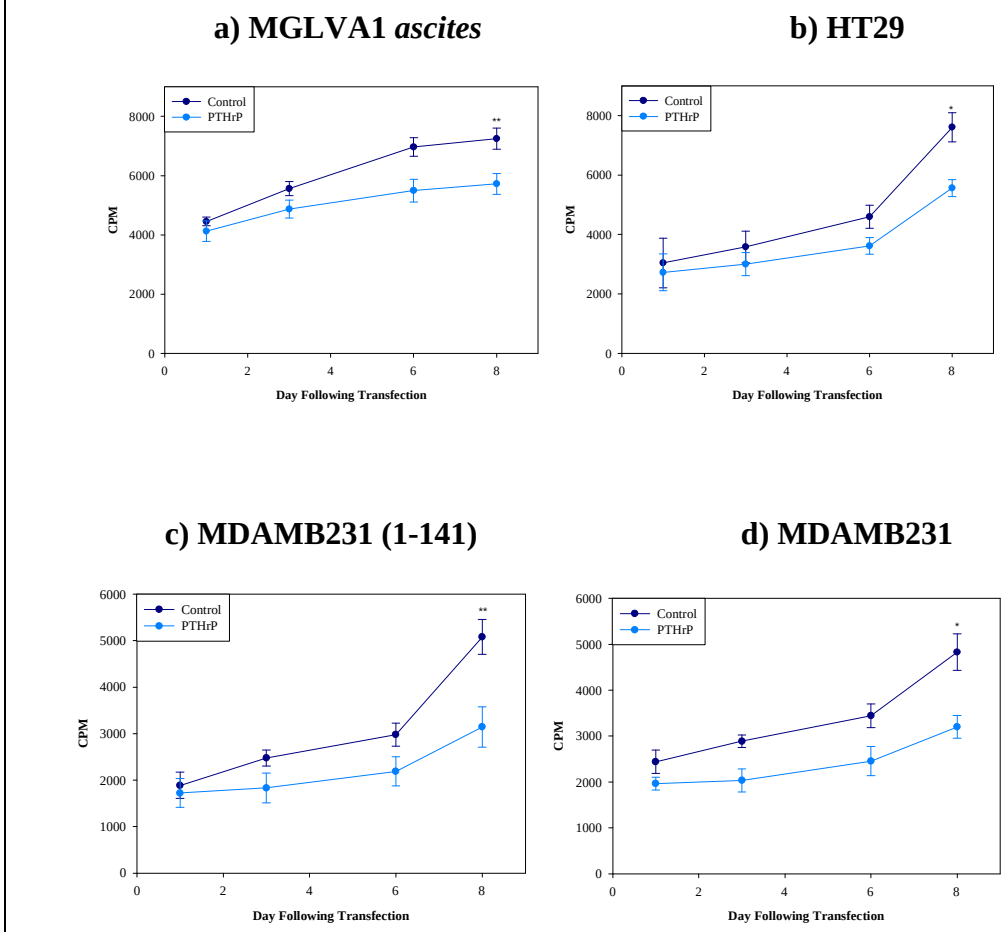


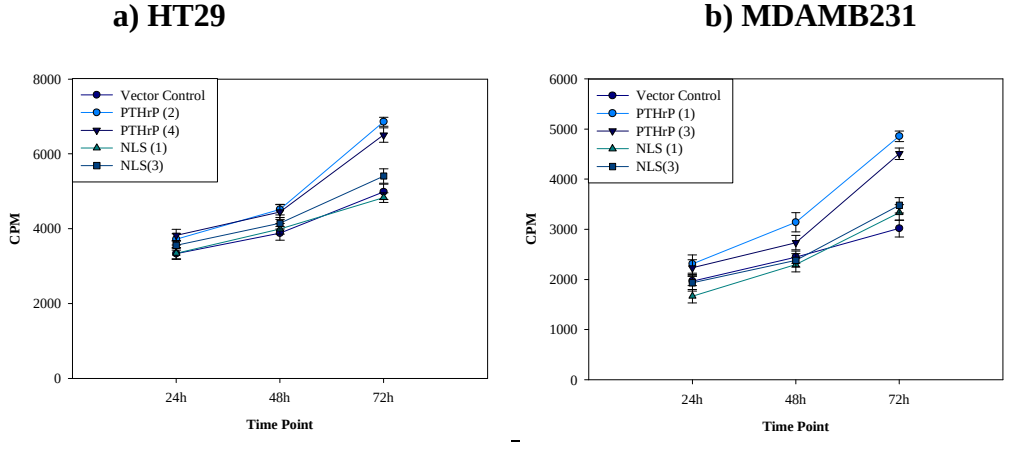
Table 4.2 Percentage inhibition of proliferation

Cell Line	Maximal Inhibition of Proliferation (% of Control)
MGLVA1 *ascites*	29.03
HT29	26.9
MDAMB231 (1-141)	38.09
MDAMB231	33.75

4.4.3.2 Effect of PTHrP over-expression on proliferation

Proliferation was also examined in the PTHrP over-expressing cell lines and compared with cells containing the vector only. Figure 4.10 demonstrates that after 72 hours the HT29 clones PTHrP (2) and (4) exhibited a significant 37% and 30% increase in proliferation, respectively. When the MDAMB231 clones PTHrP (1) and (2) were similarly examined there was an increase in proliferation of 61% and 49%, respectively (One-way ANOVA, $p<0.0001$). Figure 4.10 also shows that despite demonstrating a significant increase in PTHrP expression, the rate of proliferation in all of the NLS mutant clones was not significantly different compared to vector control.

Figure 4.10 Effect of PTHrP over-expression on cell proliferation, measured using [^{3}H]-thymidine.

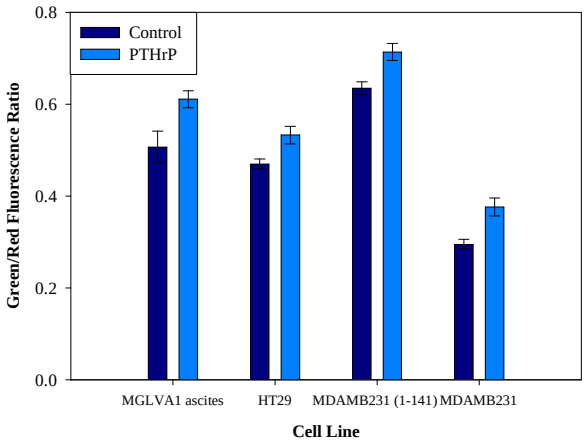


4.4.4 PTHrP Silencing and Apoptosis

Examination of cell proliferation revealed a significant decrease in [^{3}H]-thymidine incorporation in cells transfected with the PTHrP siRNA compared with scrambled control. PTHrP has also been shown to be anti-apoptotic so the effect of PTHrP silencing on apoptosis was investigated.

Apoptosis was examined five days subsequent to siRNA transfection, when the decrease in PTHrP protein expression was known to be maximal. Figure 4.11 shows that the silencing of PTHrP expression resulted in a significant increase in the green/red fluorescent ratio of JC-1, an indicator of mitochondrial membrane potential, in each of the four cell lines examined, indicative of an increase in apoptosis (Students t-test, $p<0.05$). The increase in apoptosis was greater in siRNA-treated MGLVA1 *ascites* compared with HT29 cells with an increase of 20% and 13%, respectively, and corresponds with the greater decrease in PTHrP expression observed in MGLVA1 *ascites*. Conversely, transfected MDAMB231 (1-141) cells displayed only a 12% increase in apoptosis vs. a 27% increase in MDAMB231 cells, which is not consistent with the greater decrease in PTHrP expression detected in the MDAMB231 (1-141) cell line.

Figure 4.11 Mitochondrial membrane potential of cells transfected with either PTHrP siRNA or scrambled control. Results shown are the average values of three replicate assays.



4.5 DISCUSSION

The results presented here demonstrate that using an siRNA homologous to PTHrP it is possible to not only induce post-transcriptional gene silencing but this leads to a reduction in protein expression and proliferation, and an increase in apoptosis.

Three siRNA were tested for their ability to induce post-transcriptional gene silencing. Whilst all three were able to induce a significant decrease in PTHrP gene expression, the siRNA designated Target 28 was noticeably less effective than Targets 4 and 19. As mentioned previously, there are a variety of emerging guidelines for the design of an effective siRNA, which although not conclusive have been shown to increase siRNA potency. One of these guidelines recommends the avoidance of internal repeats as these have been shown to promote the formation of an internal fold-back structure (Reynolds, Leake et al. 2004). As the sequence of Target 28 contains an internal repeat (AAGAAAAAAACGGCGAACTCGC) it can be

speculated that this is the cause of its relatively poor ability to silence PTHrP gene expression.

The siRNA induced knock-down of gene expression is a transient effect, which is generally believed to last between four and six cell doublings. It would therefore be expected that the temporal effects of siRNA will depend on the rate of cell growth. MGLVA1 *ascites* grow relatively slowly compared with the other three cell lines used here and when the temporal effects of the PTHrP siRNA were examined gene knockdown appeared to last longer in this cell line. However, when comparing the breast cancer cell lines there was a greater inhibition of gene expression in the over-expressing cell line, which lasted longer compared with the wild-type cell line. The over-expressing cell line grows marginally faster than the corresponding wild-type line so it can be speculated that the temporal effects of siRNA are not solely related to growth rate. Indeed the longest duration of action, as well as the greatest gene silencing, occurred in the two cell lines which demonstrate the greatest endogenous PTHrP expression (MGLVA1 *ascites* and MDAMB231 (1-141)). A similar effect was demonstrated when protein expression was examined: western blotting showed a greater decrease in protein expression in the cell lines MGLVA1 *ascites* and MDAMB231 (1-141) compared with MDAMB231 and HT29.

Protein expression was examined using both flow cytometry and western blotting. There was a higher degree of sensitivity with western blotting which is consistent with previous results (see Chapter 3). However the use of flow cytometry not only provided a corroboration of the protein results obtained by western blotting but used in conjunction with a fluorescent siRNA allowed the examination of the level of

transfection and protein down-regulation. The fluorescence emitted by the control siRNA-transfected cells was similar to that emitted by the PTHrP siRNA-transfected cells, indicating a similar level of siRNA transfection. Cells transfected with the PTHrP siRNA demonstrated a decrease in mean fluorescent intensity compared with control and in addition it was sometimes possible to visualise two distinct cell populations. These populations appear to represent cells where PTHrP had been effectively silenced and cells where although there was uptake of the siRNA it was ineffective i.e. no change in PTHrP protein expression. Sorting these two populations by FACS would make it possible to determine if this is indeed the case.

PTHrP was manipulated in breast and GI cell lines both through over-expression and gene silencing and the resultant effects on proliferation examined. Both mechanisms resulted in a direct effect on cell proliferation: when PTHrP expression was silenced there was a significant decrease in proliferation and when PTHrP expression was increased the opposite effect was observed. Furthermore, the increase in proliferation observed through PTHrP over-expression was reversed upon the confinement of PTHrP to the cytoplasm. These results correspond with the results obtained with the exogenous peptides and neutralising antibody (see Chapter 3), where it was suggested that there was an autocrine and/or intracrine mechanism, and also indicate that there is an important role for nuclear PTHrP in proliferation.

The results described here indicate PTHrP is mitogenic; however, PTHrP has also been shown to be an anti-apoptotic factor. The significant increase in apoptosis observed upon PTHrP gene silencing is therefore consistent with previous studies (Aarts, Davidson et al. 2001; Tovar Sepulveda, Shen et al. 2002).

When comparing the potency of the siRNA it appears that cell lines exhibiting greater endogenous PTHrP are more sensitive to the effects of PTHrP silencing. This is demonstrated by the greater decrease in gene expression (leading to a similarly larger decrease in protein expression) as well as the greater decrease in proliferation. However the levels of apoptosis observed are not completely consistent with these earlier findings; although there was a 20% increase in apoptosis observed in MGLVA1 *ascites* compared with only a 13% increase in HT29 apoptosis, there was only a 12% increase observed in MDAMB231 (1-141) cells compared with a 27% increase in the corresponding wild-type cell line. It may be possible to attribute this effect to an artefact associated with the breast cell lines as the results obtained with the GI cell lines supports the earlier findings. It may also be possible that there was a greater increase in apoptosis in MDAMB231 (1-141) cells than was detected and that many of the dead cells may have been inadvertently removed during the washing steps giving a somewhat misleading result. This second scenario is much more likely as the JC-1 assay was carried out after 48 hours and therefore the full effects of PTHrP on apoptosis could have been missed. Consequently, repeating this assay over a number of time points and investigating apoptosis using another assay would help to confirm that PTHrP is indeed anti-apoptotic. For example Baskin *et al* used DAPI, which is excluded by viable cells, in conjunction with a fluorescent microplate reader to examine apoptosis in both attached and floating dead cells. Similarly Hauge *et al* simply quantified the number of floating and adherent cells using a counting chamber and determined the level of apoptosis using the number of floating cells as a percentage of total yield. They also confirmed their finding using acridine orange, which stains both viable and dead cells, and DNA laddering. Consequently, using an alternative method to detect

apoptosis would help to clarify these inconsistent findings and there are several approaches which could be utilised in order to do so (Baskin, Ngo et al. 2003; Hague, Hicks et al. 2005).

Clarification would also be required for some of the other results described here. For example studies have shown that PTHrP localises to the nucleus in a cell-cycle dependent manner (Lam, Olsen et al. 1997; Lam, House et al. 1999) so analysis of any changes in the cell cycle following silencing of PTHrP may have correlated with the significant decrease in [^{3}H]-thymidine incorporation, i.e. proliferation, which was observed.. Also, housekeeping genes were utilised for analysis of both gene and protein expression using HPRT and β-actin, respectively and although no change in expression of either one was observed following siRNA transfection, using the same housekeeping gene for both techniques would have allowed consistency as well as corroboration of the results obtained.

The establishment of PTHrP over-expressing cell lines as well as the development of a PTHrP specific siRNA allowed effective regulation of PTHrP expression. Not only were changes in gene and protein expression observed, but these led to important biological effects which have previously been associated with PTHrP (i.e. proliferation and apoptosis). Therefore it was felt that these two systems could be used in conjunction to further examine the role of PTHrP in cell adhesion.

Chapter 5:

PTHrP Mediated Cell Adhesion

5.1 INTRODUCTION

Several studies have demonstrated a link between PTHrP and adhesion. The first study by Ye *et al* in 2001 showed that over-expression of PTHrP in HT29 cells resulted in a significant increase in cell adhesion to collagen type I but not to fibronectin or laminin. This led the authors to suggest that PTHrP may influence cell adhesion to specific extracellular matrix proteins (Ye, Seitz et al. 2001).

A similar effect has been shown in a number of other cell lines. For example, upon stable transfection of the prostate cancer cell line PC3 with cDNA encoding for PTHrP amino acids (-5 to +139) Shen *et al* demonstrated an increase in adhesion to collagen type I, laminin and fibronectin and an increase in cell surface expression of the integrin subunits α_1, α_5, α_6 and β_4. This increase was ablated in cells when the NLS was deleted (Shen and Falzon 2003). Using the breast cancer cell line MCF-7 an increase in the expression of α_6 and β_4 was again reversed by the NLS mutation which led to an increase in cell adhesion to laminin but not to collagen type I (Shen, Qian et al. 2004). Transfection of the colonic cell line LoVo led to an increase in adhesion to collagen type I, fibronectin and laminin along with an increase in α_2, α_5, β_1, β_4 and β_6 expression (Shen and Falzon 2005).

Shen *et al*'s use of different cell lines demonstrates that PTHrP's effects on integrin expression may be tissue specific and could provide an explanation for the appearance of primary tumours to favour particular organs during the formation of metastases.

To date there have been 18 α and 6 β subunits discovered however it was beyond the scope of this study to examine all of these. Following a review of the literature only the subunits believed to be relevant were studied and are shown in Table 5.1 (Friedrichs, Ruiz et al. 1995; Zhang, Vuori et al. 1995; Gong, Want et al. 1997; Wewer, Shaw et al. 1997; Giannelli, Astigiano et al. 2002).

Table 5.1 Integrin subunits examined during the course of this study and the ligands associated with each heterodimer.

Beta Subunit	Alpha Subunit	Ligand
β_1	α_1	Collagens, Laminin
	α_2	Collagens, Laminin
	α_3	Collagens, Laminin, Fibronectins
	α_5	Fibronectin
β_1 & β_4	α_6	Laminin

Over-expression of PTHrP has been used to demonstrate a link between PTHrP and cell adhesion. However, the effects of down-regulating PTHrP have yet to be explored. Using the siRNA to silence PTHrP expression it was hoped that it would be possible to further explore the effects of PTHrP on both adhesion and integrin expression.

5.2 AIMS

- To manipulate PTHrP expression and examine the resultant effects on cell adhesion.
- To determine if PTHrP effects adhesion by regulating integrin expression.
- To identify possible tissue-specific differences in PTHrP regulation of integrin expression.

5.3 METHODOLOGY

5.3.1 siRNA Transfection

Following the optimisation steps described in Section 4.4.1.1 the PTHrP siRNA was used at a final concentration of 8nM and cells were reverse transfected with either the PTHrP siRNA or scrambled control (see Section 2.4.1).

5.3.2 Assay of Extracellular Matrix Adhesion

Cell adhesion was examined as described in Section 2.6 using collagen type I, fibronectin and laminin and cells subjected to either PTHrP silencing or over-expression.

Following siRNA transfection the decrease in PTHrP protein expression was maximal five days after transfection (see Section 4.4.1.3). Thus, cells were harvested at this time point and the effect on cell adhesion examined (Section 2.6.4).

The number of viable adherent cells were determined via MTT uptake with each assay carried out in quintuplicate (Section 2.6.4 and 2.6.5). All results shown are expressed relative to the scrambled control siRNA and are the average values from three individual transfection assays.

5.3.3 Analysis of Integrin Gene Expression

RNA was extracted, reverse transcribed and gene expression examined using RT-PCR and primers designed to detect specific integrin subunits (Section 2.7) with each RT-PCR assay carried out in triplicate. Results shown are expressed relative to scrambled control or vector control, where appropriate, and are the average values from three separate assays.

5.3.4 Analysis of Integrin Protein Expression

Cell surface expression of the integrin subunits α_2, α_5, α_6, β_1 and β_4 was examined using flow cytometry (Section 2.8.3). Results shown are mean results from three replicate assays.

5.4 RESULTS

5.4.1 Effect of PTHrP Manipulation on ECM Adhesion

5.4.1.1 Silencing of PTHrP and adhesion

The effect of decreasing PTHrP expression on cell adhesion was examined using the PTHrP siRNA. In the breast cancer cell lines there was a significant reduction in

adhesion to collagen type I, fibronectin and laminin (Figure 5.1, One-way ANOVA, ** $p<0.05$, *** $p<0.0001$). When the GI cell lines were transfected, whilst there was a significant decrease in adhesion to collagen type I and fibronectin there was no decrease in MGLVA1 *ascites* or HT29 cell adhesion to laminin (One-way ANOVA, ** $p<0.005$ *** $p<0.0001$). Table 5.1 summarises the percentage decrease in cell adhesion.

Figure 5.1 Effect of siRNA transfection on ECM adhesion.

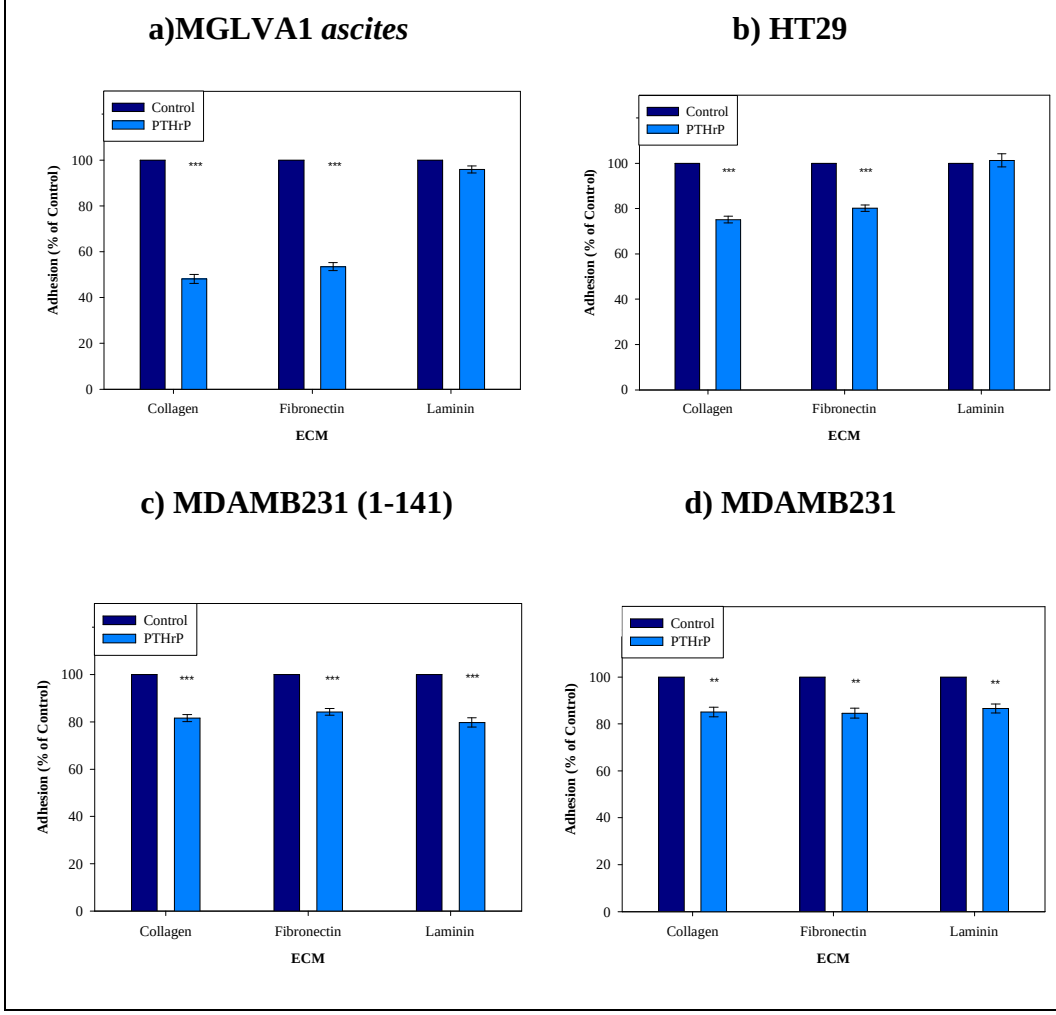


Table 5.2 Percentage decrease in ECM-cell adhesion

Cell Line	% Decrease in Cell Adhesion		
	Collagen	Fibronectin	Laminin
MGLVA1 *ascites*	51.90	46.50	4.06
HT29	24.82	19.84	8.71
MDAMB231 (1-141)	18.34	15.77	20.25
MDAMB231	14.93	15.36	13.41

5.4.1.2 Adhesion of cells over-expressing PTHrP

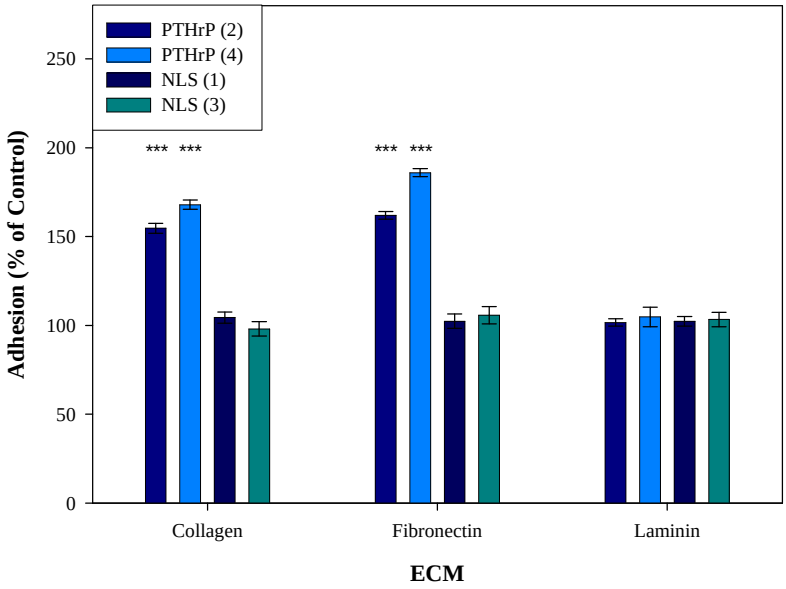
When PTHrP expression was down-regulated there was a decrease in ECM-cell adhesion however, there was no decrease in adhesion to laminin observed in the two GI cell lines. Adhesion in the breast and GI cell lines transfected to over-express PTHrP was compared to see if a similar effect was detected.

In over-expressing HT29 cells there was a significant increase in adhesion to collagen type I and fibronectin but not to laminin (Figure 5.2, One-way ANOVA, *** $p<0.0001$). This increase in adhesion was reversed in cells where the NLS had been deleted.

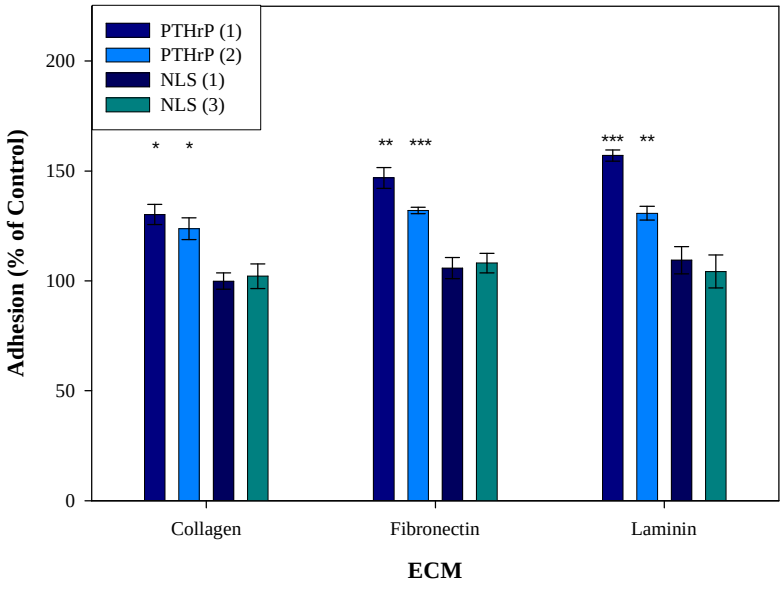
MDAMB231 cells over-expressing PTHrP demonstrated a significant increase in adhesion to collagen type I, fibronectin and laminin (One-way ANOVA, * $p<0.05$, ** $p<0.001$, *** $p<0.0001$). Again this increase was reversed in cells where the NLS had been deleted.

Figure 5.2 Effect of PTHrP over-expression on cell adhesion to collagen type I, fibronectin and laminin, with and without NLS.

a) HT29



b) MDAMB231



5.4.2 Integrin Expression

The results presented above suggest that there is a functional difference between the breast and GI cell lines which results in the variation in laminin adhesion. Previous

results suggested a link between PTHrP and integrin expression, so integrin expression was subsequently examined to determine if there were also cell-specific differences in the effect of PTHrP on integrin subunit expression.

5.4.2.1 PTHrP siRNA and integrin expression

The effect of PTHrP silencing was examined on integrin expression at both the gene and protein level.

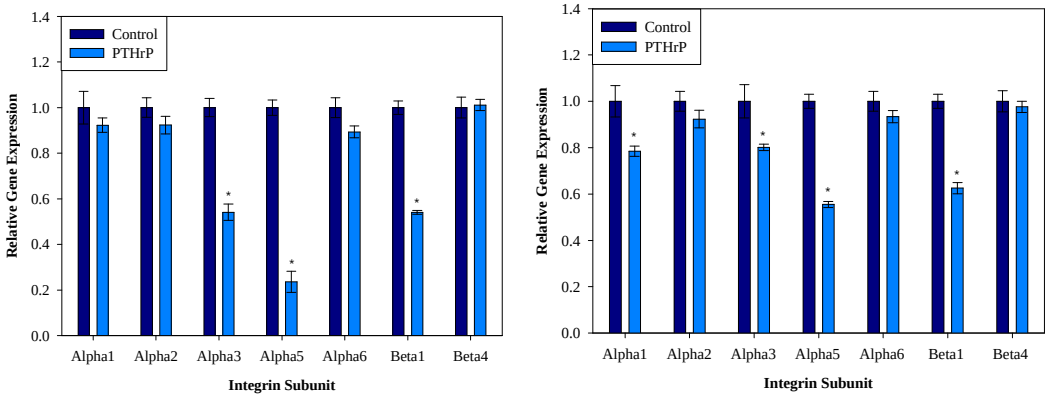
(i) Gene expression

Cells were transfected with the PTHrP siRNA and integrin expression examined five days later, when the decrease in protein expression and adhesion was maximal.

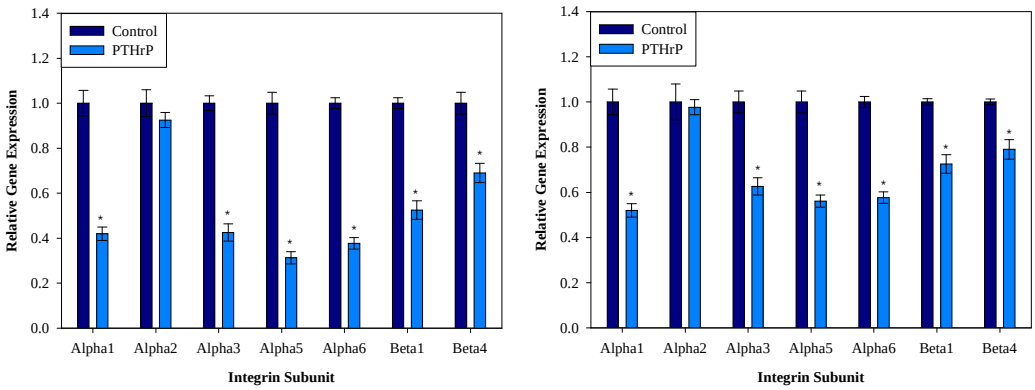
Silencing of PTHrP induced a decrease in gene expression of several integrin subunits. The results presented in Figure 5.3 show that in MGLVA1 *ascites* there was a significant decrease in expression of the subunits α_3, α_5 and β_1, whilst in HT29 cells there was a decrease in expression of α_1, α_3, α_5 and β_1. In the breast cancer cell lines there was a significant decrease in expression of α_1, α_3, α_5, α_6, β_1 and β_4 (Students t-test, * $p<0.0001$).

Figure 5.3 Effect of PTHrP silencing on integrin subunit gene expression.

a)MGLVA1 *ascites* **b) HT29**



c) MDAMB231 (1-141) **d) MDAMB231**

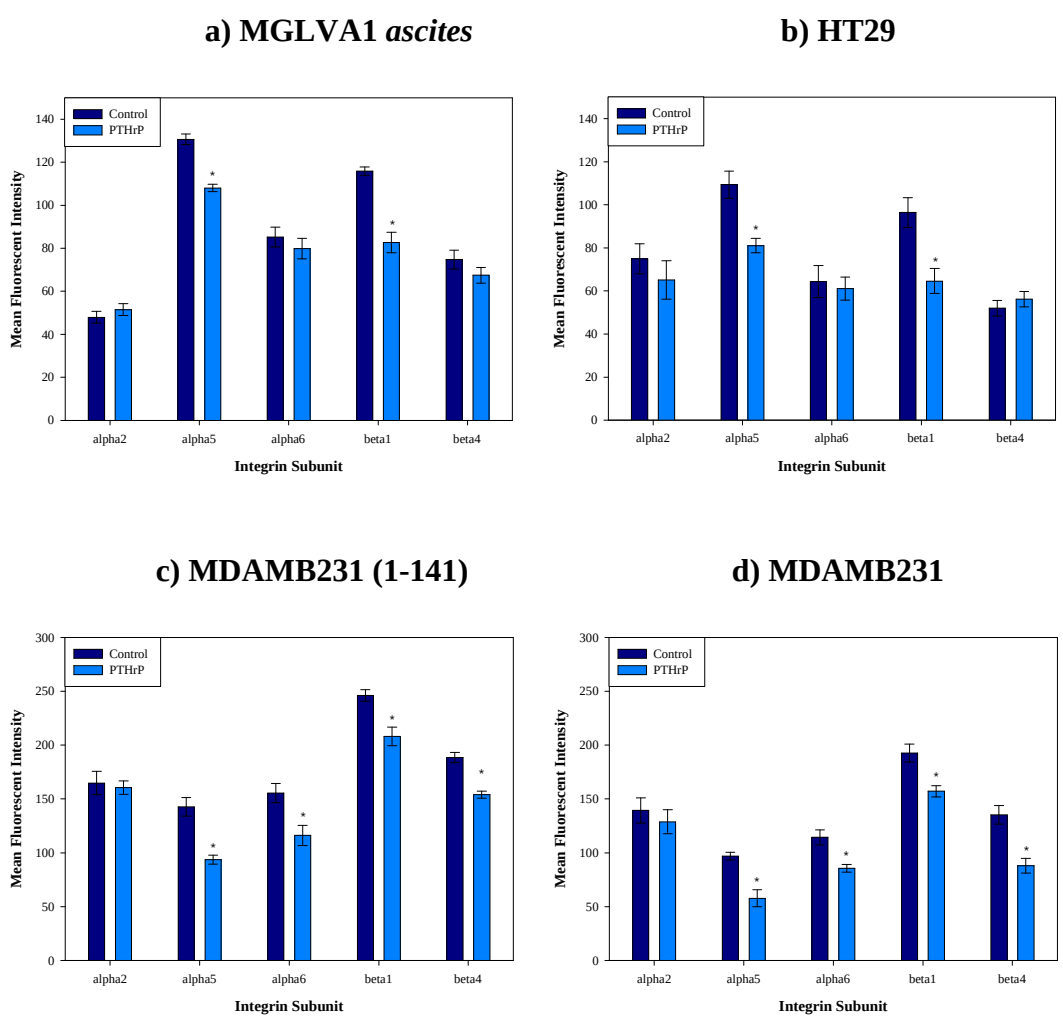


(ii) Protein expression

Protein expression of five integrin subunits were examined: the subunits which were selected included integrins which had shown no link with PTHrP expression as well as those which appeared linked to PTHrP in breast and/or GI cell lines. Analysis of protein expression of these integrin subunits using flow cytometry revealed a significant reduction in expression of only α_5 and β_1 in the GI cell lines while in the

breast cell lines there was a significant reduction in expression of α_5, $\alpha_{6,}$ β_1 and β_4 (Figure 5.4; Students t-test, * $p<0.0001$).

Figure 5.4 Effect of PTHrP silencing on integrin subunit protein expression.



5.4.2.2 PTHrP over-expression and integrin expression

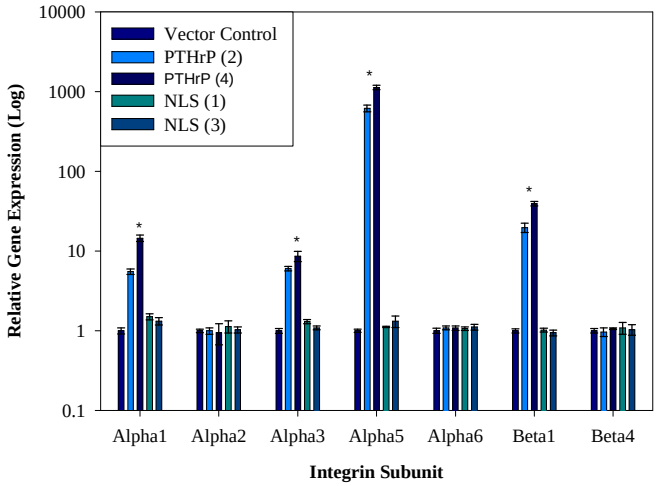
Likewise, the effect of PTHrP over-expression was also examined, again at both the gene and protein level.

(i) Gene expression

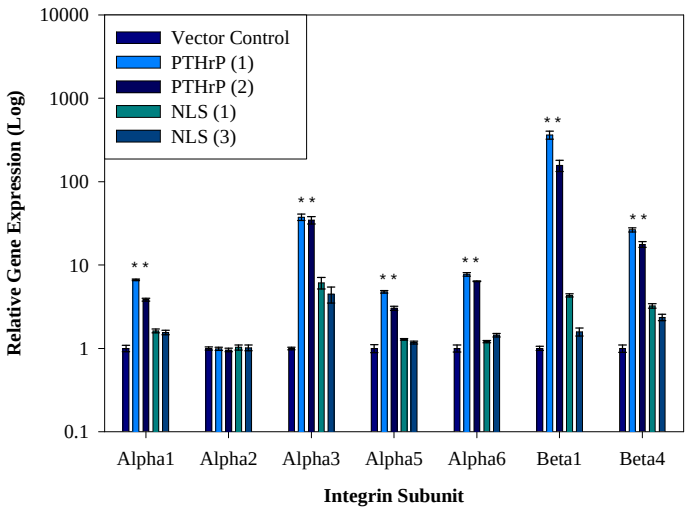
When integrin expression was then examined in the cell lines transfected to over-express PTHrP, there was a significant increase in α_1, α_3, α_5 and β_1 expression in the HT29 cells while the MDAMB231 over-expressing cells demonstrated a significant increase in α_1, α_3, α_5, α_6, β_1 and β_4 (Students t-test * $p<0.0001$). The increase in integrin expression was reversed in cells where the NLS had been deleted (Figure 5.5).

Figure 5.5 Effect of PTHrP over-expression on integrin subunit gene expression.

a) HT29



b) MDAMB231



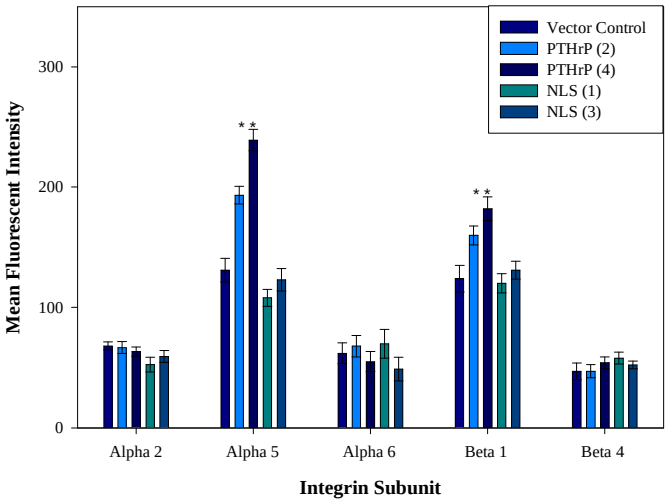
(ii) Protein Expression

When protein expression was subsequently examined, there was a significant increase in expression of α_5, α_6, β_1 and β_4 in the MDAMB231 cell line and an increase in expression of only α_5 and β_1 in the HT29 cells (Students t-test, *

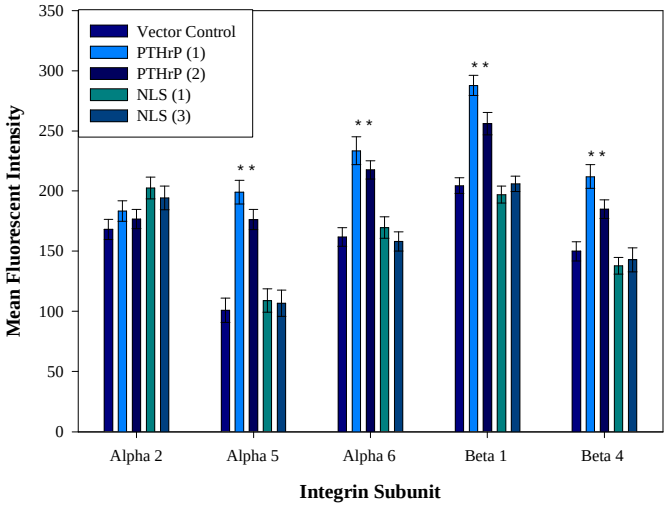
p<0.0001). Again this increase was reversed in cells where the NLS had been deleted (Figure 5.6).

Figure 5.6 Integrin cell surface in cells over-expressing PTHrP.

a) HT29



b) MDAMB231



5.5 DISCUSSION

Following the construction of HT29 and MDAMB231 over-expressing cell lines as well as the use of the PTHrP siRNA it was possible to obtain a clearer picture of the role of PTHrP in cell adhesion. By directly comparing up- and down-regulation of PTHrP in the same cell lines and observing the resultant effects it was possible to determine that PTHrP positively regulates cell adhesion i.e. a significant increase in cell adhesion was observed upon over-expression whereas silencing of PTHrP induced the opposite effect. By utilising cell lines derived from two different tissues (breast and colon) it was also possible to observe some phenotypic differences i.e. PTHrP regulated adhesion of both cell lines to collagen type I and fibronectin but only breast cancer cell adhesion to laminin.

In contrast to these results however, the MDAMB231 (1-141) over-expressing cell line used in the earlier studies showed no apparent increase in adhesion to any of the ECM proteins (see Section 3.4.4). Although the results from this study provide no definitive explanation for these differences in adhesion the most obvious difference between the cell lines is the vectors used to induce PTHrP over-expression. The cells obtained from Teresa Guise's laboratory were created using a vector encoding for PTHrP (1-141) whilst the vector obtained from Miriam Falzon encodes for PTHrP (1-139). It is therefore possible that PTHrP's effects on adhesion are mediated by the (1-139) isoform and not the (1-141) isoform. The PTHrP siRNA was able to decrease adhesion of these cells to the ECM, however although these cells over-express PTHrP (1-139) they also endogenously express the other two isoforms therefore the observed decrease in adhesion can be attributed to the siRNA decreasing expression of the endogenous PTHrP.

This hypothesis is supported by a study by Bouizar *et al* who examined the expression of the different PTHrP isoforms and correlated this expression with the formation of bone metastases from primary breast tumours. They found no expression of the (1-173) isoform in breast tumours but did demonstrate that expression of (1-139) was greater than (1-141) in breast tumours. They went on to show that expression of (1-139) was also greater in patients who developed bone metastases compared with non-metastatic tumours. Expression of (1-141) on the other hand was similar in both metastatic and non-metastatic tumours (Bouizar, Spyratos et al. 1999).

Bouizar *et al*'s study suggests that PTHrP (1-139) but not (1-141) plays a role in breast cancer progression. Although this would be consistent with the adhesion results obtained with both of the MDAMB231 PTHrP over-expressing cell lines there is no evidence in this thesis to support such a hypothesis.

When Ye *et al* examined PTHrP over-expression and adhesion using HT29 cells they demonstrated an increase in adhesion to collagen type I but not fibronectin or laminin, which is in contrast to the results described here where an increase in adhesion to both collagen type I and fibronectin was observed. There are a number of differences between the two studies which may explain this disparity. Firstly Ye *et al* used crystal violet staining to quantify adhesion rather than MTT uptake, which was used during this study. Secondly the authors do not state where they obtained their ECM proteins therefore it is possible that the fibronectin used in their study differs from the fibronectin used here. Thirdly, there may be phenotypic differences between the HT29 clones used here and the clone used by Ye *et al* i.e. following

propagation different clonal populations may have arisen following mutations in individual cells. This may have resulted in the gain, loss or mutation of individual genes and proteins and thereby affected the observable behaviour of the cell population.

Shen *et al* used the breast cancer cell line MCF-7 (Shen, Qian et al. 2004) rather than MDAMB231; therefore, it is not possible to directly compare and contrast their results with those described here. However if PTHrP does affect adhesion in a tissue-specific manner then similar results would be expected. Whilst Shen *et al* described an increase in adhesion to only laminin, over-expression of PTHrP in MDAMB231 cells led to an increase in adhesion to collagen type I, fibronectin and laminin. The same expression vector was used in both studies so any differences between the studies cannot be attributed to isoform differences or to differences in ECM proteins as Shen and colleagues also obtained their ECM proteins from BD Biosciences. As with the comparison of Ye *et al*'s study it may be possible to explain the discrepancy through differences in quantification of adhesion which Shen *et al* determined by acid phosphatase activity. In spite of this it is more likely that the differences in adhesive properties can be attributed to the establishment of these cell lines from different sources, as this has almost certainly led to a number of phenotypic differences between the two cell lines.

Such phenotypic differences may be especially relevant when considering integrin expression, due to the high degree of redundancy with these receptors i.e. it is not necessary for each subunit to be expressed as others are often able to perform the same function. When Shen *et al* observed only an increase in cell adhesion to

laminin they also observed a change in expression of only two integrin subunits - α_6 and β_4. As these subunits form a heterodimer which mediates adhesion to laminin this correlates with their cell adhesion results. Similarly, during this study when integrin expression in MDAMB231 cells was examined following regulation of PTHrP expression there was a significant change in expression of α_1, α_3, α_5, α_6, β_1 and β_4 which correlates with the changes in adhesion to collagen type I, fibronectin and laminin as these subunits form heterodimers responsible for adhesion to these ECM proteins.

By comparing the GI and breast cell lines it was hoped that it would be possible to elucidate some tissue-specific differences. When adhesion was examined following regulation of PTHrP expression there was a significant change in adhesion to laminin in the breast cell lines whilst neither GI cell line demonstrated any change in adhesion to this ECM protein. When integrin expression was subsequently examined following both PTHrP silencing and over-expression one of the common observations was the change in expression of α_6 and β_4 in only the breast cancer cell lines. As previously mentioned this integrin mediates adhesion to laminin and therefore correlates with the aforementioned changes in laminin adhesion.

Another important finding was the reversal of the increase in adhesion and integrin expression upon confinement of PTHrP to the cytoplasm. Not only does this correlate with Shen *et al*'s findings but also suggests that there is an intranuclear mechanism responsible for PTHrP's role in integrin expression. Furthermore, as both integrin gene and protein expression were examined it appears that the changes

observed at the gene level are translated to parallel changes in protein expression which suggests that PTHrP regulates integrin expression at the transcriptional level.

It is therefore possible to make a number of conclusions from these results. Firstly, expression of PTHrP is related to cancer cell adhesion. Secondly, this link is indirect and is mediated through changes in integrin expression. Furthermore, PTHrP's control of integrin expression is regulated in a tissue-specific manner and finally this is mediated via an intranuclear mechanism.

Chapter 6:

Silencing Integrin Expression

6.1 INTRODUCTION

By correlating PTHrP and integrin interactions through genetic manipulation of PTHrP expression levels (gene knock-in and knock-down), there was strong evidence suggesting that PTHrP and integrin expression were inter-related in mediating cell adhesion. By using cell lines where the NLS had been deleted, it was also confirmed that the actions of PTHrP were dependent on its presence within the nucleus.

To further support these findings and to confirm PTHrP's indirect role in cell adhesion it was decided to design siRNAs targeted against two different integrin subunits for use in conjunction with the PTHrP over-expressing cell lines.

The integrin subunits α_5 and α_6 were chosen for investigation since nuclear PTHrP appears to affect α_5 expression in both the breast and colon cancer cell lines whereas α_6 expression is only affected in the breast cancer cell line.

Studies by Elbashir and colleagues resulted in the use of a double-stranded 21nt siRNA with a two base over-hang as the use of longer siRNA was believed to induce the interferon response. However, recent work by Kim *et al* has refined this approach following their observation that increasing siRNA length also increases potency (Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001; Kim, Behlke et al. 2005).

Kim *et al* examined a range of RNA duplexes of different lengths and observed that the optimal siRNA length was 27nt. The authors also examined Dicer cleavage in their range of duplexes and demonstrated that the 21nt duplex was the only siRNA not cleaved indicating that the 27nt siRNA is cleaved to produce a 21nt siRNA. The authors went on to propose that Dicer may cleave the 27nt siRNA in several different places thereby producing a range of different 21nt products, increasing the number of siRNA homologous to the target sequence and thereby increasing potency. However when they tested this theory by synthesising each possible 21nt siRNA that could be produced from the 27nt siRNA, none were as effective as the longer siRNA either when used individually or when pooled.

Another interesting finding was the observation that increasing the length of an ineffective 21nt siRNA to 27nt resulted in a significant decrease in gene expression. Additionally, the use of the longer siRNA increased the temporal effect with gene suppression lasting for up to ten days compared with a suppression of around four days using the corresponding 21nt siRNA.

Due to the number of advantages associated with the 27nt siRNA it was decided that this new design would be applied to the integrin siRNA designed in this study.

6.2 AIMS

- To design and construct an effective siRNA targeted against the integrin subunits α_5 and $\alpha_{6.}$
- To compare the efficiency of 27nt siRNA to a classical 21nt siRNA in gene down-regulation.

- To examine the effect of PTHrP over-expression on siRNA-induced α_5 and α_6 gene silencing and the subsequent effect on cell adhesion.

6.3 METHODOLOGY

6.3.1 siRNA Construction and Transfection

Using Dharmacon's siRNA Target Finder, several different siRNA for both α_5 and α_6 were designed and then constructed using the *Silencer* siRNA Construction Kit, as described in Section 2.2. Cells were forward transfected, as previously described (see Section 2.4.1).

6.3.2 Analysis of Gene Expression Following siRNA Transfection

Changes in gene expression were examined following RNA extraction, reverse transcription and RT-PCR analysis using primers designed to detect α_5, α_6, STAT and OAS gene expression (Section 2.7). Each RT-PCR assay was carried out in triplicate. Unless otherwise stated results shown are expressed relative to α_5 scrambled control and are the average values from three separate transfection assays.

6.3.3 Analysis of Protein Expression Following siRNA Transfection

Integrin cell surface expression was examined by flow cytometry using specific α_5 and α_6 primary antibodies and PE labelled rabbit anti-mouse secondary antibodies (Section 2.8.3). Results shown are mean results from three replicate transfections.

6.3.4 Assay of ECM Adhesion

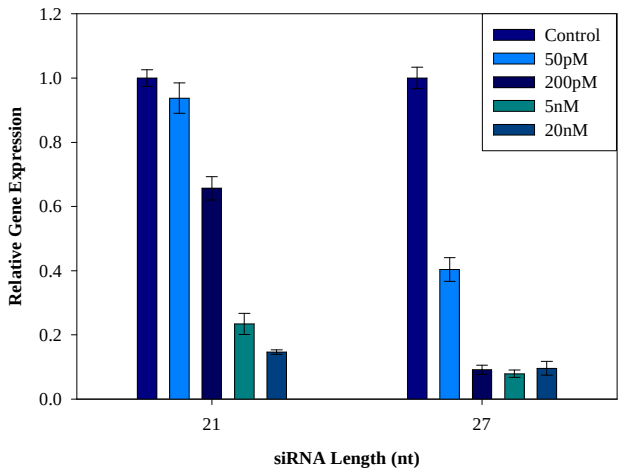
Adhesion to the ECM components collagen type I, fibronectin and laminin following siRNA transfection was measured as previously described in Section 2.6.4.

6.4 RESULTS

6.4.1 Comparison of siRNA Length

When searching for potential α_5 siRNA targets, two of the proposed siRNA overlapped and provided an ideal opportunity for investigating the potential of a 27nt siRNA. Gene expression was examined 24 hours after transfection and Figure 6.1 shows that both the 21nt and 27nt siRNA were able to significantly reduce α_5 gene expression using 20nM siRNA (Students t-test $p<0.0001$). However, unlike the 21nt siRNA, the longer siRNA was able to induce a significant decrease in gene expression at even the lowest concentration examined (50pM).

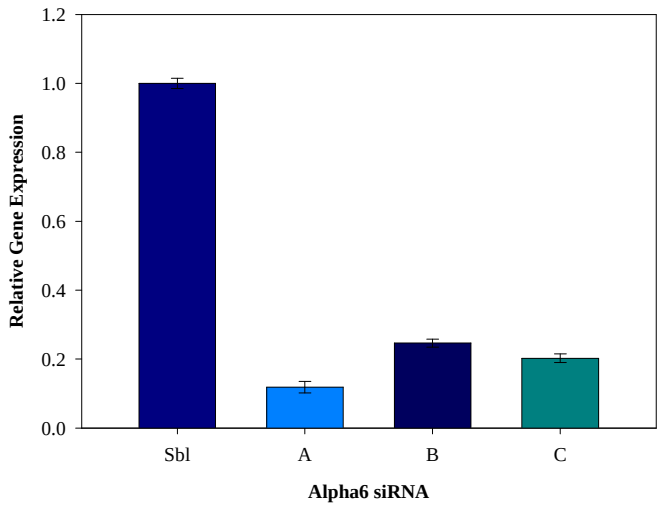
Figure 6.1 Comparison of gene silencing induced by α_5 siRNA in MDAMB231 cells. Results are expressed relative to the PTHrP scrambled control siRNA and are average values from two separate transfection assays.



When designing the α_6 siRNA it was also hoped to construct a 27nt siRNA, however it was initially necessary to construct and test several 21nt siRNA in order to find the most effective one prior to modification. The three siRNA chosen for analysis were designated α_6A, α_6B and α_6C.

Figure 6.2 shows that each of the siRNA were able to significantly reduce gene expression compared with the scrambled control (Students t-test, $p<0.0001$), however α_6A was the most effective, inducing a significantly greater reduction in gene expression compared with either α_6B or α_6C (Students t-test, $p<0.005$). α_6A was therefore subsequently altered to create a 27nt siRNA.

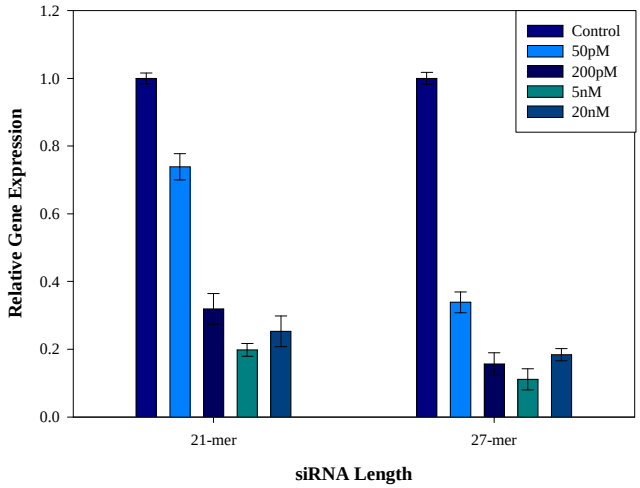
Figure 6.2 Gene silencing in MDAMB231 cells transfected with three different α_6 siRNA. Results shown are expressed relative to PTHrP scrambled control siRNA and are average values from two replicate assays.



When the corresponding 21nt and 27nt α_6 siRNA were both titrated, there was a dose-dependent increase in gene silencing. However the 27nt siRNA was much

more effective at silencing gene expression and was able to induce gene silencing of more than 60% even at the lowest concentration used (Figure 6.3).

Figure 6.3 Titration and comparison of α_6 siRNA length in MDAMB231 cells. Results shown are expressed relative to PTHrP scrambled control siRNA and are the mean values from two replicate assays.



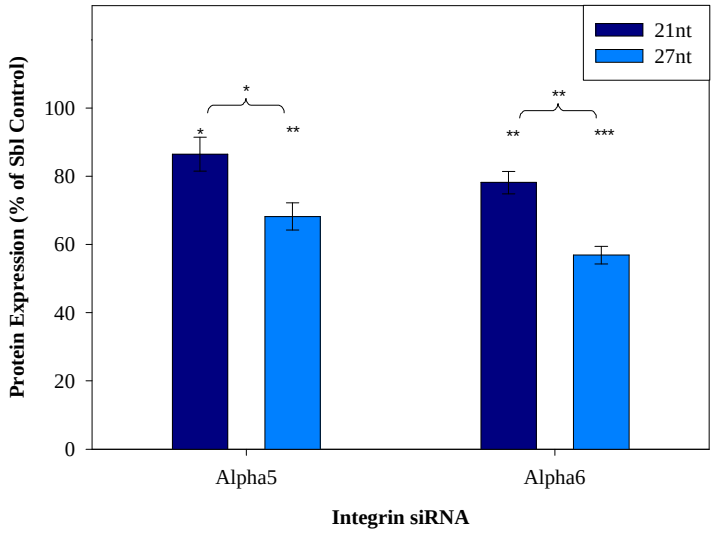
Both the α_5 and α_6 siRNA were most effective at a final concentration of 5nM and were subsequently used at this concentration in all future studies. A scrambled form of the 27nt α_5 siRNA was also constructed and used as a control in each of the subsequent experiments.

6.4.2 Effect of Gene Silencing on Protein Expression

The 21nt and 27nt siRNA were also compared for their ability to reduce protein expression. Similar to the gene expression results, there was a significant decrease in protein expression with all four integrin siRNA compared with scrambled control (Students t-test, * $p<0.01$, ** $p<0.001$, *** $p<0.0001$). As with gene silencing the 27nt siRNA were more effective at reducing protein expression compared with the corresponding 21nt siRNA. Figure 6.4 demonstrates that the 21- and 27nt α_5 siRNA

produced a 14% and 32% decrease in protein expression, respectively, whilst the α_6 siRNA induced a 22% and 44% reduction in protein expression. Comparison of the corresponding 21- and 27nt siRNA revealed that the longer α_5 and α_6 siRNA induced a significantly greater decrease in protein expression compared with the corresponding 21nt siRNAs (Students t-test * $p<0.01$, ** $p<0.001$).

Figure 6.4 Protein downregulation in MDAMB231 cells transfected with either 21- or 27nt siRNA.



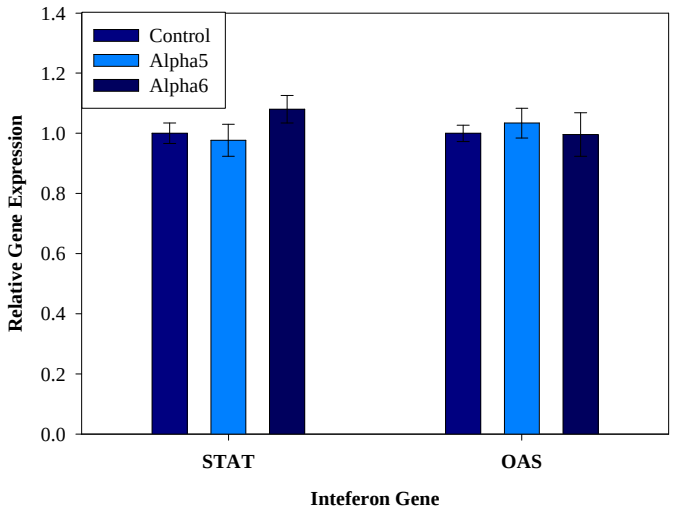
6.4.3 Integrin siRNA and the Innate Inteferon Response

The expression of two interferon genes, STAT and OAS were examined in order to determine if the innate interferon response had been induced as a result of siRNA transfection.

Figure 6.5 shows that there was no significant difference in expression of either interferon gene following siRNA transfection (STAT α_5 $p=0.5580$, α_6 $p=0.072$;

OAS α_5 p=0.3601, α_6 p=0.9307), so it can be surmised that the innate interferon response was not induced following integrin siRNA transfection.

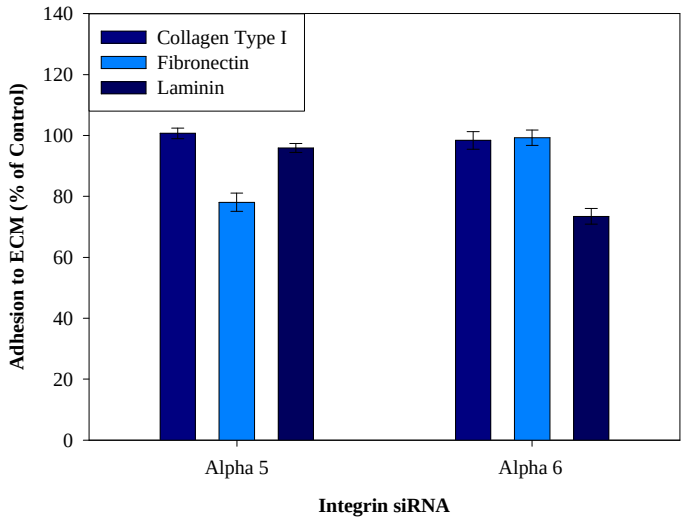
Figure 6.5 Induction of innate interferon response in MDAMB231 cells transfected with integrin siRNA. Results shown are expressed relative to α_5 scrambled control and are average values from two separate transfection assays.



6.4.4 Effect of α_5 and α_6 Silencing on ECM Adhesion

It can be seen in Figure 6.6 that following transfection with the α_5 siRNA there was a significant reduction in cell adhesion to fibronectin compared with the scrambled control but no decrease in cell adhesion to either laminin or collagen type I (One-way ANOVA, p<0.0001). Similarly using the α_6 siRNA, adhesion to laminin significantly decreased with transfection of the target siRNA compared with scrambled control (One-way ANOVA, p<0.0001), whilst there was no decrease in adhesion to collagen or fibronectin. These results indicate that the downstream effects of silencing either integrin siRNA were specific to the targeted ligand only.

Figure 6.6 Effect of integrin siRNA transfection on MDAMB231 cell adhesion to the ECM.



6.4.5 Duration of Action of Integrin siRNA

Following siRNA transfection, integrin gene and protein expression were both examined at several time points in order to assess the duration of action of both siRNA.

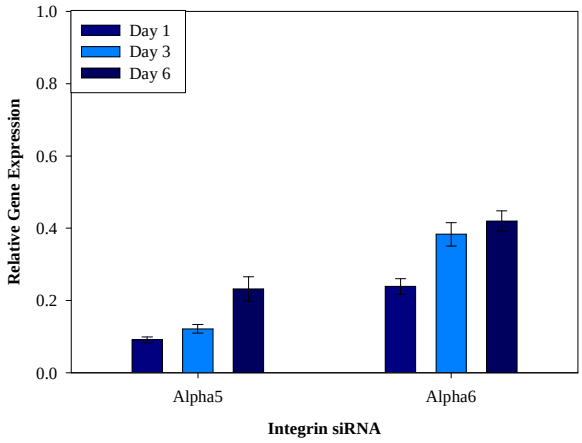
6.4.5.1 Gene expression

As with the PTHrP siRNA both integrin siRNA produced maximal gene silencing at day one and then proceeded with a gradual decrease in silencing (Figure 6.7).

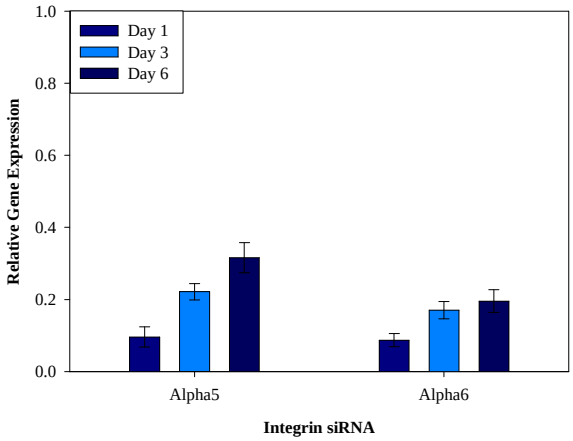
The duration of action appears to last longer compared with the 21nt PTHrP siRNA; however it is not possible to make an accurate comparison due to differences not only in the target but also in the potential transfection efficiency caused by the use of different transfection reagents and this was not investigated.

Figure 6.7 Duration of action of integrin siRNA.

a) HT29



b) MDAMB231

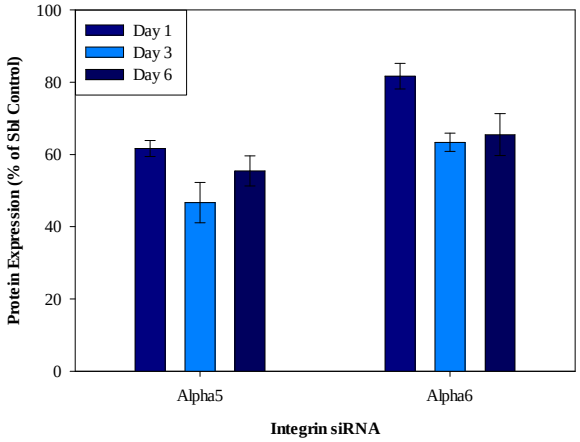


6.4.5.2 Protein expression

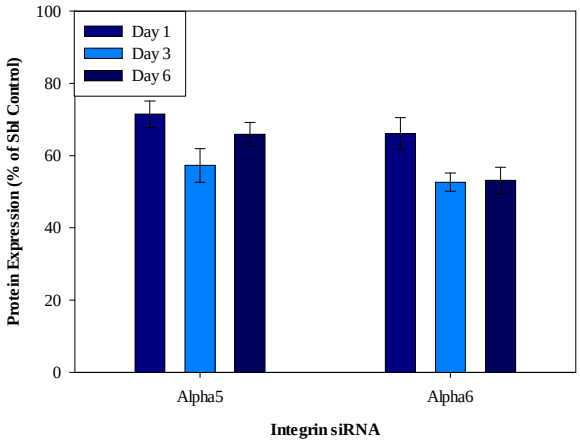
When protein expression was examined the decrease in protein expression was maximal three days after transfection in both cell lines and with both siRNA (Figure 6.8).

Figure 6.8 Integrin cell surface expression in cells transfected with integrin siRNA.

a) HT29



b) MDAMB231



6.4.6 Effect of PTHrP Over-expression on Silencing of Integrin Expression

It was predicted that over-expression of PTHrP would reduce the degree of integrin siRNA-induced silencing by being 'out-competed' by enhanced mRNA levels and that the absence of nuclear PTHrP would reverse this effect.

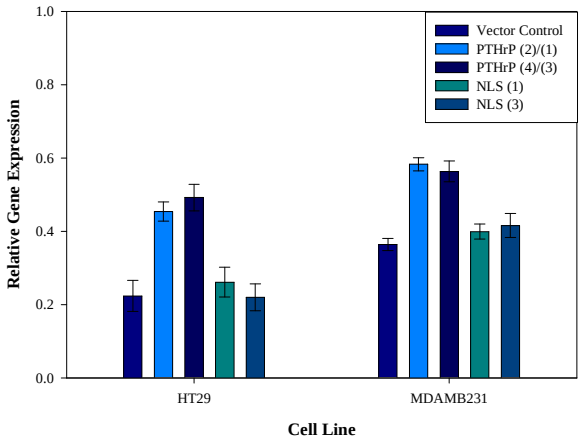
The decrease in protein expression was maximal at day three and as this translates into a comparable effect on adhesion this time point was subsequently chosen for simultaneous examination of adhesion, gene and protein expression.

6.4.6.1 Integrin gene expression

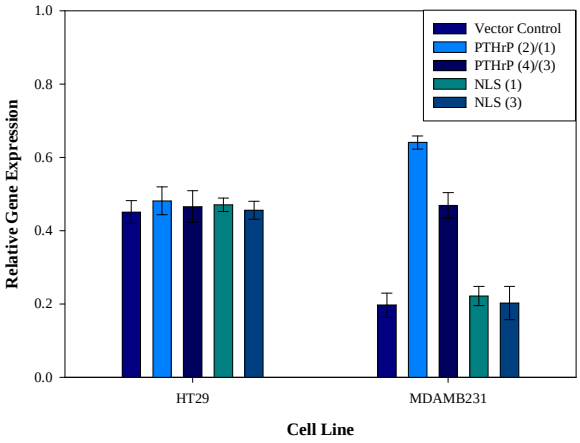
When α_5 and α_6 gene expression were examined following integrin siRNA transfection there was a significant decrease in gene expression in each of the cell lines examined when compared with cells transfected with scrambled control siRNA (Figure 6.9, Students t-test, $p<0.0001$). However Figure 6.9a demonstrates that in the cells over-expressing wild-type PTHrP there was less down-regulation of α_5 gene expression compared with vector control containing cells. In the MDAMB231 vector control cells, α_5 expression was reduced to ~40% of that seen in the scrambled control-transfected cells, whereas in the cells over-expressing wild-type PTHrP α_5 expression was reduced to ~60%. Similarly, in the HT29 PTHrP over-expressing cells, α_5 expression was reduced to ~50% compared with a reduction to only ~20% in vector control and NLS deleted cells. Similarly, Figure 6.9b demonstrates that α_6 expression was reduced to only ~20% in MDAMB231 vector control cells whereas in cells expressing wild-type PTHrP α_6 expression was reduced to 50-60% of that seen in scrambled control-transfected cells. In MDAMB231 cells where the NLS had been deleted there was a similar level of gene down-regulation as vector control cells in α_5 and α_6 siRNA-transfected cells. The decrease in α_6 gene expression was similar in each of the HT29 cell lines examined, thus providing further evidence of the cell-specific actions of PTHrP.

Figure 6.9 Effect of PTHrP over-expression on siRNA induced α_5 and α_6 gene silencing three days following transfection.

a) HT29 and MDAMB231 cell lines transfected with α_5 siRNA. Results expressed relative to scrambled control-transfected cells.



b) HT29 and MDAMB231 cell lines transfected with α_6 siRNA. Results expressed relative to scrambled control-transfected cells.



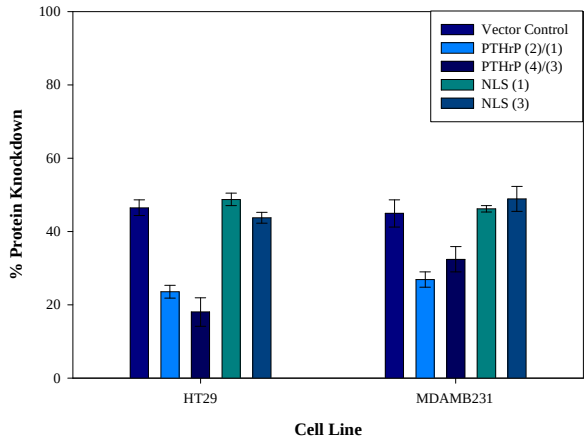
6.4.6.2 Protein expression

The same effect was observed when protein expression was examined i.e. there was a significant decrease in protein expression in each of the cell lines examined (Figure 6.10, Students t-test, $p<0.0001$). Over-expression of wild-type PTHrP in HT29 cells resulted in a reduction in the degree of α_5 knockdown in these cells compared with vector control and NLS deleted cells but had no effect on silencing of the α_6 subunit.

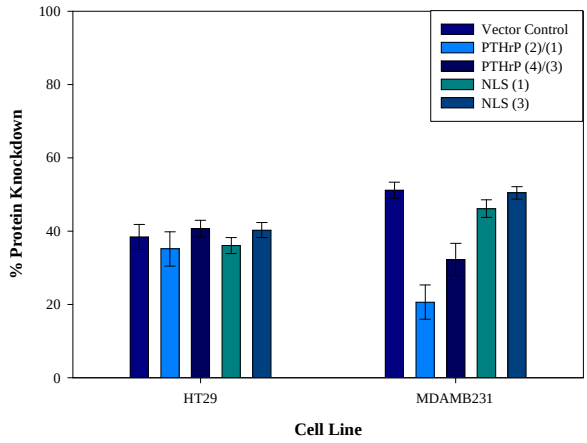
Figure 6.10a shows that in HT29 vector control cells there was a ~50% knockdown in protein expression in α_5 siRNA-transfected cells compared with scrambled control-transfected cells whereas there was only a ~20% knockdown in wild-type expressing cells. Likewise, in MDAMB231 cells expressing wild-type PTHrP there was a knockdown of α_5 expression of only ~30% compared with a knockdown of ~50% in vector control cells. Figure 6.10b demonstrates that the protein knockdown following α_6 siRNA transfection was similar in all of the HT29 cell lines whereas in the MDAMB231 cells there was a knockdown of ~50% in vector control cells and only a knockdown of ~20% in wild-type over-expressing cells. (Students t-test, $p<0.0001$).

Figure 6.10 Effect of PTHrP over-expression on siRNA induced α_5 and α_6 cell surface expression, three days following transfection.

a) HT29 and MDAMB231 cell lines transfected with α_5 siRNA. Results expressed relative to scrambled control-transfected cells.



b) HT29 and MDAMB231 cell lines transfected with α_6 siRNA. Results expressed relative to scrambled control-transfected cells.



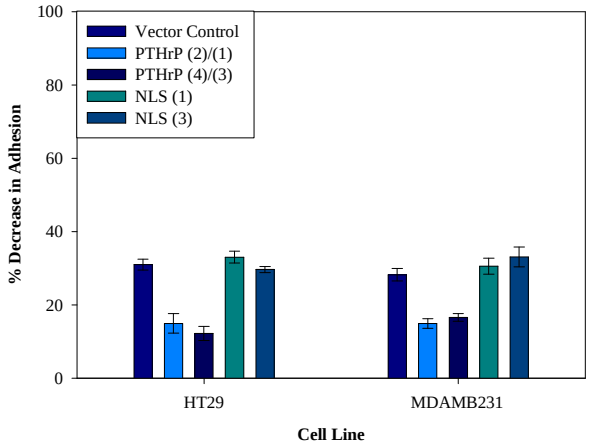
6.4.6.3 Cell Adhesion

Figure 6.11 shows that when the effect of α_5 and α_6 siRNA transfection on cell adhesion was examined, whilst there was a significant decrease in adhesion in each of the cell lines examined, the nuclear translocation of PTHrP caused a smaller reduction in adhesion of α_5 transfected HT29 cells to fibronectin (~15% decrease

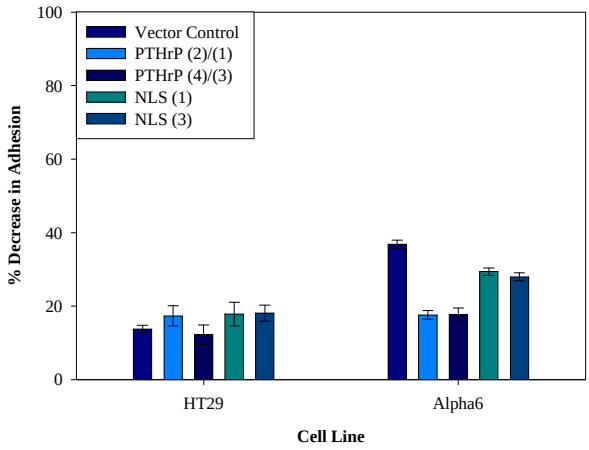
compared with a 30% decrease in adhesion in vector control cells), whilst adhesion to laminin was similar in each of cell lines examined. Both integrin siRNA caused a significant reduction in MDAMB231 cell adhesion to fibronectin and laminin and again the presence of PTHrP within the cell nucleus appears to diminish the reduction of siRNA induced adhesion. Both siRNA reduced adhesion to fibronectin and laminin by ~15% in wild-type over-expressing cells, whereas adhesion to laminin was reduced by up to 40% in vector control cells (One-way ANOVA, $p<0.0001$).

Figure 6.11 Effect of integrin siRNA transfection on ECM-cell adhesion.

a) Cell adhesion to fibronectin following transfection with α_5 siRNA. Results expressed relative to scrambled control-transfected cells.



b) Cell adhesion to laminin following transfection with α_6 siRNA. Results expressed relative to scrambled control-transfected



6.5 DISCUSSION

During the course of this study a link between PTHrP and cell adhesion has been shown. The results have also suggested that this link is mediated via changes in integrin expression. In order to confirm this indirect mode of action it was decided to examine the effect of PTHrP over-expression on silencing of the integrin subunits α_5 and α_6. If PTHrP plays a direct role in cell adhesion then it was predicted that its over-expression would have no effect on integrin silencing.

Following the successful design and construction of siRNA homologous to either α_5 or α_6, gene suppression was induced and there was a decrease in protein expression which led to a decrease in cell adhesion. α_5 forms a heterodimer with β_1 to act as a receptor for fibronectin while α_6 forms a heterodimer with either β_1 or β_4 and interacts with laminin (Hynes 2002). When cell adhesion to the ECM proteins collagen type I, fibronectin and laminin was examined following gene silencing of either α_5 or α_6 there was a significant decrease in adhesion to only fibronectin or laminin, respectively. The downstream effects of integrin silencing were therefore specific and consistent with expectations.

When the effect of PTHrP over-expression was subsequently examined, both the MDAMB231 and HT29 cells over-expressing full length PTHrP demonstrated a smaller decrease in α_5 silencing compared with both the vector control cell lines and the cell lines lacking the NLS. When α_6 silencing was similarly examined, whilst the MDAMB231 cell lines also demonstrated a smaller decrease in silencing

compared with the vector control cell lines, there was no discernable difference between the HT29 clones upon α_6 silencing. As subsequent examination of protein expression and cell adhesion demonstrated a similar effect it can be seen that PTHrP counter-acts the effects of the integrin siRNA.

Consequently, these results provide further evidence of PTHrP's indirect role in cell adhesion. They also correlate with the previous findings that PTHrP's regulation of integrin expression is dependent on nuclear localisation and the observations regarding the absence of a relationship between α_6 and PTHrP expression in the HT29 cell line and thereby support the hypothesis that there is tissue-specific regulation of integrin expression by PTHrP.

The use of siRNA to induce post-transcriptional gene silencing is a relatively new discovery and a rapidly expanding field. As such it is not known how Kim *et al*'s study examining the use of longer siRNA will fit into the RNAi story and if it will be applicable to a wide range of targets. However, the results described in this thesis support Kim *et al*'s findings and as 27nt siRNA are less reliant on adherence to the siRNA design 'rules' and are significantly more potent than the corresponding 21nt siRNA this will not only increase siRNA usability but will also enhance their potential as therapeutic agents (Kim, Behlke et al. 2005).

Chapter 7:

PTHrP and Integrin

Transcription

7.1 INTRODUCTION

The results previously described demonstrate that PTHrP regulates integrin expression at the transcriptional level which is then translated to changes in protein expression. Gene expression can be regulated through a variety of mechanisms including transcription, translation and mRNA stability.

7.1.1 Transcription

The NLS of PTHrP is homologous to the NLS of the transcription factors *c-jun*, *c-fos* and p53, which has led to suggestions that PTHrP may be a transcription factor. It has also been suggested that as PTHrP is able to move between the cytoplasm and nucleus, PTHrP may act as a nuclear export factor for mRNA whereby it would help to transport mRNA between the nucleus and cytoplasm (Fiaschi-Taesch and Stewart 2003).

7.1.2 Translation

PTHrP has previously been shown to interact with RNA by Aarts *et al*. They demonstrated the interaction of PTHrP with RNA within the nucleolus of COS-1 cells and went on to show that PTHrP down-regulates ribosomal RNA (rRNA) synthesis. rRNA is involved in translation; therefore, by decreasing synthesis, PTHrP is able to prevent translation and arrest the cell cycle. Aarts *et al* demonstrated that in doing so PTHrP was able to protect serum-deprived cells from apoptosis (Aarts, Levy et al. 1999; Aarts, Davidson et al. 2001).

However, although this would explain the effects of PTHrP on proliferation and apoptosis it does not explain the ability of PTHrP to affect adhesion and integrin expression.

7.1.3 mRNA Stability

The steady-state concentration of mRNA within a cell is dependent not only on transcription but also on mRNA degradation. As mentioned earlier PTHrP has been shown to interact with RNA, and RNA-binding proteins are known to protect mRNA from degradation. Similarly several hormones have shown an ability to regulation gene expression, a good example being oestrogen. Oestrogen is able to stabilise and destabilise the same mRNA in the same cell type in different species. For example in avian liver oestrogen destabilises vitellogenin mRNA, whilst in amphibian liver it stabilises this mRNA. Oestrogen is also able to stabilise different mRNA within the same cell e.g. ovalbumin and conalbumin in chick oviduct (Ing 2005).

Hence, it can be seen that there are a variety of mechanisms whereby PTHrP can have an effect on gene expression, with the most likely being manipulation of transcription or mRNA stability.

Calculating mRNA half-life is a difficult task as mRNA translation and degradation is not entirely stochastic. It was therefore decided to first explore the possibility that PTHrP is a transcription factor of integrin gene expression. The integrin subunits α_2, α_5 and α_6 were chosen for analysis; α_2 demonstrates no apparent link with PTHrP expression in the cell lines examined here and therefore provided a suitable negative

control, expression of both α_5 and α_6 was related to PTHrP expression in the breast cancer cell lines, and α_5 but not α_6 appeared to be linked to PTHrP in the GI cell lines. Consequently using all three integrin subunits it was hoped that it would be possible to identify whether PTHrP was a transcription factor and provide further evidence of a tissue-specific mechanism of action.

7.2 AIMS

- To create reporter constructs containing the promoter sequences of the integrin subunits α_2, α_5 and α_6.
- To determine if PTHrP increases integrin gene transcription.

7.3 METHODOLOGY

7.3.1 PCR Amplification of the Integrin Promoter Regions

The promoter region of the integrin subunits α_2, α_5 and α_6 were amplified from genomic DNA using specific primers in conjunction with PCR (See sections 2.3.2, 2.7.3 and 2.7.4). Primers for amplifying the α_2 promoter region were designed using Primer3, while the α_5 and α_6 promoter regions were amplified using primers previously published by Klein *et al* and Lim *et al*, respectively (Klein, Bikfalvi et al. 1996; Lim, Lee et al. 2001).

7.3.2 DNA Extraction and Purification from Agarose Gel

Using a 2% agarose gel, PCR products were examined and visualised using ethidium bromide staining. The DNA was then excised and purified using the reagents provided with the QIAquick Gel Extraction Kit, as described in Section 2.3.5. The purified products were then sent for sequencing at the School of Biomedical Sciences, University of Nottingham (see Section 2.3.9).

7.3.3 Ethanol Precipitation

DNA was concentrated by ethanol precipitation, by incubating the DNA with two volumes of 100% ethanol and 1/10th volume of sodium acetate. Following a one hour incubation at -20°C the DNA was pelleted by centrifugation and washed twice with 70% ethanol. Once dry the pellet was re-suspended in RNase and DNase free water (Section 2.3.4).

7.3.4 DNA Digest and Ligation

To allow ligation of the promoter sequences into the reporter vector, the primers used for PCR amplification contained restriction sites for the relevant restriction enzymes and these enzymes were incubated with either the promoter DNA or the pGL3-Basic vector over-night to allow digestion (Section 2.3.3).

The digested vector and promoter DNA were combined with a T4 ligase enzyme and incubated overnight at 4°C to promote ligation (Section 2.3.6).

7.3.5 Bacterial Transformation and Plasmid DNA Extraction

The ligated promoter DNA and vector were transformed into XL-1 Supercompetent bacterial cells by heat-shock. The transformed bacteria were then grown overnight on LB Agar plates containing 50µg/mL ampicillin. The resultant colonies were then selected and grown further in NZY LB medium (Section 2.3.7).

A GenElute Plasmid Mini-prep kit was used to extract the plasmid DNA from the overnight bacterial culture (Section 2.3.8).

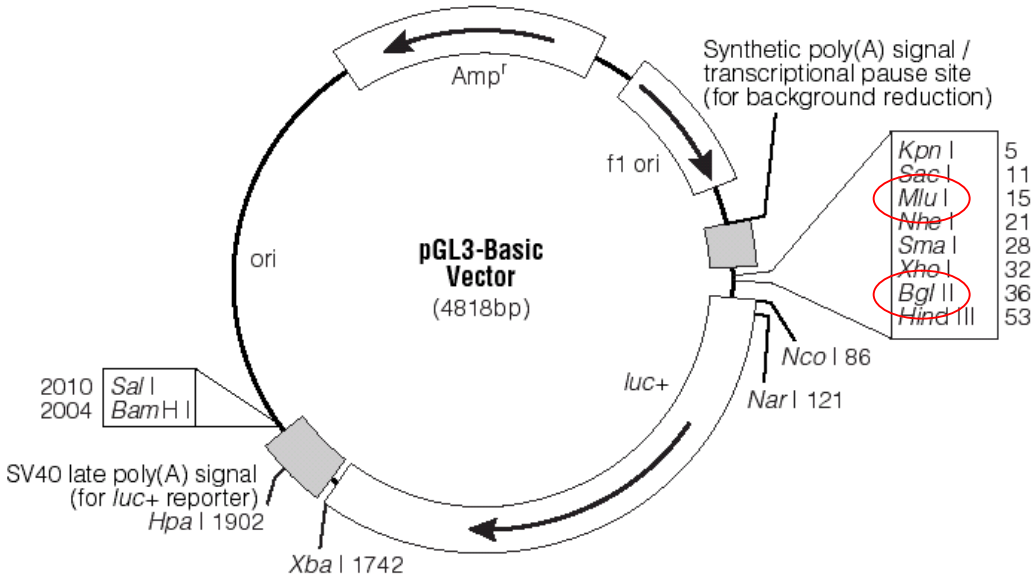
7.3.6 Plasmid Transfection

Cells were seeded into 6-well plates one day prior to transfection. The plasmid DNA was then combined with Lipofectamine 2000 and the resultant complex added to the cells, which were then incubated over-night to promote transfection (Section 2.4.2).

7.3.7 Luciferase Reporter Assay

The pGL3-Basic vector contains the cDNA of firefly luciferase (Figure 7.1). Coupling the promoter sequence to this indicator allows quantification of promoter activity as transcription of the promoter also results in transcription of the luciferase cDNA. Changes in promoter/luciferase activity can consequently be measured following addition of luciferin substrate, with an increase in luminescence indicating an increase in transcription.

Figure 7.1 pGL3-Basic vector circle map, with multiple cloning sites.



24 hours following plasmid transfection, cells were lysed, and the cell debris pelleted by centrifugation. The supernatant was transferred to a black solid 96-well plate and mixed with an equal volume of luciferase assay substrate. Luminescence was then detected following a 5-minute incubation at room temperature (Section 2.10).

7.4 RESULTS

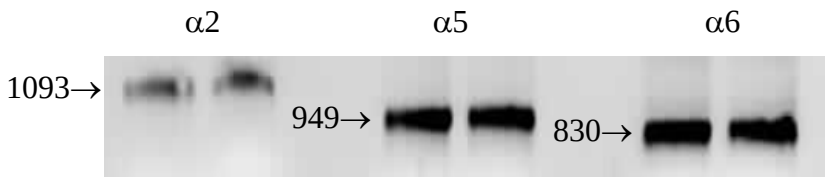
7.4.1 Amplification of Promoter Sequence

Following PCR amplification, the sequences of the PCR products were verified by the automatic sequencing service provided by the School of Biomedical Sciences, University of Nottingham. The DNA sequence for α_2 and α_5 matched the information from the Entrez Nucleotide database (Appendix 1).

Lim *et al*'s 2001 paper described the construction of an α_6 promoter/luciferase reporter, and the published primers were used for amplification of the α_6 promoter region. However, despite obtaining a PCR product of approximately the correct size, when analysed the sequence did not match the information within the Entrez Nucleotide database. Upon further analysis of the reverse primer, there was no homology with any sequence within the human genome.

A reverse primer for the α_6 promoter region was subsequently designed using the information within the Entrez Nucleotide database and the promoter region amplified by PCR. The PCR products of all three promoter regions can be seen in Figure 7.2.

Figure 7.2 α_2, α_5 and α_6 PCR products following PCR amplification, visualised by ethidium bromide staining.



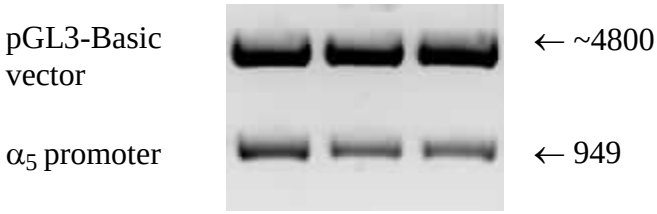
7.4.2 Cloning and Transformation

In order to allow cloning into the pGL3-Basic vector, forward primers contained sequences for the restriction site *Mlu* I while the reverse primers for α_5 and α_6 included sequences for the restriction site *Bgl* II. The promoter sequence for α_2 contained a restriction site for *Bgl* II therefore the reverse primer for this subunit was designed to contain the restriction site for *Bam* HI as *Bgl* II and *Bam* HI have compatible cohesive ends.

The promoter DNA was subsequently digested and ligated with the pGL3-Basic vector. However, following bacterial transformation none of the resultant bacterial colonies contained promoter DNA.

The restriction enzyme *Mlu* I has very low cleavage activity, with a percentage cleavage of approximately 50%. It was suspected that this may result in insufficient cleavage of the promoter DNA and vector to allow ligation. To investigate whether this was indeed the situation, the α_5 forward primer was redesigned and the *Mlu* I restriction site replaced with *Kpn* I, which has a cleavage of >90%. Following digestion, ligation and bacterial transformation a number of bacterial colonies were produced which contained the α_5 promoter DNA (Figure 7.3).

Figure 7.3 α_5 promoter and pGL3 vector following restriction digest with *Kpn* I and *Bgl* II restriction enzymes.



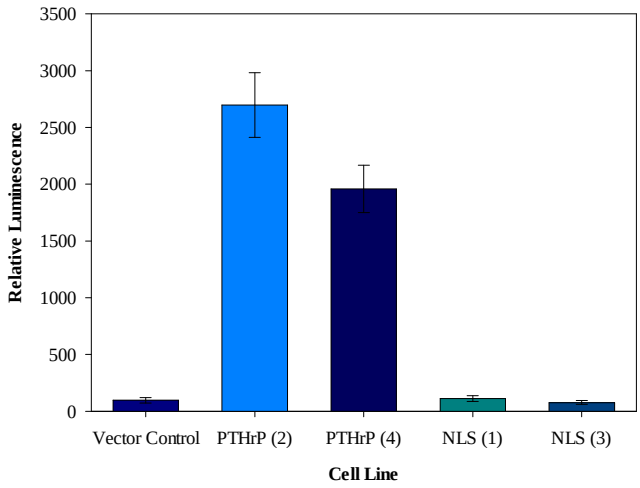
7.4.3 Promoter Activity

Once the α_5 promoter DNA had been successfully cloned into the pGL3-Basic vector, the plasmid was then transfected into HT29 and MDAMB231 cells over-expressing PTHrP and the level of α_5 promoter activity examined by measuring luciferase activity.

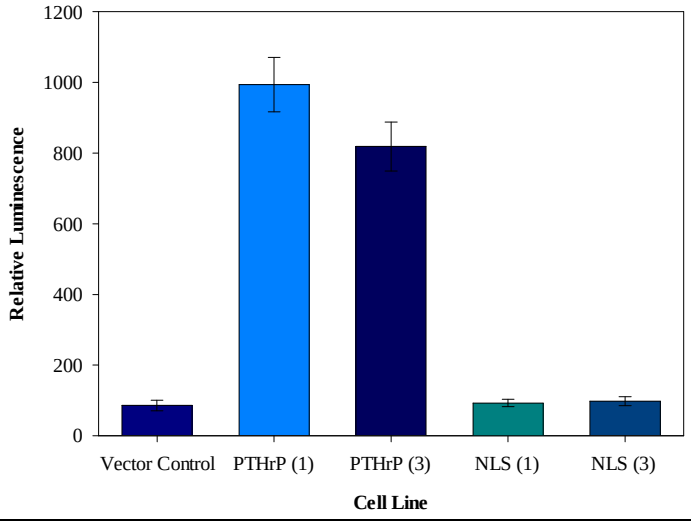
Figure 7.4 shows a significant increase in promoter activity in the cells over-expressing PTHrP, compared with vector control cells. Cells expressing PTHrP where the NLS had been deleted exhibited a level of luminescence similar to control cells (One-way ANOVA, $p<0.0001$).

Figure 7.4 Promoter activity in cells transfected with pGL3-Basic vector containing α_5 promoter DNA. Results shown are average values from three replicate assays.

a) HT29



b) MDAMB231



7.5 DISCUSSION

It was hoped that using reporter vectors containing the promoter regions for integrins α_2, α_5 and α_6 it would be possible to verify that not only does PTHrP regulate integrin gene transcription but that it does so in a tissue-specific manner.

Although it was not possible to create reporter constructs containing the promoter regions for α_2 or α_6, using the α_5 construct, a significant increase in promoter activity was demonstrated in the cell lines over-expressing PTHrP. This increase was reversed in the cell lines where the NLS had been deleted and therefore supports the other findings described in this study.

The multiple cloning sites included in the pGL3 vector allow a degree of choice when selecting the sites to be used during cloning; *Mlu* I and *Bgl* II were subsequently chosen as these restriction enzymes could be used with all three integrin subunits. However the low cleavage by *Mlu* I caused difficulties during cloning and one of the alternative cloning sites was therefore sought and successfully used in the cloning of the α_5 construct.

Although *Kpn* I could also be used during cloning of the α_2 construct, the promoter region of the α_6 promoter contains a restriction site for *Kpn* I thus preventing its use during the cloning of this vector. Consequently it would be necessary to choose an alternative cloning site and both *Xho* I or *Hind* III could be utilised as each of these enzymes have a cleavage of >75% and neither site can be found within the α_6

promoter region. Unfortunately time limitations prevented such a course of action being pursued.

Although the PTHrP-induced increase in α_5 promoter activity indicates an increase in transcriptional activity it does not necessarily support the hypothesis that PTHrP is a transcription factor. PTHrP may increase integrin transcription indirectly by stimulating a known transcription factor such as Sp1, NF-κB or the *ets* family of proto-oncogenes (Birkenmeier, McQuillan et al. 1991; Nishida, Kitazawa et al. 1997), rather than interacting with the promoter region directly. It would be possible to examine the interaction of PTHrP with the integrin promoter DNA by using a protein-DNA interaction assay such as DNase I footprinting or electrophoretic mobility shift (Fried and Crothers 1981; Garner and Revzin 1981; Brenowitz, Senear et al. 1986) and thereby conclusively ascertain whether or not PTHrP is indeed an integrin transcription factor.

Chapter 8:

Discussion

8.1 INTRODUCTION

PTHrP is emerging as a multi-functional protein. Even after almost 20 years and numerous studies into its expression and function, neither its role or mechanisms of action have been fully elucidated (Philbrick, Wysolmerski et al. 1996; Lam, Thomas et al. 2000; Clemens, Cormier et al. 2001; Fiaschi-Taesch and Stewart 2003).

In spite of this it is clear that PTHrP plays a major role in tumour biology and is involved in various facets of cancer. Whether as a mitogen or an anti-apoptotic factor or by manipulating cell adhesion it is evident that targeting this protein presents an invaluable opportunity in the treatment of multiple tumour types (Asa, Henderson et al. 1990; Wysolmerski and Broadus 1994; Nishihara, Ito et al. 1999; Deftos 2000; Akashi, Fuse et al. 2002; Richard, Luchin et al. 2003). Further examination of the regulation of PTHrP expression as well as its mechanism of action would help to refine the targeting of this protein and thereby enhance its potential as a therapeutic target and this has been the main goal of this thesis.

Tumour progression leads to numerous complications in cancer patients and is a major obstacle to effective therapy; many primary tumours can be removed by surgery and remaining tumour cells eradicated by chemo- or radiotherapy. The establishment of secondary tumours however significantly reduces patient survival with the majority of cancer deaths attributed to the development of metastases. For example, a 2001 study used immunohistochemistry analysis to detect the presence of occult tumour cells in lymph nodes and bone marrow aspirates obtained from breast cancer patients deemed to be metastases-free. The disease free survival and overall survival rates in patients where occult tumour cells were present were significantly

lower compared with patients where occult tumour cells were not detected (Gerber, Krause et al. 2001). Similarly 70% of patients with colorectal cancer liver metastases have a median survival of only 15 months, even with chemotherapy (Belluco, Mammano et al. 2005).

Tumour progression is a multi-step process with adhesion of tumour cells to the ECM and the exchange of biological information one of the key steps (Fidler 1990). It has often been observed in cancer patients that primary tumours have a partiality for one particular organ during metastasis, for example breast tumours metastasise to bone (Powell, Southby et al. 1991) while GI tumours predominantly metastasise to the liver (Fidler 1990; Belluco, Mammano et al. 2005). PTHrP has previously been associated with tumour progression and linked to tissue-specific metastasis (Ye, Seitz et al. 2001), however the mechanism by which this occurs has not yet been elucidated. This present study examined the role of PTHrP in both breast and GI cancer cell adhesion and investigated the role of this molecule in cell adhesion to various components of the ECM and the role that integrins play in this process.

8.2 EXPRESSION OF PTHrP

Ye *et al*'s 2001 study indicated that PTHrP was an important factor in GI cell adhesion and numerous other studies have demonstrated that PTHrP plays an important role in breast cancer progression (Southby, Kissin et al. 1990; Powell, Southby et al. 1991; Wysolmerski and Broadus 1994). It was thus decided to initially examine PTHrP expression in a number of GI cell lines to further define the role of PTHrP in this tumour type and to use a breast cancer cell line induced to over-express PTHrP (Guise, Yin et al. 2002) in conjunction with the corresponding

wild-type cell line in order to allow direct correlation between PTHrP expression and downstream effects in breast and GI tumour cells (Ye, Seitz et al. 2001).

Initial investigations used a panel of tumour-derived cell lines and examined PTHrP expression at both the gene and protein level. The panel included gastric, colorectal and pancreatic cell lines. Although PTHrP gene expression was not detected in all of the cell lines examined, protein expression was detected in the entire panel suggesting wide-spread PTHrP expression.

Post-transcriptional and translational regulation (e.g. gene transcription rate, transcript stability, translational regulation, protein degradation) often results in differences between gene and protein expression (Paradet-Balade, Boulme et al. 2001). Such disparities between mRNA and protein expression have been previously described, for example Gygi *et al* examined the correlation between mRNA and protein expression in yeast and although they showed a general trend whereby increased mRNA expression resulted in increased protein expression they also found cases where similar mRNA levels resulted in a variation in protein expression of up to 30-fold (Gygi, Rochon et al. 1999).

8.3 PTHrP AND CELL GROWTH

8.3.1 Mitogenic actions of PTHrP

Post-translational processing results in the production of various PTHrP peptides, with the main three believed to be PTHrP (1-36), (38-94) and (107–139) (Fiaschi-Taesch and Stewart 2003). These peptides are subsequently packaged and secreted

from the cell and are more commonly expressed by cells than the unprocessed peptide (Philbrick, Wysolmerski et al. 1996).

There have been a number of studies examining the effects of exogenous PTHrP on cell proliferation and this has resulted in conflicting views as to the role of PTHrP. For instance PTHrP (1-34) has been shown to be both mitogenic (Villaneuva-Penacarrillo, Cancelas et al. 1999; Asadi, Faraj et al. 2001) and anti-mitogenic (Luparello, Romanotto et al. 2001), with other studies demonstrating that exogenous PTHrP has no effect on proliferation (Ye, Seitz et al. 2001). One of the objectives of the present study was to compare the effects of different post-translational fragments of PTHrP on proliferation and down-stream signalling events.

Of the various peptides used as part of this study only PTHrP (1-34) increased cell proliferation in GI tumour-derived cell lines. Similarly when antibodies directed towards different regions of PTHrP were used to neutralise PTHrP, the antibody directed towards PTHrP (1-34) resulted in a significant reduction in cell proliferation whereas the antibody directed towards PTHrP (38-64) had no effect. These results, therefore, suggest that only amino-terminal PTHrP is involved in cell proliferation and are consistent with Asadi *et al* and Villaneuva-Penacarrillo *et al* studies which demonstrated that PTHrP (1-34) caused an increase in proliferation in a prostate and pancreatic cell line, respectively. Similarly Cataission *et al* demonstrated that a PTHrP neutralising antibody directed against amino acids (1-34) caused a dose-dependent decrease in proliferation. However, these results are in contrast to several studies including those led by Claudio Luparello where PTHrP (1-34), (38-94)-amide, (67-86) and (107-139) have all been shown to decrease breast cancer cell

proliferation (Luparello, Burtis et al. 1995; Cataission, Lieberherr et al. 2000; Luparello, Romanotto et al. 2001).

For example, Luparello *et al's* 1995 study demonstrated a decrease in 8701-BC cell proliferation using PTHrP (1-34), (34-68), (67-86) and (107-138) at concentrations similar to those used in the present study: all four peptides induced a decrease in proliferation at 100nM. In a later study Luparello and colleagues (2001) used a panel of different breast cancer cell lines to examine the effect of mid-region PTHrP on growth, invasion and tumourigenesis. Two of the six cell lines used (Hs578T and T47D) demonstrated a biphasic response to PTHrP (38-94)-amide whereby at lower concentrations (0.01nM) there was an increase in proliferation but at higher concentrations (1nM) this switched to a decrease. The authors were unable to explain this response but suggested that it could be the product of activation of different signalling pathways at different peptide concentrations. Although a receptor for the mid-region of PTHrP has not yet been identified, it is known that PTH1R is linked to both the adenylyl-cyclase/PKA and PLC/PKC pathways and Fortino *et al* demonstrated that activation of the PKA pathway in human skin fibroblasts results in a decrease in proliferation whereas activation of the PKC pathway increases proliferation (Luparello, Burtis et al. 1995; Luparello, Romanotto et al. 2001; Fortino, Torricelli et al. 2002).

A disparity in results obtained between different studies is not uncommon. For example when Hoey *et al* examined the role of PTH1R in breast cancer cell proliferation using exogenous PTHrP and the breast cancer cell line MCF-7 their results conflicted with a similar study by Maioli and Fortino who questioned their

results in a letter to the British Journal of Cancer. Hoey *et al*'s study used MCF-7 cells induced to over-express PTH1R and using 125nM of PTHrP (1-34) they induced an increase in cAMP production and proliferation in the over-expressing cell line whereas the vector control cells were unresponsive to PTHrP. In their study, Miaoli and Fortino induced an increase in wild-type MCF-7 cell proliferation using 640nM PTHrP (1-34) and led them to suggest that the concentration Hoey *et al* used was too low to induce a response in their wild-type cells. Hoey *et al* replied and suggested that the disparities were the result of "differences in experimental approach" (Hoey, Sanderson et al. 2003; Hoey, Sanderson et al. 2004; Maioli and Fortino 2004).

The peptide concentration used during the present study was 100nM and could therefore be the cause for the differences in results obtained here and those described elsewhere. A previous in-house study had shown a reduction in proliferation of other breast cancer cell lines at this peptide concentration and repeating this assay using a range of concentrations may induce a greater, or biphasic response, not only to PTHrP (1-34) but also to the other peptides.

Examining the activity of the two different PTH1R transduction pathways following peptide stimulation would allow the cellular actions of PTHrP to be linked to these pathways, and allow clarity on this complex issue. For instance there may be a threshold where the transduction pathway switches from the PKC to PKA signalling pathways and if receptor density differs between cell lines, and indeed different clones, this could result in the previously observed disparities between studies (Hoey *et al* & Maioli and Fortino) and would also be consistent with Luparello *et al*'s

findings. Using a radioligand binding assay to examine the number and affinity of receptors in different cell lines would help to explain differences in the cellular response to exogenous PTHrP. Similarly investigating PKC and PKA signalling and the resultant downstream effects at various peptide concentrations would also help to clarify the role of exogenous PTHrP on cell proliferation. Similarly, even though a mid-region receptor has not yet been cloned, analysis of second messenger signalling upon peptide stimulation would allow a similar study to be carried out for mid-region PTHrP.

It has also been shown that PTHrP localises to the nucleus in a cell-cycle dependent manner (Lam, Olsen et al. 1997; Lam, House et al. 1999). Investigation into whether the PTHrP siRNA affected the cell cycle was not part of this study but should be conducted in order to assess what effects, if any, PTHrP had on the cell cycle.

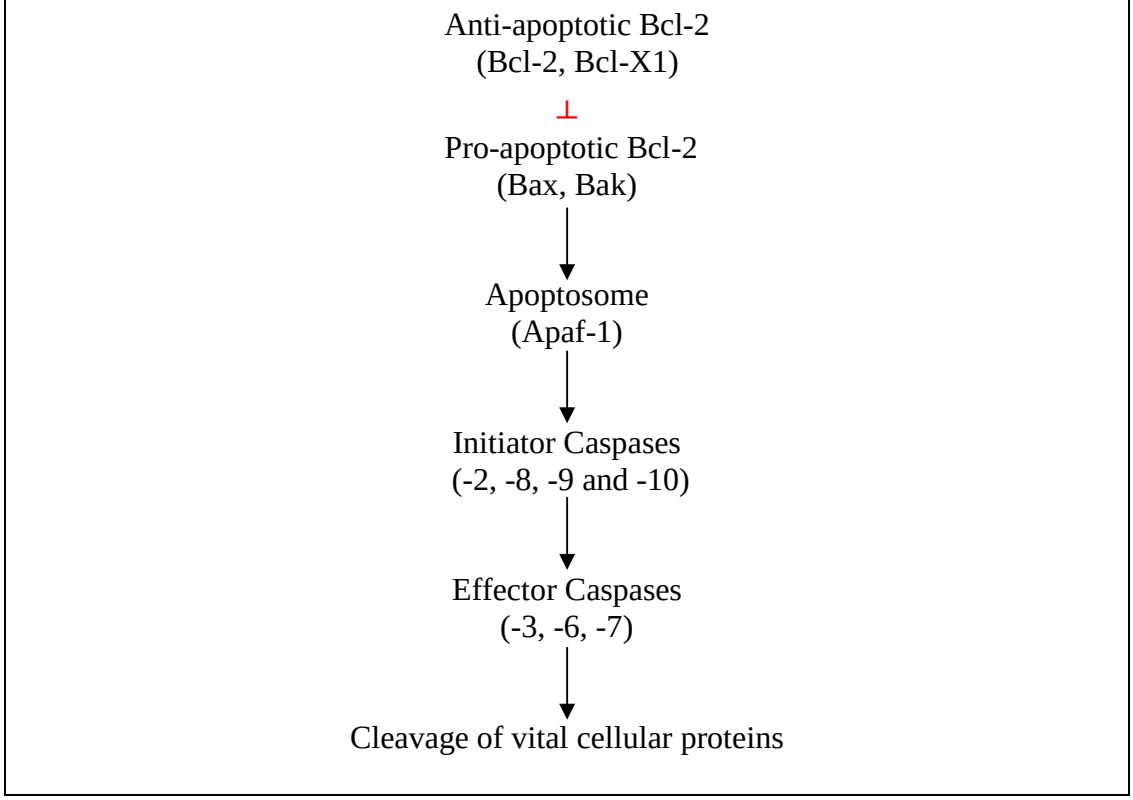
When clonagenicity assays were carried out using PTHrP over-expressing cell lines, elevated PTHrP expression resulted in a significant increase in proliferation which was reversed in cells where the NLS had been deleted. This is consistent with other studies where intranuclear PTHrP has also been shown to affect proliferation (Tovar Sepulveda and Falzon 2002; Shen and Falzon 2005). It is not, however clear whether the autocrine/paracrine PTH1R pathway is distinct from the intranuclear mechanisms: PTHrP may be released, internalised after binding to PTH1R before translocation towards the nucleus. It is clear that further investigation is necessary in order to clarify which mechanisms are involved.

8.3.2 PTHrP as an anti-apoptotic factor

Aarts and colleagues demonstrated that PTHrP binds to RNA within the nucleus and that this is dependent on the NLS. They went on to show this action protects chondrocytes from apoptosis induced by serum starvation and consequently suggested an anti-apoptotic role for intranuclear PTHrP. Similarly, Tovar Supulveda and Falzon demonstrated that not only does intranuclear PTHrP increase mitosis but it also protects cells against apoptosis. As growth rate is a balance between proliferation and apoptosis it can therefore be speculated that the pro-proliferative effects of intranuclear PTHrP are mediated by both an increase in proliferation as well as a decrease in apoptosis (Aarts, Levy et al. 1999; Aarts, Davidson et al. 2001; Tovar Sepulveda, Shen et al. 2002).

Apoptosis is a complex process and a simple representation of the intrinsic Bcl-2 regulated pathway can be seen in Figure 8.1. Briefly, a change in the ratio of the pro- and anti-apoptotic proteins in the Bcl-2 family of apoptotic proteins can result in mitochondrial membrane permeabilisation. This not only disrupts key cellular functions but also results in the release of apoptotic proteins including caspases, proteases responsible for the cleavage of cellular proteins and the ultimate cause of cell death (Jin and El-Deiry 2005).

Figure 8.1 Bcl-2 regulated apoptosis (adapted from (Jin and El-Deiry 2005)



When apoptosis was examined following antibody neutralisation during this study there was a significant increase in apoptosis observed with the PTHrP (1-34) antibody, as measured by mitochondrial membrane potential. Similarly when the effect of PTHrP silencing was examined there was a significant increase in apoptosis in cells treated with the PTHrP siRNA compared with the scrambled control. These results therefore suggest that PTHrP is an anti-apoptotic factor and are consistent with several other studies investigating PTHrP and apoptosis.

For example, Miyaji *et al* also used an anti-PTHrP monoclonal antibody raised against amino acids (1-34) and demonstrated a dose-dependent increase in chondrosarcoma cell apoptosis, measured using the TUNEL assay. They also detected decreased levels of procaspase-3 which they attributed to cleavage of the procaspase to the active form, as well as a dose-dependent decrease in Bcl-2 and an

increase in Bax expression. Similarly, Tovar Sepulveda *et al* demonstrated that PTHrP over-expression caused a decrease in serum deprivation induced apoptosis in breast cancer cells, which was dependent on the NLS, and went on to demonstrate that this was mediated through changes in the Bcl-2 and Bcl-x_L to Bax ratio (Tovar Sepulveda, Shen et al. 2002; Miyaji, Nakase et al. 2003).

Apoptosis was measured in the present study using an indicator of early stage apoptosis (mitochondrial membrane permeabilisation). The results obtained are consistent with both Miyaji *et al* and Tovar Sepulveda *et al*'s studies which also demonstrated that PTHrP acts at an early stage in the apoptosis pathway. The present study has also demonstrated that PTHrP regulates expression of certain integrins. Integrins are known to regulate the activity of members of the Bcl-2 family, for example ligation of $\alpha_5\beta_1$ increases transcription of the *bcl-2* gene in CHO cells (Matter and Ruoshlahti 2001). As such rather than acting on Bcl-2 directly, PTHrP's anti-apoptotic effects may instead be mediated through modulation of integrin expression. Further study will be required in order to determine if this is indeed the case and could be carried out through the use of RNAi of integrin subunit expression and examination of any resultant changes in apoptosis, in particular Bcl-2 expression.

8.4 PTHrP AND ADHESION

8.4.1 Changes in cell adhesion

The main focus of the present study was to investigate the role that PTHrP plays in adhesion. The exogenous PTHrP peptides (1-34), (67-86), (67-101), (107-139),

(140-173) had no effect on cell adhesion, which is consistent with other studies by Ye *et al* and Shen and Falzon. Ye *et al* used exogenous PTHrP (7-34), (67-86) and (107-139) during their investigation but did not demonstrate any effect on HT29 cell adhesion. Similarly Shen and Falzon examined the effect of PTHrP (1-34) on PC-3 cell-surface integrin expression and PTHrP (1-34), (67-86), (107-139) and (141-173) on MCF-7 cell adhesion, migration, invasion and cell surface integrin expression but demonstrated no significant effects (Ye, Seitz et al. 2001; Shen and Falzon 2003; Shen, Qian et al. 2004).

Both the PTHrP (1-34) and (38-64) neutralising antibodies used in this study caused a significant reduction in adhesion to collagen type I and fibronectin in both the breast and GI cell lines and a reduction in breast cell adhesion to laminin. To date no one has examined the effect of antibody addition on cell adhesion so it is not possible to compare these results. However the results obtained did indicate that PTHrP was involved in adhesion and it was decided to further investigate the mechanism whereby PTHrP regulated adhesion.

Although statistically significant, the reduction in adhesion obtained with the PTHrP antibodies was modest and proved to be an inefficient means of inhibiting the biological actions of PTHrP. It was therefore felt necessary to find a system whereby PTHrP could be more effectively regulated. As RNAi has been shown to be an effective yet simple way of inducing post-transcriptional gene silencing (Bertrand, Pottier et al. 2002; Ramaswamy and Slack 2002) it was decided to design an siRNA specific for PTHrP.

Three siRNAs (all targeted within exon VI of the PTHrP gene, which is common to all three PTHrP isoforms) were initially tested for their ability to reduce PTHrP gene expression and the most effective of the three was chosen for further study. Once it had been established that this siRNA (Target 19) was able to significantly reduce both gene and protein expression, the resultant effects on adhesion were investigated. Following gene silencing, there was a significant reduction in adhesion to collagen type I and fibronectin in each of the breast and GI cell lines examined. However, whilst there was a reduction in adhesion to laminin in the breast cancer cell lines there was no alteration in GI cell adhesion to this ECM protein.

The pattern of changes in adhesion was consistent when either siRNA or antibodies were used i.e. there was no change in GI cell adhesion to laminin with either approach. However, compared with the antibodies, the siRNA was able to induce a much greater decrease in adhesion. For example, whereas the anti-PTHrP antibodies induced a ~15% decrease in MGLVA1 adhesion to collagen type I, the siRNA caused a ~52% decrease.

When PTHrP was over-expressed in a GI and breast cell line there was an increase in adhesion to both collagen type I and fibronectin whereas only breast cancer cells demonstrated an increase in adhesion to laminin, again consistent with previous observations. This selective regulation of adhesion by PTHrP may relate to differences between the sites of metastases for cells of GI and breast origin during cancer progression.

Paget's "seed and soil" hypothesis proposes that cancer cells metastasise to an area

which has a favourable microenvironment. Primary tumours appear to exhibit a partiality for one particular organ during metastasis, for example breast cancer cells predominantly metastasise to bone (Powell, Southby et al. 1991) whilst gastrointestinal tumours tend to establish secondary tumours in the liver (Fidler 1990; Belluco, Mammano et al. 2005). PTHrP expression in MDAMB231 cells demonstrated a correlation with adhesion to collagen type I, fibronectin and laminin, which may be linked to the composition of bone which is comprised of 95% collagen type I and contains fibronectin and laminin within the bone marrow ECM (Vituri, Alvarez-Silva et al. 2000), with laminin also a key component of the sinusoidal basement membrane (Gu, Kortesmaa et al. 2003). Hepatic sinusoidal endothelial cells do not possess a basement membrane but both fibronectin and collagen type I are prevalent in the space of Disse, which separates hepatocytes from sinusoids (Martinez-Hernandez and Amenta 1995), so cells which have an increased ability to adhere to fibronectin and collagen type I but not laminin (i.e. GI cells), may exhibit preferential metastasis to the liver rather than bone (Shah, Haddad et al. 1997).

8.4.2 Regulating Integrin Expression

The cell adhesion receptors, integrins, are the largest group of adhesion molecules (Hynes 2002) and were considered to be the best target for elucidating the pathway linking PTHrP to cell adhesion, an idea supported by Shen and Falzon's studies. Integrin expression was therefore examined in relation to PTHrP expression and comparisons of endogenous PTHrP and integrin expression revealed a parallel increase in integrin expression alongside an increase in PTHrP expression (Shen and Falzon 2003; Shen and Falzon 2003; Shen, Qian et al. 2004; Shen and Falzon 2005) .

Subsequent manipulation of PTHrP expression (over-expression and silencing) supported these early findings and several subunits were identified as potential targets (specifically α_1, α_3, α_5, α_6, β_1 and β_4). The results shown in Chapter 5 also suggested that PTHrP was able to selectively regulate integrin expression i.e. α_6 and β_4 expression was affected in the breast but not the GI cell lines. As α_6 combines with either β_1 or β_4 to form a laminin receptor these results are consistent with the previously described changes in cell adhesion where only breast cancer cell adhesion to laminin was affected following manipulation of PTHrP.

These findings are also consistent with those described by Shen and Falzon who examined MCF-7 cell adhesion and only detected an increase in adhesion to laminin in association with an increase in expression of α_6 and β_4 expression. Although the MDAMB231 cells used in the present study also demonstrated changes in adhesion to collagen type I and fibronectin these results suggest that adhesion to laminin is a key aspect of PTHrP's role in breast cancer metastasis to bone (Shen, Qian et al. 2004).

Some of the results described in the present study are comparable with those previously described in the literature, specifically the increase in adhesion and integrin expression as a result of PTHrP over-expression. However there are some disparities. For example Ye *et al*'s 2001 study used HT29 cells over-expressing PTHrP and, whereas they demonstrated an increase in adhesion to collagen type I and no change in adhesion to laminin, they did not detect any change in adhesion to

fibronectin which is in contrast to the results described here. Ye *et al* used the same cDNA encoding PTHrP (-36-136) but measured the increase in PTHrP expression by the level of immunoreactive PTHrP secreted into the culture medium, the transfected cells demonstrating an 80% increase in PTHrP secretion compared with vector control. PTHrP expression in this study was measured by RT-PCR and not PTHrP secretion and it is therefore not possible to directly compare the fold increase in PTHrP expression between the two studies (Ye, Seitz et al. 2001).

It may be possible to explain the discrepancy between Ye *et al*'s study and the results shown here by differences in experimental protocol, as Ye *et al* used crystal violet to quantify cell adhesion compared with MTT uptake used in the present study. Similarly Shen and Falzon also used a different method to quantify cell adhesion (alkaline phosphatase activity) and their examination of integrin expression correlated with their observed changes in cell adhesion which was also demonstrated here. This therefore suggests that the method used to quantify cell adhesion may not significantly affect the results obtained.

Although Shen and Falzon used different cell lines to the ones used in the present study they did use cell lines derived from breast and colon tissue. Therefore, a similar adhesion profile would be expected, but was not observed. Consequently these disparities may be indicative of a more general problem in the varied integrin expression in immortalised cell lines. Kiefer and Farach-Carson noted this possibility in their 2001 study; using the prostate cancer cell line PC-3 to examine integrin expression in PC-3 cells they compared their results to the findings from another group and found different expression levels of certain integrins.

Specifically, where Kiefer and Farach-Carson found cell surface expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ Kostenuik *et al* found only low expression of $\alpha_1\beta_1$ and $\alpha_3\beta_1$. Kiefer and Farach-Carson attributed these inconsistencies to either "subtle phenotypic differences" in the different PC3 cell clones used or the different monoclonal antibodies used in the two studies. If phenotypic differences exist between clones derived from the same parental cell line then there will certainly be phenotypic differences between distinct cell lines (Kostenuik, Singh et al. 1997; Kiefer and Farach-Carson 2001).

Such phenotypic differences are especially relevant when examining integrin expression as even a small difference in integrin subunit phenotype may significantly alter the adhesion profile of cells due to the numerous interactions that each α subunit can have with each β subunit e.g $\alpha_1\beta_1$ binds to collagens and laminin whereas $\alpha_4\beta_1$ and $\alpha_4\beta_7$ both bind to fibronectin (Hynes 2002).

8.4.3 PTHrP as a Transcription Factor

In their 2003 review on the intracrine actions of PTHrP, Fiaschi-Taesch and Stewart suggested a number of possible roles for PTHrP within the nucleus, including a role as a nuclear export factor for mRNA, whereby PTHrP would shuttle mRNA between the nucleus and cytoplasm. They also suggested that PTHrP may bind to other nuclear or cytoplasmic proteins in order to carry out its nuclear actions as well as indicating that PTHrP may be a transcription factor due to the homology of PTHrP's NLS to the NLS of several transcription factors that are known to bind directly to DNA (e.g. *c-jun, c-fos* and p53) (Fiaschi-Taesch and Stewart 2003).

The results described in Chapters 5 and 7 of this study suggest that PTHrP plays a role in transcriptional regulation of integrins. As both integrin gene and protein expression were altered following PTHrP silencing and over-expression this suggests that PTHrP regulates integrin expression at the transcriptional level which is then translated to similar changes in protein expression. In addition the increases in both adhesion and integrin expression upon PTHrP over-expression were reversed when the NLS of PTHrP was deleted, suggesting that PTHrP must locate to the nucleus to carry out this role.

It was therefore decided to explore the role of PTHrP in integrin transcription by examining integrin promoter activity. As discussed in Chapter 7 it was not possible to construct an α_2 or α_6 reporter vector, however using the α_5 vector it was possible to demonstrate a significant increase in promoter activity in the cell lines over expressing PTHrP, but not in those cell lines lacking a NLS. This is consistent with the previous results demonstrating that intranuclear PTHrP increases expression of specific integrins and in turn, affects cellular adhesion.

To further define the role of PTHrP as a transcription factor a number of additional studies are required. Transcription factors bind directly to specific sites within the promoter or enhancer region to regulate transcription; consequently analysing whether PTHrP directly interacts with the integrin promoter region would help to conclusively determine the nature of PTHrP's role in integrin expression. Employing a DNA footprinting assay or electrophoretic mobility shift assay to demonstrate that PTHrP binds directly to the integrin promoter region would clarify

the nature of PTHrP's role in integrin transcription. Alternatively PTHrP may act indirectly by regulating a known transcription factor and as the promoter regions of the majority of integrins have been characterised (e.g. α_5 (Birkenmeier, McQuillan et al. 1991; Nishida, Kitazawa et al. 1997) and α_6 (Nishida, Kitazawa et al. 1997)), investigation of the interaction of PTHrP with transcription factors which bind to these promoters would be another possible approach.

PTHrP has also been shown to affect different integrin subunits in different cell types which suggests that PTHrP is able to selectively regulate integrin expression Similarly, PTHrP isoform expression is selectively regulated by the growth factors TGF-β and EGF, which are able to increase expression of the PTHrP isoforms (1-141) and (1-139), respectively (Sellers, Luchin et al. 2004).

Alternative splicing of the PTHrP gene results in the transcription of three different isoforms and varied expression levels have been shown in different cancers. For example Sellers *et al* (2004) demonstrated that while PTHrP (1-141) was the only isoform expressed in normal lung tissue, all three isoforms were detected in adjacent neoplastic samples suggesting that PTHrP expression is altered during carcinogenesis. The PTHrP gene contains three different promoters, suggesting a degree of transcriptional control and a complex regulation of expression. The use of alternative promoters may therefore allow tissue-specific gene transcription. For example, Richard *et al* demonstrated a change in the ratio of promoter-initiated transcripts in carcinoma samples compared with healthy tissue; the ratio of P1:P2:P3 changed from 3:74:23 to 21:39:40 (Richard, Luchin et al. 2003; Sellers, Luchin et al. 2004).

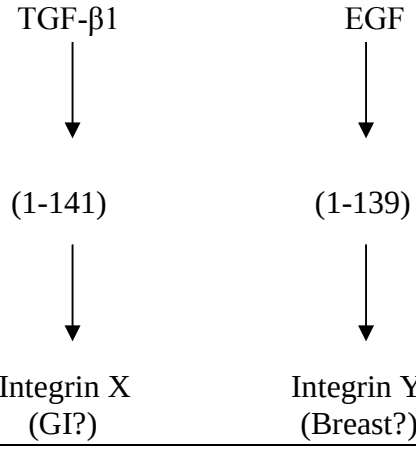
Similarly Bouizar *et al*'s study of PTHrP isoform expression in relation to the formation of bone metastases in primary breast tumours also examined the usage of the three promoter sites and found that the P3 promoter was used more frequently in patients who developed metastases. This study also demonstrated greater expression of PTHrP (1-139) than (1-141) in breast tumours as well as showing that (1-139) expression was greater in patients who developed bone metastases compared with non-metastatic tumours (Bouizar, Spyratos et al. 1999).

During the early stages of this study the breast cancer cell line MDAMB231 induced to over-express PTHrP was used as a positive control for PTHrP expression. The cells were obtained from Dr Guise's laboratory where they had been stably transfected with cDNA encoding for PTHrP (1-141). During this study despite demonstrating a marked increase in gene expression there was only a small increase in adhesion and integrin expression. In comparison, when the same wild-type cell line was stably transfected with cDNA encoding for PTHrP (1-139) there was not only a significant increase in integrin expression, but this resulted in a significant increase in adhesion to the extracellular matrix. These differences may therefore be indicative of isoform-specific regulation of cell adhesion. For example Bouizar *et al*'s study suggested that whereas PTHrP (1-139) played an important role in breast tumour progression PTHrP (1-141) was less important. Based on their study and the results presented here it can be hypothesised that each PTHrP isoform may selectively regulate transcription of different integrin subunits. For example, if PTHrP (1-139) is expressed at higher levels in breast cancer than the other two isoforms and it selectively regulates transcription of either α_6 or β_4 this would in turn

increase adhesion to laminin and thereby aid metastasis to bone (Guise, Yin et al. 2002).

TGF-β1 and EGF have not only been shown to modulate PTHrP expression but they have also been linked with integrin expression. For example Kostenuik *et al* found that TGF-β1 increases binding of PC3 cells to a type I collagenous matrix by increasing integrin α_2 expression whilst Adelsman *et al* stimulated EGF receptor activation in a breast cancer cell line using EGF and HRGβ and revealed an increase in integrin β_1 activity resulting in an increase in cell adhesion and migration. A link between EGF, TGF-β1, PTHrP and integrin expression can thus be proposed (Figure 8.2). If TGF-β1 and EGF can selectively regulate PTHrP expression, this may then result in selective regulation of individual integrin subunits. Such a hypothesis could be tested by silencing EGF or TGF-β1 and then analysing the resultant changes in both PTHrP and integrin expression (Kostenuik, Singh et al. 1997; Adelsman, McCarthy et al. 1999).

Figure 8.2 Tentative model describing the pathway which could lead to tissue-specific regulation of PTHrP and in turn integrin expression



8.5 PTHrP AND THERAPY: ANTIBODY vs. SMALL INTERFERING RNA

Following its initial discovery, several studies targeted PTHrP in an attempt to treat HHM. For example Kukreja *et al* used antibodies to block the biological activity of PTHrP, producing a significant decrease in serum and urine calcium levels as well as urine cAMP. However, serum calcium levels did not normalise in all animals which the authors attributed to an insufficient concentration of antibody to fully neutralise PTHrP activity. In a similar study Talon *et al* also used antibodies to neutralise the effects of PTHrP *in vivo* and following an initial study found it necessary to increase the frequency of the treatments in order to induce a response. Both of these studies support the results presented here where only a modest decrease in PTHrP activity was induced by the anti-PTHrP antibodies (Kukreja, Shrevin et al. 1988; Talon, Lindner et al. 2005). Several other *in vivo* studies using anti-PTHrP agents have been described including antibody based approaches (Guise, Yin et al. 1996; Massfelder, Lang et al. 2004) and a recent study published by Chugai Pharmaceutical Company described the effective use of an anti-PTHrP monoclonal in a human xenograft model of bone metastasis where the antibody, directed against PTHrP (1-34), reduced osteolytic bone metastasis. This led the authors to conclude that the anti-PTHrP antibody could be used as an effective new treatment for bone metastasis in breast cancer (Saito, Tsunenari et al. 2005).

Other more novel approaches have also been used. For example, nucleotide analogues of guanine have been used to target PTHrP (Gallwitz, Guise et al. 2002) and PTHrP peptides have also been used to vaccinate mice against PTHrP. Another alternative approach was described by Arima *et al* who used PTHrP (102-111) or (110-119) peptides to generate cancer-reactive cytotoxic T lymphocytes from

peripheral blood mononuclear cells derived from patients with different tumour types, including gastric, colon and renal cancer. The authors suggested that PTHrP could be targeted in specific immunotherapy (Arima, Matsueda et al. 2005).

However, despite numerous *in vivo* studies there have yet to be any human clinical trials with an anti-PTHrP agent and it remains to be seen whether an effective anti-PTHrP therapeutic can be developed.

RNAi is a relatively new discovery; however, siRNA have been shown to be powerful tools for the induction of posttranscriptional gene silencing. Use of the PTHrP siRNA described in this thesis resulted in significant reduction in proliferation, apoptosis and cell adhesion. In all cases the effects were greater than those observed with neutralising antibodies. These results therefore suggest that siRNA could be an effective therapeutic tool. As RNAi is a relatively new field the mechanisms of action are being further investigated and continually deduced so it is therefore unlikely that the therapeutic potential of siRNA has yet been fully exploited. Indeed, although a number of weaknesses associated with siRNA have been identified, as many of the problems are similar to those encountered in anti-sense technology (e.g. uptake) most have been addressed and quickly overcome. For instance siRNAs have a relatively short period of action and unmodified duplex siRNA are quickly degraded by nucleases within the body and filtered through the kidneys (Lieberman, Song et al. 2003) but cationic polymers and liposomes have been shown to increase siRNA half-life *in vivo* and chemical modifications to siRNA have been shown to prevent nuclease degradation. In general the RNAi process is more efficient than anti-sense; for example, the RISC complex is recycled thereby

allowing it to target further RNA molecules and lower concentrations are needed in order to induce an effective response (Kurreck 2004).

The use of RNAi for therapeutic purposes is still in its infancy however there are a number of promising RNAi-based therapeutics currently in development. The first RNAi-based drug filed with the US Food and Drug Administration for clinical trials was Cand5, developed by Acuity Pharmaceuticals to treat age-related macular degeneration (a form of blindness). Cand5 decreases VEGF in human retinal pigment epithelial cells thereby reducing the growth and leakage of abnormal blood vessels. It has been successfully used in a primate model of age-related macular degeneration (Tolention, Brucker et al. 2004) and with Phase I trials completed, Phase II trials began early in 2006 (www.acuitypharma.com). To date the trial has produced positive results and Cand5 has shown "dose dependent efficacy without discernable adverse effects" (http://www.acuitypharma.com/press/release13.pdf).

Other companies are using RNAi to target diseases such as HIV (www.internationaltherapeutics.com; www.benitec.com.au), Parkinson's disease (www.alnylam.com) and diabetes/obesity (www.benitec.com.au, www.cytrx.com). A number of companies are also using RNAi to target cancer. For example Atugen AG are exploring the use of siRNA to target hepatocellular, prostate, pancreatic and lung cancer (www.atugen.com). Since PTHrP is a mitogen and an anti-apoptotic factor, silencing PTHrP could also be used to both reduce tumour growth and induce apoptosis as part of a multi-pronged attack.

However, the transient effects of siRNA result in gene knock-down rather than knock-out. Consequently, combining a PTHrP siRNA with another established treatment designed to kill cells may produce a more effective, synergistic effect. For example Stoeltzing *et al* used a novel inhibitor of $\alpha_5\beta_1$ (ATN-161) in conjunction with continuous 5-FU infusion and was able to significantly reduce the size and number of colorectal liver metastases in mice, as well as increasing apoptosis and overall survival. Neither ATN-161 or 5-FU achieved this individually (Stoeltzing, Liu et al. 2003).

A selective but effective delivery mechanism will also prove vital; the success of Cand5 can be attributed to the local delivery mechanism whereby it is injected directly into the eye.

8.6 FUTURE RESEARCH DIRECTIONS

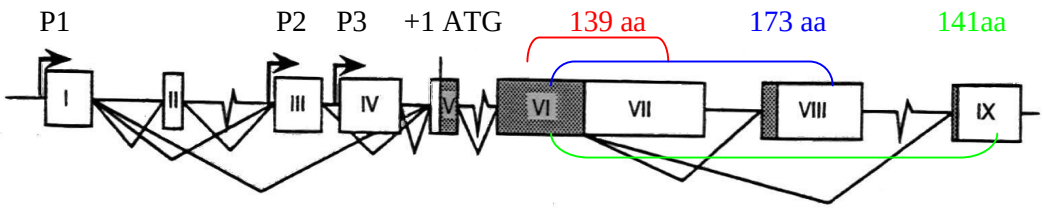
An increase in transcription of the α_5 subunit was demonstrated but time constraints prevented the construction of a reporter vector containing the promoter region of the α_6 subunit. Demonstrating an increase in promoter activity of the α_6 subunit in breast but not colon cell lines would add weight to the suggestion that PTHrP is able to selectively regulate integrin expression and thereby adhesion. As such exploring the transcriptional activity of this subunit in the PTHrP over-expressing cell lines would provide further evidence for the tissue-specific effects of PTHrP. Similarly co-transfecting cells with the reporter vectors and PTHrP siRNA would also support the previous results regarding the role of PTHrP in integrin transcription.

In order to determine if PTHrP is a transcription factor it would first be necessary to isolate the region responsible for mediating the effects of PTHrP on transcription by cloning smaller sections of the promoter region into the reporter vector. It would then be possible to conduct a DNA footprinting assay to determine if PTHrP interacts directly with that region.

The possibility that the tissue-specific effects of PTHrP are mediated via the three different PTHrP isoforms could be explored by not only examining isoform expression in a panel of tumour-derived cell lines from different tissues and then comparing this with the endogenous levels of integrin expression but also by stimulating cells with either EGF or TGF-β1 and observing any resultant changes in isoform expression.

Similarly transfecting cells encoding for PTHrP (1-141) and (1-173) would also allow a further exploration of the PTHrP's regulation of cell adhesion. Alternatively siRNA could be used to selectively silence each of the three isofoms. The PTHrP siRNAs used here were targeted against exon VI as this encodes for all three PTHrP isoforms. Targeting siRNA against exons VII, IX and VIII would allow the silencing of the (1-139), (1-141) and (1-173) isoforms, respectively, and make it possible to elucidate any isoform specific activities of PTHrP (Figure 8.3).

Figure 8.3 Gene structure of PTHrP, highlighting the different exons which could be silenced to selectively decrease isoform expression



Unfortunately it would not be possible to examine protein expression of the different isoforms due to the small differences in length i.e. there is only a two amino acid difference in length between the (1-139) and (1-141) isoforms, however it would be hoped that examination of gene expression would provide sufficient insight.

Reports from different groups have used the same cell lines but obtained small yet significant differences compared with previously published data (Kiefer and Farach-Carson 2001; Maioli and Fortino 2004). Such reports imply phenotypic differences between cell lines and suggest that these groups are using different clonal populations of the same cell line. These different populations have likely arisen after years of propagation following immortalisation. As such, the use of immortalised cell lines may be an unreliable tool when investigating protein function and signalling pathways. Consequently contrasting EGF/TGF-β1, PTHrP and integrin expression in tumour and normal tissue may provide a more accurate picture of the role of these proteins in tumour progression as well as an indication of a possible pathway connecting these proteins. The use a tissue array would allow us to investigate whether expression of EGF, TGF-β1 or the integrin subunits correlates with that of PTHrP.

The results described here show for the first time that an siRNA can be used effectively to silence PTHrP expression. RNAi is an ever expanding field and with several siRNA currently being tested in clinical trials there is increasing evidence for their potential as effective therapeutic agents. As such an *in vivo* study using the PTHrP siRNA would allow the examination of the therapeutic potential of a PTHrP siRNA as an inhibitor of metastasis and tumour growth. Increasing the length of the PTHrP siRNA to 27nt may also increase the potency of the PTHrP siRNA (Kim, Behlke et al. 2005) and should consequently be tested *in vitro* prior to embarking on such a study.

In order to establish if the composition of bone and liver helps to dictate which tumour types metastasise to which organs it would be necessary to use an invasion assay using a chemoattractant (e.g. collagen, fibronectin or laminin) and examining whether the cells invade into the Matrigel and also whether regulation of PTHrP expression (i.e. knock-in or knock-down) affects any resulting invasion.

8.7 SUMMARY

The data presented here demonstrate that PTHrP is not only a mitogen and anti-apoptotic factor but is also a key protein in facilitating cell adhesion. Additionally it has been shown that this may be mediated via changes in integrin expression and more importantly has demonstrated the mechanism by which this occurs - through transcriptional activation of the cell adhesion molecules integrins.

Chapter 9:

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Chapter 10:

Appendices

10.1 APPENDIX 1

DNA sequence for integrin subunit α_2
(Accession Number L24121)

GGTTTCAGCAATGTGCTGGAAATTTGTGGCCAAACTTTTGGATAAAACAT
AAGATTCAGACAAGAAAAATTAATATCCTATTTCTTAGGTTTTTAACTAA
AAATCTCGGATATAGAATGTTCTCTTTCCAGATGTGAGACTGAAATAATG
GAAGAAAATGGTCACAGTCTATGTCACGGTGTAATGTAGACAGGGAGAG
TCACAAGTGCATGTATGGGCTCTCAGCCTTTTCTCTGGCCTTGAAGTTAA
TCTCCTAAACTCAGTCATTAAATCAGCTCTTAATTATCTCGGAGGAGAGG
AAGTAATGATGATTTCTCTCTCTCTGTCTCTCTGTCACACACACACACAC
ACACACACAGCTCTTGCAGCAGGTATTGCTTAAATATCACCTTGGATAAT
CATAACTTGTGAGCAGATCTTCTTTCCTGATGAGTTAGTTTCTAACCTGGT
CATTCTGCGCTTATTTTTGTCCCTTTCTCCACCCACTTAGGAAAAACAGAG
AAAGGGACGCACCGCGCAGCCCCTAGGCACTGTGGTTTAGGGCTAGTGC
CCTCGGACCCCGCTGCCAGGAGCCGGGCGCTGCCAAGGGCTGCGGAGGG
GCCACGTTCTCCCGGGGACTGGGGCATCTCCTGCGTACTGGCGACAGGCT
CGCGGGGGCGGAGTGGTGCCAGGGCGGGCGCTCGCCCGTCCGGATATGC
CCACCCGTCCCGTCCAGGGCAGGAAAGCCTGCCAGGGCGCCATCCCCAT
CCCCACCGCCTCCAGGCTGCCGGGGCTGGGCCGCTGTACGGGAGCCAAG
GTCGGTGCCCCGCGTGTGGACGAGCCGAGGTGCAGCCCGCGGGGCCGCA
GGGCCGGGGTGGGGCGGGCGCGGCCGGAGCAGATCCGGTGTTTGCGGAA
TCAGGAGGGGCGGGCCGGGGCGGGCCCTCGGCGCTGCAGGAGCTGCCCA
GAAACTTTTCCCTGCTCTCACCGGGCGGGGGAGAGAAGCCCTCTGGACA
GCTTCTAGAGTGTGCAGGTTCTCGTATCCCTCGGCCAAGGGTATCCTCTG
CAAACCTCTGCAAACCCAGCGCAACTACCGTCCCCCCGTCAGACCCAGG
ATGGGGCCAGAACGGACAGGGGCCGCGCCGCTGCCGCTGCTGCTGGTGG
TAGCGCTCAGTCAAGGTAAGCGGGGATTTC

10.2 APPENDIX 2

DNA sequence for integrin subunit α_5
(Accession Number U48214)

GTTTACACCGATTAGGAGCTGAAGGTTTGGGTCCCTTTCGAAGACTTTCC
ACCACCACCACCACCTCTCACAATTCTCCAGGTTTGTAGGAACCCGGCCA
GTTTAATCAAGCAGGTACTGAGGGGCCAGCCACTCGCAGGCTTTTGGTCA
ACTGTCAGATCCAAGGAAGGGGCTCAGCAGAGGTTTACATACCCTAGGA
CCAAGAAGGGGGCAAACTGGAGGAGAATCCTCCTGATGAATGACCCCAA
ACTCCTGGGTTTTCAATACTGAAGAAGCTTGGAGGAGGAAGCAGCGGAA
CTTGAGGAGCCCGTGTAGGTCCTCTAGCAAGCTGACTGCGAACGCTGTCT
CTGTACCTCAACTGTTGTGTCCCAAATCCAGCTGGCCAGAACCCAGGCAC
CCGGCGGCCCCGGAAGGCAAGGGGGAATCCCAGTTGGCCGGACGCCAG
AGGAGTCTCCTGTCTCTTTAGACCCCAAAGTCTCCTCCCTAAAGCACTGA
GGGAGACCCCACACACATATACAGGCTTCCTCCGCCCACCAGAGGTGAT
TCCTTTCCTCATTAGGAAATTCTCCGCTCCCTTTTCCGACTCGTTTTCCGA
GCGTTTACGTTGTACATCTGGAAAGGAATGGGGAGGAAGGAGGGGGAA

GCAGAGAGGAGGGGGAAGAACCCAAACCCGCCCAGTCTAACCCAGTCC
AGACAACCGGCTTCCAGCTGGGGCTGGGGAAAGGGGGTTGGAGGGGTGC
GCCCCCCCCCCACGCCCCTTAGGGGTGGGGGACGCGGGCTCAGAGTTTC
CAGGGACCCAGGAATGCCCCCCCGCCAGCCCCCTCGGCAGGCGGGGGGA
GGGCTCAGCCGGGAGTTTGGCAAACTCCTCCCCGCGTTGAGTCATTCGCC
TCTGGGAGGTTTAGGAAGCGGCTCCGGGTCGGTGGCCCCAGGACAGGGA
AGAGCGGGCGCT

10.3 APPENDIX 3

DNA sequence for integrin subunit α_6

(Accession Number AF078694)

CCCGGGTTAAGAACCCTGCAGGATAAGGTTGCCCCTAGGGCCAACACCT
CACTGGAGCCCCGCCTCATGTCCTGGAGGCAGGAGCCTTCATGCCACCTA
CACAGAACTCGGAGCTGTCTCTTGGCCCAGCAGTTCTCCCCACAACTAGT
TCTGAAAGTCCGGGGCTAGGAAAGAACGGCATCGTCGCCTGAGCTCCTG
GCGCCACTTTAAACAACCCATCCTTGACTTGCGTGACTTCTTCCACAAGC
TCTCCTGGTCACCTGGGCAAAATCCTAGGTGATCTGGGGACAAGGCGGA
ACTTCGGTTTTCTGATCTGCAAAAAGAAACACCTACCTCATAGGACTGTT
AGGATGAATTGAGGTAATGGCTGGGTTGTGTACATTATACAGCACTATCA
AGGTGTGCGCTGTGATCATTTTGAGGGTTGTTAGGTGTTTGAGGACCCAG
AACAGTCTACACAGCTGTAGTCCCCAAGTGTTGGGCACGCCTTAAGCGCT
CCATAAACACCTGTAGAAATGAATGAATGACGTATGCATCTGCACGTGG
GCCCACATCTGCAAGAACAGGCTGCTCAGGCCATGAGGCCCGGTGCATC
ACCTGCACTTCTCTTTATAACGGGTAGTAAAGTCTCCCTCGCTCTGTGCTA
CTCGGCAACCACAATTCTGTCCACAGAGGGCGGCGCAGTGGGGCTGCTT
CGCCGCGAGCTCGCCTCCGGGGCTCCCACGTCGTGGCTTCCGGGCAGGTA
CCGGGCAGCTGGAGACGCCAGAGCCGGCGGGTAAGGTGCGGGCGGTGC
GCCGGGCCGCGGGCGCGCAAGGAGGGGCGAGAGGGTGGGGAGGGGCGG
GGCCGGCGTCCTCGTCACTTGATAAAACGCCTGCGAGTCTCCAGAGAAC
AACGGGCTCATTCAGCGGTCGCGAGCTGCCCGCGAGGGGGAGCGGCCGG
ACGGAGAGCGCGACCCGTCCCGGG