

REGULATION OF PARATHYROID-HORMONE RELATED
PEPTIDE IN A SQUAMOUS CERVICAL CARCINOMA
CELL LINE, CASKI

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**REGULATION OF PARATHYROID-HORMONE RELATED PEPTIDE IN A
SQUAMOUS CERVICAL CARCINOMA CELL LINE, CASKI**

By

© Joy Ann Buckle

**A thesis submitted to the School of Graduate
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ABSTRACT

PTHrP is produced by primary and established cervical keratinocytes. However, the factors involved in the expression and regulation of PTHrP in malignant cervical cells has not been characterized. The ectocervical epithelium is normally under the control of the sex steroid hormones, estrogen and progesterone. Here I report the effects of known regulators of PTHrP (EGF, dexamethasone and 1,25-dihydroxyvitamin D₃) as well as the effects of potential tissue-specific regulators, 17 β -estradiol and progesterone, on PTHrP expression and secretion in the human papillomavirus type 16 (HPV-16) infected squamous cervical cancer cell line, CaSki, using Northern analysis and radioimmunoassay.

A dose-dependent increase in PTHrP mRNA levels was observed in response to EGF in the absence of FCS, with maximal stimulation (normalized to cyclophilin) at 20 ng/mL and a 3-fold increase in immunoreactive (i) PTHrP at 24 hours. 17 β -Estradiol produced a dose-dependent increase in PTHrP mRNA expression with maximal stimulation observed at 10⁻⁸ M. At this concentration, 17 β -estradiol produced a 2.5-fold increase in iPTHrP at 24 hours. Progesterone did not produce a significant dose-dependent change in PTHrP mRNA expression. Nevertheless, the effects of progesterone were examined in time course studies at a concentration of 10⁻⁸ M. A dose-dependent inhibition of PTHrP mRNA expression was observed for both dexamethasone and 1,25-dihydroxyvitamin D₃, with maximal effects at

10^{-8} M. Both produced a 50% reduction in iPTHrP relative to control values at 24 hours in the presence of FCS.

Time course studies were subsequently performed using those doses which showed maximal effects in dose-response experiments. An early increase in PTHrP mRNA expression (relative to control) was observed in response to EGF with a peak of 4.5-fold at 2 hours. Both dexamethasone and 1,25-dihydroxyvitaminD₃ produced only slight increases in PTHrP mRNA expression, relative to the time zero (basal), which were maximal at 2 and 4 hours, respectively. However, 24 hours after stimulation, a 50% inhibition of PTHrP mRNA expression was observed (relative to control) for both hormones. Stimulation with progesterone resulted in a 2-fold increase in PTHrP mRNA expression with peak effects at 2 hours. Treatment of the cells with 17 β -estradiol produced a 3.5-fold increase in PTHrP mRNA expression with peak effects at 6 hours.

In HPV-16 established human ectocervical cells (HEC-16), it had been previously demonstrated that known regulators as well as tissue-specific regulators (progesterone and 17 β -estradiol) modified PTHrP production. The pattern of expression of PTHrP in CaSki cervical carcinoma cells was similar to that observed in HEC-16 cells. However, the level of iPTHrP secretion was significantly less in CaSki cells. This suggests a dysregulation of

PTHrP in malignant cervical cells. The response to the tissue-specific sex steroid hormones, progesterone and 17β -estradiol, suggests an interaction with PTHrP and thus a possible regulatory role for PTHrP in the control of cellular proliferation and differentiation of cervical tissue. These results suggest that this model of cervical carcinoma could be used to assess the effects of other potential modulatory factors such as selective estrogen agonists or progesterone antagonists.

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ABBREVIATIONS

AA	Antibiotic-Antimycotic
AREs	AU-Rich Elements
bFGF	basic Fibroblast Growth Factor
CIN	Cervical Intraepithelial Neoplasia
CNS	Central Nervous System
dATP	Deoxyadenosine triphosphate
DBD	DNA Binding Domain
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
E ₂	17 β -Estradiol
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal Growth Factor

EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ER α	Estrogen Receptor alpha
ER β	Estrogen Receptor beta
ERE	Estrogen Response Element
FCS	Fetal Calf Serum
FGF-3	Fibroblast Growth Factor-3
GRE	Glucocorticoid Response Element
HBSS	Hank's Balanced Salt Solution
HEC-16	Human Papillomavirus type 16 immortalized ectocervical cells
HHM	Humoral Hypercalcemia of Malignancy
hPTHrP	human Parathyroid Hormone-related peptide
HPV	Human Papillomavirus
HPV-16	Human Papillomavirus type 16
HRE	Hormone Response Element
IGF-II	Insulin-like Growth Factor II
IL-2	Interleukin-2
iPTHrP	immunoreactive PTHrP
JE gene	monocyte chemoattractant protein -1

kb	kilobases
kDa	kilodaltons
KGM	Keratinocyte Growth Medium
MgCl ₂	Magnesium chloride
MOPS	Morpholinopropanesulfonic acid
mRNA	messenger Ribonucleic Acid
MSF	Migration Stimulating Factor
NaCl	Sodium chloride
NaH ₂ PO ₄	Sodium phosphate monobasic
Na ₂ HPO ₄	Sodium phosphate dibasic
ng eq	nanogram equivalents
nGRE	negative Glucocorticoid Response Element
NLS	Nuclear Localization Signal
NP-40	Nonidet P-40
nVDRE	negative Vitamin D Response Element
OBBP	Outdated Blood Bank Plasma
OD	Optical Density
PBS	Phosphate Buffered Solution
PR	Progesterone Receptor
PRA	Progesterone Receptor A

PRB	Progesterone Receptor B
PRE	Progesterone Response Element
Prog	Progesterone
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-related Peptide
RIA	Radioimmunoassay
rRNA	ribosomal Ribonucleic acid
S	Synthesis phase of the cell cycle
SDS	Sodium dodecyl sulfate
SEM	Standard Error of the Mean
SIL	Squamous Intraepithelial Lesion
SREs	Serum Response Elements
SSC	Sodium chloride and Sodium citrate Solution
TCA	Trichloroacetic acid
TE Buffer	Tris-EDTA Buffer
TGF- β	Transforming Growth Factor-beta
TGF- γ	Transforming Growth Factor-gamma
TRE	Thyroid Response Element
Tris	Tris Base

Tris-HCl	Tris-hydrochloric acid buffer
Tyr	Tyrosine
UTRs	Untranslated Regions
VDR	Vitamin D receptor
VDRE	Vitamin D Response Element
1,25(OH) ₂ D ₃	1,25-dihydroxyvitamin D ₃
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1: INTRODUCTION

1.1 GENERAL BACKGROUND

Parathyroid hormone (PTH) was initially hypothesized as the tumour product that was responsible for hypercalcemia observed in many cancer patients. The clinical syndrome, termed Humoral Hypercalcemia of Malignancy (HHM), was found to be due to the secretion by the tumour of a calcemic factor acting on the skeleton to increase bone resorption and on the kidney to decrease urinary calcium excretion. The PTHrP gene was cloned and sequenced. It has sequence homology with PTH and can therefore interact with the PTH receptor and mimic the actions of PTH.

1.2 HISTORICAL REVIEW

The existence of a parathyroid hormone-like factor that was responsible for the hypercalcemia associated with malignancy was postulated nearly sixty years ago by Fuller Albright (1941). The theory of "ectopic" PTH production by the tumour was favoured, but it was not until the 1960's, with the development of radioimmunoassay (RIA) techniques, that this hypothesis was tested. In 1966, Bernson and Yalow published results of the first RIA for PTH, in which they found significant elevation of PTH levels in the circulation of a number of hypercalcemic patients suffering from lung cancer. In the early 1970's, there was some doubt that PTH itself was the major factor involved in producing the clinical and biochemical features of this clinical syndrome. More comprehensive clinical and biochemical

investigations followed and indicated that the manifestations of HHM were mediated via PTH receptors in bone and kidney (Mundy GR and Martin TJ, 1982). A sensitive cytochemical bioassay for PTH in kidney cells was able to detect PTH-like bioactivity in the serum of patients in whom immunoreactive PTH was undetectable (Goltzman D *et al*, 1981). Studies in PTH-responsive ROS 17/2.8 osteoblastic cells showed that the conditioned medium of three different cell lines (including human prostate carcinoma: PC3, rat Leydig cell tumour: Rice-500 and rat carcinoma cell line derived from the hypercalcemic variant of the Walker tumour: WRC-256) could each stimulate adenylate cyclase in this system (Rodan SB *et al*, 1983). In this study, peptide antagonists of PTH blocked biological activity, but preincubation with PTH antisera did not prevent PTH antagonists from blocking biological activity (Rodan SB *et al*, 1983). This suggested that the active material acting on PTH receptors in ROS 17/2.8 cells was immunologically distinct from PTH. Messenger RNA (mRNA) for PTH could not be detected in any of the tumours associated with HHM (Simpson EL *et al*, 1983). This active material also had a higher molecular weight than PTH (Benson Jr. RC *et al*, 1974). These observations led investigators to conclude that the substance responsible for HHM was different from PTH.

In attempts to identify and isolate the "PTH-like factor", animal models of HHM were developed and cell lines were established from human tumours associated with HHM (Strewler GJ *et al*, 1983; Gkonos PJ *et al*, 1984; Ikeda K *et al*, 1988). This led to the

isolation and purification of a protein from human cancer cell lines which revealed the existence of PTH-related peptide (PTHrP). In 1987, the peptide was isolated from a human lung cancer cell line (Moseley JM *et al*, 1987), a human renal carcinomacell line (Strewler GJ *et al*, 1987) and a breast tumour (Stewart AF *et al*, 1987). Subsequently, three groups identified clones encoding this novel protein (Suva LJ *et al*, 1987; Mangin M *et al*, 1988; Thiede M *et al*, 1988).

1.3 PTHrP GENE

1.3.1 Primary Sequence:

PTHrP has a molecular weight of 17 to 18 kDa (Moseley JM *et al*, 1987). Amino-terminal sequence analysis of purified material pointed to significant N-terminal homology with PTH. Eight of the first thirteen amino acid residues are identical to those of PTH, but beyond this no significant homology was observed (Moseley JM *et al*, 1987; Stewart AF *et al*, 1987; Strewler GJ *et al*, 1987) (Figure 1.1).

PTHrP	A	V	S	E	N	C	L	L	H	D	R	G	R	S	I	Q	D	L	R	R	A	F	F	L	N	N	L	I	A	R	I	N	T	A
PTH	S	V	S	E	I	C	L	N	H	F	L	G	R	N	L	K	S	H	E	R	V	E	W	L	N	K	K	L	Q	D	V	N	N	F

Figure 1.1: Amino acid sequence of PTH(1-34) and PTHrP(1-34).

(Adapted from Bilezikian JP, ed, 1994)

The pronounced homology between PTH and PTHrP at the N-terminus led to the discovery that established linear sequential organization of functional domains in PTH (Potts Jr. JT, 1971) is also present in PTHrP (Kemp BE *et al*, 1987; Yates AJ *et al*, 1988). This homology allowed PTHrP to interact with the PTH receptor and cause PTH-like effects (Jueppner H *et al*, 1991; Abou-Samra AB *et al*, 1992). Studies have shown that the first 34 amino acids of both PTH and PTHrP constitute a fully active region of these hormones for activities relevant to PTH-like effects on mineral metabolism (Kemp BE *et al*, 1987).

The structural organization of the chicken, mouse and rat PTHrP genes have been determined, and all share substantial homology with the human PTHrP (hPTHrP) gene. Mature PTHrPs have been cloned in the human, chicken, rat and mouse, and there is a remarkable conservation of the primary sequence up to amino acid residue 111 (Mangin M *et al*, 1989; Yasuda T *et al*, 1989a; Karaplis AC *et al*, 1990; Mangin M *et al*, 1990a; Thiede MA *et al*, 1990b). Mouse and rat differ from hPTHrP by only three amino acids, whereas chicken differs by seventeen amino acids in the corresponding region. Beyond amino acid 111 there is considerable sequence divergence. This abrupt demarcation of sequence conservation implies that the region PTHrP(1-111) is crucial for the biological function of PTHrP, whereas, the residues C-terminal to 111 either are not biologically relevant or could possibly have unique species-specific functions. Alternatively, these residues could be involved in stabilization of the protein.

1.3.2 PTH versus PTHrP:

Chromosomes 11 and 12 are thought to have a common ancestral origin because they are of similar size, centromere index and banding pattern (Comings DE *et al*, 1972; Mannens M *et al*, 1987). The hPTHrP gene is located on chromosome 12 (12p11.2-p12.1) whereas the human PTH gene is located on chromosome 11 (11p15.4) (Mangin M *et al*, 1989; Suva LJ *et al*, 1989). The PTH and PTHrP genes appear to have arisen from an ancient chromosomal duplication event and represent two members of a small gene family (Philbrick WM *et al*, 1996). The genes for several other closely related proteins have been demonstrated on these chromosomes, consistent with such an ancestral relationship (Martin TJ *et al*, 1997). For example, insulin-like growth factor II and insulin-like growth factor I are located on chromosomes 11 and 12, respectively (Martin TJ *et al*, 1991). The PTH and PTHrP genes have clearly evolved separately although they have a similar exon/intron organization as well as a highly homologous sequence at the N-terminus of the peptide (Philbrick WM *et al*, 1996). This may well account for the shared biological targets of PTH and PTHrP, as well as the interaction with a common PTH/PTHrP receptor.

The PTH protein is normally produced by only two tissues, the parathyroid gland and the central nervous system (CNS), and it functions primarily to regulate systemic calcium homeostasis through its skeletal and renal actions (Bilezikian JP, ed, 1994). In contrast, PTHrP is produced by virtually every cell and tissue within the body and has a broad range

of functions, most of which under normal circumstances have little to do with calcium homeostasis. The PTHrP gene has a complex structure and its product(s) seem to function in an autocrine and/or paracrine (Philbrick WM *et al*, 1996) and/or intracrine (Kaiser SM *et al*, 1994) fashion. There is mounting evidence to suggest that PTHrP may function predominantly, if not exclusively, in an autocrine and/or paracrine fashion (Halloran BP and Nissenson RA, eds, 1992) and that it has a limited role as an endocrine hormone. Growth factor-like properties of PTHrP have been demonstrated in fibroblasts (Insogna KL *et al*, 1989), osteoblasts (Centrella M *et al*, 1989) and embryonal carcinoma cells (Chan SD *et al*, 1990). PTHrP has less in common functionally with PTH than with epidermal growth factor (EGF), transforming growth factor beta (TGF- β), insulin-like growth factor I (IGF-I), or any of a number of developmental factors. A more appropriate name might be TGF- γ or a name that reflects its developmental and physiological roles (Wysolmerski JJ and Stewart AF, 1998).

PTH and PTHrP are equipotent in most of their acute effects and in receptor binding, despite the divergence of the primary sequence in the 14-34 amino acid domain (Orloff JJ *et al*, 1989). PTH(14-34) and PTHrP(14-38), fragments with entirely divergent sequences, both compete for PTH receptor binding, albeit weakly (Abou-Samra AB *et al*, 1989). Although PTH and PTHrP are dissimilar in their primary structures in the 14-34 region, they are

highly similar in conformational or three-dimensional terms (Philbrick WM *et al*, 1996). This is an unusual and important example of shared function due to similarity of secondary/tertiary structure, in the face of a remote relation of primary amino acid sequence.

1.3.3 Human PTHrP Gene:

The hPTHrP gene is a complex transcriptional unit spanning approximately 15 kb (Suva LJ *et al*, 1989; Yasuda T *et al*, 1989b). It can produce multiple PTHrP mRNA species, and these, in turn, encode more than one product (Suva LJ *et al*, 1989; Yasuda T *et al*, 1989b; Kaiser SM and Goltzman D, 1993). Although there is no uniform nomenclature for the exonic organization of the PTHrP gene, it has been generally accepted that the gene is composed of nine exons (Martin TJ *et al*, 1991; Moseley JM and Gillespie MT, 1995) (Figure 1.2).

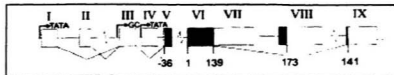


Figure 1.2: Organization of the human PTHrP gene. The coding regions (closed boxes), untranslated regions (open boxes), promoters (arrows) and splicing events (below the map) are shown. (Adapted from Martin TJ *et al*, 1997)

The gene is transcribed by functionally distinct promoters, and mRNA transcripts can be alternatively spliced both at the 5' and 3' ends (Mangin M *et al*, 1989; Yasuda T *et al*, 1989b; Vasavada RC *et al*, 1993). Through alternative splicing, the multi-exonic PTHrP gene gives rise to three initial translation products: PTHrP(1-139), PTHrP(1-141) and PTHrP(1-173) (Bilezikian JP, ed, 1994). These undergo extensive posttranslational processing giving rise to a family of mature secretory forms of the peptide, each with its own physiological functions (Orloff JJ *et al*, 1994; Wysolmerski JJ and Stewart AF, 1998). For example, PTHrP(1-36) initiates multiple events including vasorelaxation, bone resorption and cellular proliferation (Wysolmerski JJ and Stewart AF, 1998). PTHrP(107-139) inhibits bone resorption (Fenton AJ *et al*, 1991). The physiological functions of the mature secretory forms of PTHrP are the subject of ongoing investigations. The PTHrP gene is far more complex than the PTH gene, which has only three exons. However, they possess similar

exonic regions encoding the prepro- sequence (Moseley JM and Gillespie MT, 1995). PTHrP exists as a single copy gene (Yasuda T *et al*, 1989b).

Exons I through IV are noncoding exons, and can be fused in various ways as a result of promoter usage and/or alternate splicing patterns to generate 5' untranslated regions (UTRs) that differ among PTHrP mRNA species (Suva LJ *et al*, 1987; Mangin M *et al*, 1990b). Canonical TATA promoters have been found 5' to exons I and IV, while 5' to exon III is a GC-rich promoter region. These promoters have been identified by primer extension, S1 protection and transient transfection analyses (Thiede MA *et al*, 1988; Mangin M *et al*, 1989; Suva LJ *et al*, 1989; Campos RV *et al*, 1992; Vasavada RC *et al*, 1993). The three spatially distinct promoters are responsible for transcription of the human gene, and appear to be differentially regulated (Mangin M *et al*, 1990b; Vasavada RC *et al*, 1993; Southby J *et al*, 1995). The existence and use of alternative promoters suggests that tissue-specific expression of the gene may occur as a function of the use of differing promoter regions and points to complex gene regulation (Martin TJ *et al*, 1991). Exons V and VI are invariant in mature PTHrP mRNA species described to date. Exon V encodes most of the thirty nine amino acid prepro- or signal sequence of PTHrP. Exon VI encodes the majority of the coding region for the mature protein, up to residue 139, where a splice donor is located; this exonic organization is similar to that of the PTH gene (Martin TJ *et al*, 1991). A key "hinge" sequence occurs at the end of the coding region in exon VI. This four base pair sequence,

GTAA, plays the dual role of a splice donor site (GT consensus) or stop codon (TAA) (Bilezikian JP, ed, 1994). Read-through of exon VI and VII results in the introduction of a terminal signal, thus producing a protein product 139 amino acids in length. Splicing of exon VI to exon VIII or exon VI to exon IX results in C-terminal extensions of PTHrP and produces mature isoforms of 173 and 141 amino acids, respectively (Yasuda T *et al*, 1989b; Mangin M *et al*, 1990b). Thus, alternative 3' splicing produces proteins with variable carboxyl termini, which are of unknown biological significance at present. The 3'-untranslated sequences of some genes may play a role in development or in tissue-specific gene expression whereas PTHrP has been determined to be necessary for normal development (Cramer SD *et al*, 1996), investigators have not found any tissue-specific gene expression to date.

1.3.4 Early Response Gene:

The promoter region of the PTHrP gene contains nucleotide sequence motifs similar to members of the early response gene family. Immediate-early response genes include a number of transcription factors such as c-myc and c-fos or cytokines such as M-CSF and JE (Jones TR, 1987; Wilson T *et al*, 1988; Vakalopoulou E *et al*, 1991) with half-lives of approximately 30 minutes. These genes, like PTHrP (Orlowsky A and Stanley ER, 1987), are induced when resting cells are treated with mitogens, which suggest that they may be involved in a cascade that initiates progression through the cell cycle. Thus, their targets are

likely to be involved in initiating or promoting growth (Felig P *et al*, eds, 1995). Hence, one would expect an increase in the activity of these genes to be associated with oncogenesis. Comparative analysis and transient transfection of promoter constructs have revealed that many of these genes contain serum response elements (SREs) in their 5' flanking sequences, and that these SREs are essential for induction in response to growth factors (Christy B and Nathans D, 1989).

The immediate-early genes also contain multiple copies of an AUUUA motif in their 3'-untranslated sequences. Messenger RNAs involved in cellular proliferation or differentiation commonly have motifs rich in adenine and uracil (AU) in their 3'-untranslated regions (Clemens MJ, 1987), which contribute to instability and rapid turnover of the mRNA (Shaw G and Kamen R, 1986; Kaiser SM and Goltzman D, 1993). Each exon specifying the 3'-untranslated sequence of PTHrP mRNA contains multiple copies of this instability motif (exons VII and IX each contain 4 copies of the AUUUA motif, while exon VIII contains 2 copies) (Yasuda T *et al*, 1989b; Mangin M *et al*, 1990b). These motifs are highly conserved in the untranslated regions of all PTHrP mRNAs from chicken to man, but are absent in PTH (Heinrich G *et al*, 1984; Mangin M *et al*, 1989; Yasuda T *et al*, 1989b; Karaplis AC *et al*, 1990; Mangin M *et al*, 1990b).

These findings suggest that rapid mRNA turnover is critical for proper regulation of PTHrP

mRNA expression and biological function (Halloran BP and Nissenson RA, eds, 1992). It appears that the expression of PTHrP mRNA is under a tight control through a combination of transcriptional and posttranscriptional mechanisms. This pattern of control presumably accounts for the low level of PTHrP mRNA that is present in most, if not all, cells that express the gene constitutively, as well as the rapid and transient PTHrP mRNA responses that have been observed in a number of tissues following stimulation (Thiede MA *et al.* 1990a; Kremer R *et al.*, 1991). These kinetic features would appear to be highly relevant to the proposed autocrine, paracrine and intracrine actions of PTHrP observed in cells that secrete the peptide via the constitutive pathway; how these features might affect control of PTHrP gene expression in a regulated secretory cell is unclear (Halloran BP and Nissenson RA, eds, 1992). PTHrP mRNA has been found to have a short half-life in the range of thirty minutes to three hours (Philbrick WM *et al.*, 1996), supporting the notion that the presence of the AUUUA sequence produces mRNA instability. In transient transfection experiments in which copies of this sequence were deleted from the 3'-untranslated sequence of c-fos or c-myc, mRNA transcripts were stabilized (Jones TR *et al.*, 1987; Wilson T *et al.*, 1988). Inhibition of protein synthesis as a result of cycloheximide treatment produces mRNA stability and suggests that the AUUUA motif may direct the degradation of PTHrP mRNA. In addition, cycloheximide transcriptionally induces PTHrP expression in a variety of cell lines (Allinson ET and Drucker DJ, 1992; Kiriya T *et al.*, 1993). This phenomenon is also observed with other members of the early response gene family (Greenberg ME *et al.*, 1986).

Therefore, the expression of the PTHrP gene has the kinetics of a so-called immediate-early gene. The regulation of PTHrP mRNA expression is reminiscent of the control of a number of cytokine mRNAs and is consistent with that of a predominantly constitutively secreted product with powerful regulatory effects. Therefore, PTHrP has features which suggests its involvement in cellular growth and differentiation (Kaiser SM *et al*, 1992; Kaiser SM *et al*, 1994).

Another feature of PTHrP which is also seen in other members of the early response gene family is the presence of a 7-mer sequence, TTTTGTA, or its inverted sequence, TACAAAA, in the 3'-untranslated region. The PTHrP gene contains this sequence within exon IX, the inverted repeat is found in exon VII and the inverted repeat with one nucleotide mismatch (TAGAAAA) within exon VIII (Thiede MA *et al*, 1990b). A number of transcription factors including c-jun, jun, junD, c-fos, fosB and the cytokines JE, IL-2 and M-CSF have this sequence in their 3'-untranslated regions (Moseley JM and Gillespie MT, 1995). The sequence is not involved in polyadenylation, splicing or destabilization of transcripts. However, as all immediate-early response genes are induced by serum, it has been postulated that this sequence along with the SRE may be essential for trafficking of mRNA for protein synthesis (Freter RR *et al*, 1992).

1.3.5 Post-Translational Processing:

As soon as the three cDNA-predicted PTHrP amino acid sequences were ascertained, it was clear that PTHrP could undergo extensive posttranslational processing and might serve as a prohormone or polyprotein from which a family of peptide hormones could be derived. That is, the cDNA sequences encode a typical prepro- or signal sequence with a hydrophobic core of 10-15 amino acids flanked upstream and downstream by charged amino acids. The pre- sequence is generally thought to be removed as the nascent peptide enters the cisternae of the endoplasmic reticulum and is unlikely to persist in the cell (Liu B *et al*, 1995). Intracellular conversion of pro-PTHrP to the mature peptide is required, and removal of the pro- sequence most likely occurs in the Golgi apparatus (Liu B *et al*, 1995). Moreover, the primary sequence of the mature PTHrP peptide contains multiple clusters of basic amino acids in the conserved region (that is, up to amino acid 111) arranged in pairs, triplets and quadruples (Philbrick WM *et al*, 1996). These clusters are potential sites for the action of processing enzymes, several of which exhibit the features of convertase (or dibasic processing endoproteases) target sites (Gillespie MT and Martin TJ, 1994). Although it has not been proven, this raises the possibility that tissue-specific posttranslational processing events will be important in characterizing the circulating forms of PTHrP as well as understanding the biological actions of these peptides.

1.4 AUTOCRINE AND PARACRINE FUNCTIONS OF PTHrP

Autocrine secretion is defined as a mechanism by which a cell secretes a hormone-like substance for which the cell itself has functional external receptors. Paracrine regulation involves the secretion of factors (such as hormones, growth factors, and cytokines) by one cell, which diffuse through the extracellular space to their ultimate target cells. PTHrP has diverse biologic activities in many normal tissues which are mediated by the PTH/PTHrP receptor. At times, PTHrP is secreted via the classical secretory pathway and interacts with cell surface receptors in an autocrine/paracrine fashion which suggests that PTHrP has multiple effects. Targeted expression of PTHrP delays endochondral bone formation (Schipani E *et al*, 1997). More recently, PTHrP has been found to directly signal to the PTH/PTHrP receptor on proliferating chondrocytes to slow their differentiation (Chung UI *et al*, 1998). It is essential in numerous developmental events including induction of differentiation toward parietal endoderm in F9 embryonal carcinoma cells (Verheijen MH *et al*, 1999), epidermal differentiation in adult mice (Foley J *et al*, 1998) and mammary gland development (Dunbar ME *et al*, 1999). It is involved in apoptosis in various systems such as the insulin-producing beta cells of the pancreatic islet and the vascular smooth muscle cells of the arterial wall (Vasavada RC *et al*, 1998). It is undisputable that PTHrP has many important autocrine and paracrine functions. Scientific research is finding and continues to find new roles for PTHrP.

1.5 PTHrP RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

As described in 1.3.5, there is abundant evidence that PTHrP is a polypeptide that is cleaved/processed into multiple products. Since PTHrP is posttranslationally processed to a range of mature secretory forms with distinct intrinsic bioactivities, it is clear that multiple receptors for PTHrP must exist. The multiple products can be divided into three categories:

1. An amino-terminal PTHrP secretory form, PTHrP(1-36), which interacts with the classical PTH/PTHrP receptor (type 1 cell surface receptor) (Orloff JJ *et al*, 1994), and possibly with an as yet structurally undefined amino-terminal PTHrP receptor (Orloff JJ *et al*, 1995). PTHrP is thought to exert its biological effects by binding to and activating the type 1 surface receptor (Orloff JJ *et al*, 1994; Philbrick WM *et al*, 1996). This mediates at least some of the local paracrine/autocrine actions of PTHrP (Nguyen MT and Karaplis AC, 1998) including vascular smooth muscle cell relaxation and cellular proliferation (Orloff JJ *et al*, 1995). The most well established effects of PTHrP are mediated via this receptor including promotion of urinary cyclic AMP and phosphate excretion and a reduction in calcium excretion (Martin TJ *et al*, 1991). There is also evidence that PTHrP employs its own unique receptors in addition to the classical PTH/PTHrP G-protein coupled receptor.
2. Mid-region PTHrP appears to have its own receptor which mediates activities such as placental calcium transport (Rodda CP *et al*, 1988) and inhibition of the growth of a breast carcinoma cell line (Luparello C *et al*, 1995). This putative midregion receptor appears to signal exclusively via the calcium messenger system (Philbrick WM, 1998).
3. The carboxyl-

terminal PTHrP peptide, PTHrP(107-139), appears to act via a separate receptor and mediates other actions such as inhibition of osteoclastic bone resorption (Fenton AJ *et al*, 1991). Less is known regarding the receptors for the mid-region and carboxyl-terminal hPTHrP peptides. These receptors are the subject of ongoing investigations.

1.6 MECHANISMS OF PTHrP SECRETION

PTHrP is unusual in that it has features of both a neuroendocrine peptide and a growth factor or cytokine (Martin TJ *et al*, 1997). Peptide hormones are small proteins. Their mRNA encodes initial translation products which are larger than the secretory form of the peptide. That is, mRNA is translated into protein precursors, which are then processed by a series of proteolytic cleavages to yield the final secretory product. Growth factors are polypeptides which regulate cell proliferation through binding to specific receptors. They constitute a large group of hormones with prominent actions on cell proliferation, as opposed to the effects on differentiation and secretion that are typical of many other peptide and protein hormones.

PTHrP is like a neuroendocrine peptide in that it undergoes extensive posttranslational processing in a fashion analogous to proopiomelanocortin or somatostatin and it is the product of a broad variety of neuroendocrine cell types, including pancreas, pituitary cells and the central nervous system (Orloff JJ *et al*, 1994). PTHrP enters the classical secretory

pathway, beginning with signal peptide-mediated entry into the endoplasmic reticulum (ER), it then transverse the Golgi stacks and the trans-Golgi network. Subsequently, it enters the transport vesicles of the constitutive secretory pathway or the secretory granules of the regulated secretory pathway (which varies in different tissues), and finally it undergoes posttranslational glycosylation and cleavage by prohormone convertases (Wysolmerski JJ and Stewart AF, 1998). The PTHrP secretory protein is synthesized as precursors containing an amino-terminal signal sequence which mediates interactions between the growing peptide during translation and the membrane of the ER. In these cells, PTHrP is packaged into secretory granules (Philbrick WM *et al*, 1996).

On the other hand, PTHrP is also produced by a broad range of constitutively secreting cell types including keratinocytes, chondrocytes and vascular smooth muscle cells (Philbrick WM *et al*, 1996). In these cells, which do not contain neuroendocrine secretory machinery, PTHrP is secreted in a constitutive fashion (Martin TJ *et al*, 1997). This duality of secretory mechanisms has not been documented for other peptides in different cell types (Philbrick WM *et al*, 1996).

1.6.1 PTHrP and Nuclear Localization Signals (NLS):

Regardless of size, some proteins functioning within the nucleus possess a nuclear localization signal (NLS) which is required for recognition by and active translocation through the nuclear pore complex (DeRobertis EM *et al*, 1978; Henderson JE *et al*, 1995). Nuclear localization signals: (1) are typically short sequences, usually not longer than 8-10 amino acids; (2) contain a high proportion of positively charged amino acids (lysine and arginine); (3) are not located at specific sites within the protein; (4) are not removed following localization; and (5) can occur more than once in a given protein (Garcia-Bustos J *et al*, 1991).

It appears that some effects of PTHrP may be mediated through intracrine mechanisms via nuclear or nucleolar targeting (Kaiser SM *et al*, 1992; Kaiser SM *et al*, 1994; Henderson JE *et al*, 1995). PTHrP contains a bipartite nuclear localization signal in the midregion (amino acids 87-107) of the protein (Kaiser SM *et al*, 1992; Meerovitch K *et al*, 1997). This finding was unexpected because native PTHrP includes a prepro- (leader) sequence that would normally target the nascent protein to the endoplasmic reticulum for secretion (Nguyen MT and Karaplis AC, 1998).

The PTHrP sequence contains three clusters of basic residues in the (87-106) region that are similar in sequence and configuration to the single and bipartite nuclear or nucleolar

localization sequences in viral transcription factors, such as basic fibroblast growth factor (bFGF) and fibroblast growth factor-3 (FGF-3) (Kaiser SM *et al*, 1992; Kaiser SM *et al*, 1994). PTHrP can be identified by immunocytochemistry in the nucleus, and has been demonstrated in the nucleolus of immortalized human ectocervical cells (HEC-16) (Rahimtula M and Kaiser SM, submitted) and in COS-7 cells transfected with PTHrP (Henderson JE *et al*, 1995). Deletion of the multibasic(87-106) region prevents this nuclear localization, and deletion of the PTHrP signal peptide, which would divert PTHrP away from the secretory pathway, enhances nuclear localization (Henderson JE *et al*, 1995).

The question arises here as to how PTHrP can, under some circumstances, enter the secretory pathway and at the same time, avoid entry into the ER to remain in the cytoplasm and enter the nucleus. This phenomenon has been described for a variety of peptide growth factors including bFGF (Hill DJ *et al*, 1992) and FGF-3 (Kiefer P *et al*, 1994). These peptides are known to modulate cellular function via a dual mode of action: firstly, by extracellular activation of classical signal transduction pathways, and secondly, by nuclear actions that critically rely on an endocytosis-dependent pathway. One proposed mechanism whereby PTHrP might find its way to the nucleus is through secretion via the classical secretory pathway, followed by receptor-mediated endocytosis/internalization of the receptor-ligand complex and release of the PTHrP ligand within the cell. Once entry into the cell has been gained, nuclear localization could be mediated by nuclear or nucleolar targeting

signals (Wysolmerski JJ and Stewart AF, 1998).

A second proposed mechanism for PTHrP nuclear localization suggests that secreted proteins can be diverted from the default secretory pathway and targeted to the nucleus by alternative initiation of translation at non-AUG codons, in particular, CUG (Nguyen MT and Karaplis AC, 1998). This mechanism to bypass the secretory pathway has been described for FGF-3. Here, a CUG-initiated species was shown to have dual fates, either secretion or nuclear targeting (Kiefer P *et al*, 1994).

Another potential mechanism has been suggested whereby, under some circumstances secreted proteins might undergo recruitment via interactions with sequestering proteins or chaperones, and subsequent translocation to the cytoplasmic department (Nguyen MT and Karaplis AC, 1998). It is unclear whether this may occur by reversal of the translocation machinery or by a distinct transport system. Reversal of the early steps of protein commitment to secretion makes this pathway a plausible mechanistic alternative for the translocation of PTHrP from the ER lumen to the cytoplasm (Nguyen MT and Karaplis AC, 1998).

1.7 PTHrP EXPRESSION

Although initially discovered in malignancies, the production of PTHrP in normal tissues was first demonstrated in 1986 by Merendino JJ *et al* where a "PTH-like" activity was identified in cultures of normal human epidermal keratinocytes. Following the cloning of PTHrP cDNAs, an intriguing and extensive list of tissues which produce this molecule has been assembled. PTHrP is produced by many normal and malignant tissues, particularly those of epithelial origin (Grone A *et al*, 1996). Many histological types of cancer cells produce PTHrP (Danks AJ *et al*, 1989; Drucker DJ *et al*, 1989; Asa SL *et al*, 1990; Kaiser SM and Goltzman D, 1993). PTHrP protein and/or its mRNA have been demonstrated in the normal adult brain, uterus, cervix, placenta, amnion, lymphocytes, lactating breast, pancreatic islets, kidney, osteoblasts, chondrocytes, adrenal cortex and medulla, urinary bladder and vascular smooth muscle, as well as in numerous fetal tissues (Orloff JJ and Stewart AF, 1995; Philbrick WM *et al*, 1996). Essentially, PTHrP is produced by virtually every cell and tissue within the human body at some point in development (Wysolmerski JJ and Stewart AF, 1998).

The identification of PTHrP in a wide variety of normal cell types, the expression of PTHrP in diverse tissues during development and the presence of PTHrP receptors in many tissues suggest that PTHrP has a role in normal growth, development and differentiation in these cells and tissues (Cramer SD *et al*, 1996)

1.8 REGULATION OF THE PTHrP GENE

The PTH and PTHrP genes appear to be regulated quite differently. Physiological control of the PTH gene is limited to calcium and 1,25-dihydroxyvitaminD₃, and the control appears to be exclusively at the level of gene transcription (Okazaki T *et al*, 1992). In contrast, a wide variety of agents has been reported to influence steady-state PTHrP mRNA levels and/or gene transcription. The gene appears to be subject to tight control through a combination of transcriptional and posttranslational mechanisms (Holt EH *et al*, 1994). Some elements involved in this regulation include the three alternate promoters, a number of positive and negative regulatory elements, as well as, posttranscriptional regulation (Philbrick WM *et al*, 1996).

Two characteristic patterns of physiologic or pharmacologic stimuli have been reported, one consisting of a prolonged or plateau response, lasting many hours to days (Chan SD *et al*, 1990; Thiede MA *et al*, 1990a) and the second characterized by rapid induction-deinduction kinetics occurring over one to several hours (Ikeda K *et al*, 1990; Kremer R *et al*, 1991). The kinetics of the second type of response are reminiscent of those of a number of protooncogene and cytokine mRNAs including, for example, c-myc, c-fos and M-CSF. AU-rich elements (AREs) or instability motifs that were initially identified in these mRNAs are also present in PTHrP mRNAs, which similarly have been found to be unstable (Holt EH *et al*, 1994) as discussed in 1.3.4.

Estrogen has been reported to elicit both patterns of PTHrP mRNA response. The administration of 17β -estradiol to ovariectomized or intact rats *in vivo* leads to a transient PTHrP mRNA response in the uterus, with a peak occurring 2 hours after administration (Thiede MA *et al*, 1991). 17β -Estradiol administration to human papillomavirus type 16 (HPV-16) immortalized human ectocervical cells also produces a peak PTHrP mRNA response at 2 hours, with a return to basal levels by 72 hours (Rahimtula M and Kaiser SM, submitted). In contrast, when 17β -estradiol was administered to rat myometrial cells in primary culture, a plateau type of response was observed, with peak PTHrP mRNA levels at 24 hours, and maintenance of these levels for up to 5 days (Theide MA *et al*, 1991).

Regulation of PTHrP production in neoplastic and non-neoplastic tissues appears to differ in different tumours and tissue types. For example, elevated extracellular calcium levels lead to decreased PTHrP production by both normal and malignant keratinocytes *in vitro* (Lowik CW *et al*, 1992), but increased production in a rat Leydig cell tumour model of HHM (Rizzoli R and Bonjour JP, 1989). In addition, $1,25$ -dihydroxyvitamin D_3 increased PTHrP production in normal human keratinocytes (Kremer R *et al*, 1991), but inhibited PTHrP mRNA expression in a human C-cell line derived from a medullary thyroid carcinoma (Ikeda K *et al*, 1989). The molecular basis involved in the control of PTHrP production is unknown in most tissue types.

PTHrP expression has been shown to be regulated by multiple factors in a variety of tissues. Of the known regulators of PTHrP, glucocorticoids most profoundly down-regulate PTHrP expression and decrease mRNA and protein production (Lu C *et al*, 1989; Glatz JA *et al*, 1994). Glucocorticoids are steroid hormones that regulate the metabolism of glucose and other organic molecules. Dexamethasone, a synthetic glucocorticoid, decreases the expression of PTHrP mRNA by about 70% (Glatz JA *et al*, 1994). 1,25-Dihydroxyvitamin D₃ is the metabolically active form of vitamin D (Darwish H and DeLuca HF, 1993) and it can either up-regulate (Merryman JI *et al*, 1993) or down-regulate (Abe M *et al*, 1998) PTHrP expression. Estrogen (17 β -estradiol) has been documented to increase PTHrP gene expression in immortalized human ectocervical cells (Rahimtula M and Kaiser SM, submitted), whereas PTHrP secretion was inhibited in the presence of estrogen in an estrogen receptor-positive human breast cancer cell line, KPL-3C (Kurebayashi J and Sonoo H, 1997). The effects on expression of the PTHrP gene by these hormones are both dose- and time-dependent. PTHrP has also been reported to be regulated by other factors including serum (Kremer R *et al*, 1991), epidermal growth factor (Heath JK *et al*, 1995), cycloheximide (Allinson ET and Drucker DJ, 1992) and phorbol esters (Rodan SB *et al*, 1989). Although the biological significance of these regulators has not been fully evaluated, some of the functional consequences of their actions on PTHrP production in various tissues have been addressed.

1.9 PTHrP PHYSIOLOGY

The function and physiological roles of PTHrP can be classed into three broad categories: (1) PTHrP appears to stimulate transepithelial calcium transport in a variety of tissues, including the renal tubule (Philbrick WM *et al*, 1996) and the placenta (Rodda CP *et al*, 1988). (2) PTHrP is a smooth muscle relaxant in a broad range of tissues, including urinary bladder (Yamamoto M *et al*, 1992) and vascular smooth muscle (Mok LL *et al*, 1989). (3) PTHrP is involved in the regulation of growth and development. PTHrP affects proliferation (Kaiser SM *et al*, 1992) and differentiation (Kaiser SM *et al*, 1994) in many cell types. Disruption of the PTHrP gene (Karaplis AC *et al*, 1994) is lethal in fetal or immediate postpartum life, indicating that PTHrP is an important, sometimes necessary, developmental factor.

1.10 PTHrP AND MODULATION OF CELL GROWTH AND DIFFERENTIATION

Antisense RNA technology is a powerful tool which can be used to block the expression of a targeted gene with high specificity (Holt JT *et al*, 1986). This method was employed by Kaiser SM *et al* (1992) to investigate the possibility that PTHrP may be involved in the modulation of cellular growth in keratinocytes. The human keratinocyte serves as a paradigm for autocrine/paracrine regulation inasmuch as it has the capacity to produce a number of factors that can then modulate function through the interaction of these factors with their respective receptors. An established human keratinocyte cell line, HPK1A, was shown to

overexpress PTHrP. In this model, antisense RNA blocked the production of endogenous PTHrP and resulted in growth stimulatory effects. That is, antisense-infected cells demonstrated a shorter doubling time, an increase in [^3H]-thymidine incorporation and a greater proportion of cells were in the S (synthesis) phase of the cell cycle compared to control cells (Kaiser SM *et al*, 1992).

The same antisense technology was used to assess the potential role of PTHrP in differentiation of keratinocytes by Kaiser SM *et al* (1994). HPK1A cells have a number of well-characterized maturation indices which are sequentially expressed and indicate keratinocyte differentiation. The capacity of PTHrP to modulate keratinocyte differentiation was examined by assessing: (1) the production of high molecular weight keratins, (2) the expression of transglutaminase, a membrane-bound keratinocyte enzyme that catalyzes protein-protein cross-linking to form cornified envelopes, and (3) the production of involucrin, one of the precursors of the envelope, in the absence of endogenous PTHrP production (Kaiser SM *et al*, 1994). There was a reduction in the production of high molecular weight keratins, as well as transglutaminase and involucrin, in the absence of endogenous PTHrP production. These data suggest that endogenous PTHrP inhibited the expression of markers of keratinocyte differentiation and, therefore, in these established keratinocytes, endogenous PTHrP acted to enhance differentiation (Kaiser SM *et al*, 1994). Therefore, antisense-mediated inhibition of PTHrP production has shown that PTHrP can

modulate the growth of normal (Kremer R *et al*, 1996b) and transfected cells (Kaiser SM *et al*, 1993; Kaiser SM *et al*, 1994; Rabbani SA *et al*, 1995).

1.11 HUMAN PAPILLOMAVIRUS

The papillomaviruses are a group of small DNA viruses associated with benign squamous epithelial tumours in higher vertebrates. Human papillomavirus (HPV) infection is quite common in women and is clearly the major risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. It is in the most estrogen-sensitive cells of the genital tract that papillomavirus lesions and their malignant sequelae usually occur (Auborn KJ *et al*, 1991). HPV DNA has been detected in 80% to 90% of high-grade squamous intraepithelial lesions (SILs) and invasive cervical cancers (Duerst M *et al*, 1991). More than 70 different strains of HPV have been identified, and specific subtypes have been associated with a greater risk of progression to dysplasia and cervical cancer (Montero JA *et al*, 1997). HPV types 6 and 11 preferentially induce benign proliferative changes of the epithelium (condylomata acuminata), while others such as HPV types 16, 18, 33 and 35 are associated with precancerous lesions and squamous cell carcinomas (Duerst M *et al*, 1991). Using *in situ* hybridization, Hellberg D *et al* (1993) found that 75% of 201 cervical punch biopsies, which showed CIN lesions on histology, were infected with HPV. HPV 16, 18 and 33 were found in 33.6%, 12.5% and 8.5%, respectively, of the HPV lesions. Using the same method, Pillai MR *et al* (1998) observed a significant correlation between the presence of HPV 16 and

high-grade squamous intraepithelial lesions (SILs) or invasive cancer. In another study, 47 patients with cervical carcinoma, 55% were infected with HPV-16 and 40% were infected with HPV-18 (Konya *J et al*, 1995). These studies support the association of HPV with cervical cancer.

The E6 gene, from cancer-associated HPVs, has been shown to be involved in the oncogenicity of HPV-16. The E6 protein is most commonly detected in high-grade SILs and cervical tissue expressing either HPV 16 or 18 (Rapp L *et al*, 1998). This protein is produced by the high-risk HPV types (16 and 18) and can bind to and inactivate the tumour suppressor protein p53, leading to dysregulated proliferation and defective apoptosis, thus facilitating tumour progression (Pillai MR *et al*, 1998).

1.12 CERVIX AND THE INFLUENCE OF STEROID HORMONES

The cervix is a specialized region of the uterus that undergoes an intricate process of proliferation and differentiation under the influence of steroid hormones. It is one of the target tissues of the sex steroid hormones, progesterone and estrogen, and is subject to control by these steroids. The human cervix is lined with three types of epithelia: the ectocervix, endocervix and the junction between these two regions, the transformation zone epithelium (Sun Q *et al*, 1992). The ectocervical epithelium is further divided into three zones: the basal zone, containing stem and parabasal cells; the midzone containing more

mature cells; and the superficial zone containing the highly differentiated, granular cells (Gorodeski GI *et al*, 1990). Physiologically, sex steroid hormones are closely associated with the growth and differentiation of the cervical epithelium (Khare S *et al*, 1997). During the menstrual cycle, the ectocervical epithelium undergoes a cyclic change in differentiation that is correlated with changes in the circulating levels of estrogens and progestins (Gorodeski GI *et al*, 1990).

The squamous epithelium of the uterine cervix possesses specific binding proteins with high affinity for both estradiol and progesterone, known as estrogen receptors (ER) and progesterone receptors (PR), respectively (Cao ZY *et al*, 1983). Biochemical and immunohistochemical studies have identified ER and PR in cervical epithelium (Gaton E *et al*, 1982; Fujiwara H *et al*, 1997). Estrogens promote cervical growth and differentiation, whereas progestins inhibit maturation of the ectocervical epithelium (Macinga D *et al*, 1995). The ectocervical squamous epithelium is therefore a sex-steroid dependent tissue whose proliferation and differentiation are assumed to be regulated by sex steroid hormones via ER and PR (Fujiwara H *et al*, 1997; Kanai M *et al*, 1998).

1.13 RATIONALE AND PURPOSE OF THIS PROJECT

The majority of human cervical neoplasms are classified as squamous cell carcinomas (90%) (White JO *et al*, 1992) and the remaining 10% of tumours show a mixed squamous/glandular or pure glandular differentiation pattern (Dunne FP *et al*, 1994). Cervical squamous

carcinoma is a phenotype which has not conventionally been considered to be steroid-hormone responsive (White JO *et al*, 1992). However, the human squamous cervical carcinoma cell line, HOG-1, was found to be responsive to steroid hormones (White JO *et al*, 1992). This suggests the possibility that endocrine therapies may be of benefit in this disease.

A recent study has suggested that cancers of the cervix exhibit a high incidence of PTHrP expression as detected by immunocytochemistry. In one investigation of 121 patients, 63 of whom had cancers of the cervix, vulva or vagina, 75% exhibited positive immunostaining for PTHrP (McKenzie S *et al*, 1994). Of the remainder, which comprised endometrial cancers and Mullerian duct tumours, the only PTHrP-positive cancers were those exhibiting a squamous phenotype. In another study by Liapis H *et al* (1993), 93% of squamous cell carcinomas of the cervix showed moderate to strong cytoplasmic immunoperoxidase staining for PTHrP. Dunne FP *et al* (1994) performed immunohistochemical staining and *in situ* hybridization on invasive cervical tumours. In this study, 10 of 10 and 16 of 16 tumours examined were positive for PTHrP mRNA and PTHrP peptide, respectively. Kitazawa S *et al* (1992) found that 96% of invasive squamous-cell carcinomas were positive for PTHrP, regardless of the patients' serum calcium levels. They also found that in CIN, including koilocytic atypia, 76% of cases were positive for PTHrP as well. Collectively, these studies strongly suggest a role for PTHrP in the oncogenicity of cervical tissue.

Squamous carcinomas are the prototypical PTHrP-producing tumours and the most common cause of HHM (Burtis WJ *et al*, 1990). Some reports have indicated that although approximately one third of tumours complicated by malignancy-associated hypercalcemia are of squamous cell origin, this complication is relatively uncommon in gynecologic cancers (Tait DL *et al*, 1994) compared to other malignancies such as squamous cell lung carcinomas or breast cancers. Other reports have indicated that gynecologic neoplasms are frequently associated with hypercalcemia (Bockman RS, 1980; Holtz G, 1980). Despite the conflicting evidence, since PTHrP is believed to act in an autocrine/paracrine/intracrine fashion to modulate functions other than the mobilization of calcium, we have investigated the regulation of its production and action in a cervical cancer cell line.

In this project, the CaSki cell line, which serves as a model for the study of cervical carcinoma, was employed. This cell line is derived from an epidermoid carcinoma of the cervix (Patillo RA *et al*, 1977) and is a well characterized squamous carcinoma cell line (Grenman S *et al*, 1988). The CaSki line contains over 500 integrated copies of human papillomavirus 16 (HPV-16).

The purpose of this project was to investigate the regulation of expression as well as the secretion of PTHrP in response to various growth factors and hormones, and in particular

to study the effects of the sex steroid hormones, 17 β -estradiol and progesterone. We have compared the results obtained with these malignant cells to data previously obtained in HPV-16 immortalized cells (HEC-16).

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Growth and Maintenance of Cells:

The human epidermoid cervical squamous carcinoma cell line, CaSki, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Squamous cell carcinomas express a specific membrane antigen phenotype including keratin pearl formation, a positive reaction to pemphigus antigen as well as to pemphigoid antigen (Carey TE *et al.*, 1983; Grossman B *et al.*, 1984). CaSki cells also express this typical phenotype (Grenman S *et al.*, 1988). CaSki cells were grown as monolayers on 75 and 175 cm² tissue culture flasks (VWR, Mississauga, ON). Cells were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM, 90%, v/v) without phenol red (Life Technologies, Burlington, ON), fetal calf serum (FCS, 10% v/v; Life Technologies) and 1x antibiotic-antimycotic (Life Technologies). The 1x antibiotic-antimycotic was used to prevent contamination of the cell line from bacteria, fungi and yeast. The antibiotic-antimycotic contains 10,000 units of penicillin (antibiotic), 10,000 µg of streptomycin (antimycotic) and 25 µg of amphotericin B/mL utilizing penicillin G, streptomycin sulfate and amphotericin B as Fungizone®. DMEM was sterilized by filtration through a 0.2 µ filter (Nalgene, VWR). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

The cell line was maintained by passing cells once or twice weekly. Cells were washed with phosphate buffered solution (PBS) and trypsinized with 5% trypsin in Hank's Balanced Salt Solution (HBSS) (Life Technologies). Cells were centrifuged at 200 xg for 5 minutes, the supernatant was aspirated and the cells were replated onto tissue culture flasks at a density of approximately 2×10^5 cells/mL of fresh medium. Cells were again incubated and were allowed to grow until 70% confluent. At 70% confluence, 75 and 175 cm² flasks contained approximately 6.5×10^6 and 1.5×10^7 cells, respectively.

Stocks of CaSki cells were stored in DMEM + 10% FCS containing dimethyl sulfoxide (DMSO) (17%, v/v, Fisher Scientific, Montreal, QC) in a -70 °C freezer or under liquid nitrogen. Freshly trypsinized cells were pelleted at 200 xg for 5 minutes and resuspended in the medium/DMSO mixture at a concentration of 2×10^6 cells/mL. A 1 mL portion of this suspension was transferred to a 2 mL Corning cryogenic vial (Fisher Scientific) and stored at -70 °C overnight before placing into liquid nitrogen. To retrieve a stock, a vial of cells was thawed immediately at 37 °C and pelleted at 200 xg for 5 minutes. The pellet was resuspended in 10 mL of growth medium and plated onto a 75 cm² tissue culture flask.

2.1.2 Hormone/ Growth Factor Preparation:

All stock solutions were prepared by dissolving the hormone/ growth factor stock reagents in 100% ethanol. Ethanol at 0.1% or less has been shown not to affect growth rate, viability,

or saturation of cells over the duration of experiments (Grenman *S et al*, 1988). Epidermal growth factor (EGF, Sigma, Oakville, ON) was prepared as a stock solution at a concentration of 100 ng/mL. Progesterone (Prog), 17 β -estradiol (E₂) and dexamethasone (Dex) (all from Sigma) were prepared as stock solutions at a concentration of 10⁻³ M. 1,25-Dihydroxyvitamin D₃ (1,25 (OH)₂ D₃) was a kind gift of Dr. M Uskokovic (Hoffman-LaRoche, Nutley, NJ) and a stock solution was similarly prepared. All stock solutions were stored at -20 °C until use and were diluted in growth medium prior to use in experiments.

2.1.3 Dose Response Experiments:

CaSki cells were seeded in 6-well plates in DMEM + 10% FCS at a density of 2.4 x 10⁵ cells/well. During plating, the cell suspension was frequently and thoroughly mixed to ensure a uniform cell density in each well. When the cells had adhered to the wells and were approximately 60% confluent (about 24 hours), the growth medium was removed and replaced with serum free DMEM for 24 hours. Following this, at time zero, cells were treated with varying concentrations of the factor under investigation, and incubations were continued. At specified times after treatment, cells were harvested. Cells were trypsinized with 1 mL of 5% trypsin (in HBSS), a 200 μ L portion of the trypsinized cell solution was removed and counted. The 200 μ L cell sample was placed into 20 mL of Isoton III electrolyte solution (Coulter Corporation, Miami, FL) and counted in a Coulter Counter

(Model ZBI, Coulter Electronics, Bedfordshire, UK). The remaining cells were centrifuged at 200 xg for 5 minutes, rinsed with ice cold PBS and stored at -70 °C for subsequent RNA analysis.

2.1.4 Time Course Experiments:

CaSki cells were seeded in 6-well plates in DMEM + 10% FCS at a density of 1.5×10^5 cells/mL and incubated for 24 hours. During plating, the cell suspension was frequently and thoroughly mixed to ensure a uniform cell density in each well. When the cells were approximately 50% confluent (at 24 hours), the medium was removed and replaced with serum free DMEM for 24 hours. At time zero, cells were treated with set concentrations of factors. At specific time points, cells were trypsinized, a 200 μ L portion of the trypsinized cell mixture was removed and counted. The 200 μ L cell sample was placed into 20 mL of Isoton III electrolyte solution and counted in a Coulter Counter. The remaining cells were centrifuged at 200 xg for 5 minutes, washed with ice cold PBS and stored at -70 °C for subsequent RNA analysis.

2.2 RIBONUCLEIC ACID (RNA) ANALYSIS

Cytoplasmic RNA was isolated using the Nonidet P-40 (NP-40) method of extraction (Gough NM, 1988). Pelleted cells were thawed. Cells were resuspended in 400 μ L of NP-

40 lysis buffer consisting of cold 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1.5 mM MgCl₂ and 0.65% NP-40. The cell suspension was transferred to a microfuge tube and centrifuged in an Eppendorf microfuge (Model 5415C, Brinkmann Instruments Inc., Westbury, NY) at 1000 rpm for 5 minutes at 4 °C. The supernatant was transferred to a microfuge tube containing 200µL urea buffer (7M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5) plus 400 µL of phenol:chloroform:isoamyl alcohol (50:50:1, v/v/v). The phases were mixed by vigorous shaking for 30 seconds and stored at -70 °C for a minimum of 1 hour. The tubes were then thawed at room temperature centrifuged at 14,000 rpm for 5 minutes. The aqueous phase (upper layer) containing the RNA was transferred to a new microfuge tube and the RNA was precipitated by the addition of 40 µL of 3M sodium acetate (pH 5.2) and 1 mL ice cold 100% ethanol and this was stored at -70 °C. RNA was recovered by centrifugation at 14,000 rpm for 15 minutes. The liquid was aspirated and the pellet was washed with 1 mL of 75% ethanol. Tubes were centrifuged at 14,000 rpm for 5 minutes, the ethanol was again aspirated and RNA pellets were dried using a Speedvac (Savant Instruments Inc., Hicksville, NY) for 4 minutes. Recovered RNA was dissolved in 10 µL of 0.1% DEPC treated water. The concentration of RNA was determined spectrophotometrically and the RNA was stored at -70 °C until Northern analysis.

2.3 QUANTITATION OF RNA

To determine the quantity of recovered RNA, the RNA was heated in a 54 °C water bath (Model 180, Precision Scientific, Chicago, IL) for 5 minutes. The tubes were immediately placed on ice for 5 minutes to prevent secondary and tertiary folding within the RNA. A 1 µL portion of the RNA solution was diluted 1:1000 (with DEPC treated water) and the optical density (OD) was determined in a Beckman spectrophotometer (Model DU-64, Mandel Scientific, Guelph, ON). The OD₂₆₀ was used to calculate the concentration of RNA in the sample. An OD₂₆₀ of 1 corresponds to approximately 40 µg/mL for single-stranded RNA. The OD ratio 260/280 provided an estimate of purity of the nucleic acid solution. Pure RNA solutions have an OD 260/280 ratio of 2.0 (Maniatis T and Fritsch EF, 1987).

2.4 ELECTROPHORESIS OF RNA

RNA was size-fractionated by electrophoresis on a 1.1% agarose-formaldehyde gel. Twenty µg of RNA was combined with 3.2 parts of sample loading buffer containing 37% formaldehyde (21%, v/v), 10 X MOPS (13%, v/v, 0.4 M MOPS, 0.1 M sodium acetate pH 7.0, 10 mM EDTA pH 7.5) and formamide (66%, v/v). The formamide had been deionized by stirring with a mixed bead resin, AG 501-X8 (Bio-Rad, Hercules, CA), for one hour. RNA was denatured by incubating the tubes in a 65°C water bath for 15 minutes. The tubes

were quickly chilled on ice for 5 minutes and centrifuged briefly. Two μL of RNA loading buffer (50% glycerol, 0.25% bromophenol blue and xylene cyanol FF and 1 mM EDTA, pH 8) and 2 μL of ethidium bromide (0.1 mg/ml) were added to each sample and loaded onto the gel. Electrophoresis was conducted at a constant voltage (20 Volts) in 1 X MOPS buffer (40 mM MOPS, 10 mM sodium acetate pH 7.0, 1 mM EDTA pH 7.5) for about 16 hours (overnight). Following electrophoresis, RNA was visualized using ultraviolet (uv) transillumination to ascertain that the RNA had been electrophoresed for a sufficient time. That is, electrophoresis was stopped when the 18S and 28S ribosomal RNA (rRNA) were approximately 2 cm apart. The location of the 18S and 28S ribosomal RNA (rRNA) were determined by photographing the gel under uv light gel using the ChemiImager 4000 (Alpha Innotech Corporation, distributed by Canberra-Packard Canada Ltd., Montreal, QC).

2.5 NORTHERN BLOT ANALYSIS

2.5.1 Gel Transfer:

Following electrophoresis, RNA was transferred to a nylon membrane (Hybond-N, Amersham Canada Inc., Oakville, ON) by capillary blotting (Figure 2.1). Briefly, the membrane was wetted in 0.1% DEPC-treated water and soaked in 10X SSC (1.5M sodium chloride, 0.15 M trisodium citrate, pH 7.0) for at least 15 minutes prior to the transfer. A support was placed inside a large dish which was filled with 10X SSC to the level of the

bottom of the support. A piece of gel blot paper (Mandel Scientific), which was cut wider than the gel and long enough to draw up buffer solution from the reservoir, was placed across the support. The wells and the top right hand corner of the gel were cut and removed. The gel was placed on the gel blot paper in an inverted position. The wet nylon membrane was then placed on top of the gel and the corner of the membrane was cut to match the corner from the gel. Air bubbles were removed by rolling a glass pipet over the surface of the membrane. Cellophane wrap was placed around the nylon membrane to prevent evaporation of the 10X SSC solution. Next, two pieces of gel blot paper, wetted in 2X SSC (0.3 M sodium chloride, 30 mM trisodium citrate, pH 7.0), were placed on top of the membrane and again air bubbles were removed. A stack of paper towels, which acted as a wick, was placed on top of the gel blot paper. Next, a glass plate was placed on top of the paper towels and finally, a weight of 500 grams was placed on the stack of paper towels. The transfer was allowed to take place for 18-24 hours. The filter was then air dried and baked at 80 °C for two hours to fix the RNA onto the membrane. The positions of the 18S and 28S rRNA were marked on the nylon membrane and were used as internal markers. The filters were then stored in sealed bags at 4 °C until required for hybridization.

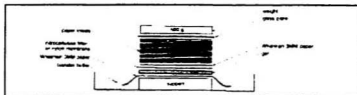


Figure 2.1: Diagrammatic representation of a capillary blotting apparatus.

(Adapted from Maniatis T and Fritsch EF, 1987)

2.5.2 cDNA Probes:

Hybridization was carried out with either a human PTHrP or a rat cyclophilin probe. The PTHrP probe was obtained from an SP65 (Promega) *in vitro* transcription plasmid into which was subcloned a 537-base pair *SacI-HindIII* restriction fragment encoding exon VI (coding region) of the PTHrP gene (Kremer R *et al*, 1991). The rat cyclophilin probe was a *BamHI* restriction fragment of rat cyclophilin from plasmid p1B15 (Danielson PE *et al*, 1988). DNA probes were labeled using the random primer method described in section 2.5.3.

2.5.3 Random Primer Method Labeling:

Double stranded DNA was labeled using the Random Primers DNA labeling kit (Life Technologies). The reaction was performed following the manufacturer's instructions and using the reagents provided in the kit. The reaction tube contained the following reagents:

Probe DNA (PTHrP or cyclophilin)

25 ng

dATP solution	2 μ L
dGTP solution	2 μ L
dTTP solution	2 μ L
Random Primer Buffer Mixture	15 μ L
[α - ³² P]dCTP (>3,000 Ci/mmol)	5 μ L
Klenow fragment	1 μ L
Distilled water (to a final volume of 50 μ L)	

[α -³²P]dCTP was purchased from Amersham (Mississauga, ON). The reaction tube was incubated for 2 hours at 25 °C and the reaction was terminated by the addition of 5 μ L of Stop Buffer. Spin-column chromatography (section 2.5.4) was performed to remove unincorporated dCTP.

2.5.4 Spin-Column Chromatography:

A 1 mL syringe (Becton Dickinson, Mississauga, ON) was plugged with fibreglass (Corning Glass Works, Corning, NY) and was placed into an empty 15 mL polystyrene centrifuge tube (Fisher Scientific). Sephadex G-50 (Pharmacia) slurry equilibrated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the syringe. This was centrifuged at 1000 rpm (200g) in a Sorvall centrifuge (Model RT6000D, DuPont, Wilmington, DE) at 4 °C for

5 minutes. More Sephadex G-50 slurry was added and centrifugation was repeated until the packed column had a volume of 1 mL. This was followed by the addition of 1 mL TE Buffer (pH 8.0) and the column was again centrifuged. The labelling reaction mixture (50 μ L) was then applied to the packed column and centrifuged for 5 minutes. The effluent (containing 32 P-labelled DNA) was collected in a microfuge tube. A 1 μ L portion of the labelled DNA probe was placed into 10 mL of ScintiVerse scintillation fluid (Fisher) and specific activity (cpm/ μ L) was estimated by scintillation counting (Model LS 3801, Beckman Instruments, Fullerton, CA). The radiolabelled DNA probe was stored at -20 $^{\circ}$ C until used.

2.5.5 Prehybridization:

The membrane was placed onto a nylon mesh (BH-9109, Bio/Can Scientific Inc., Mississauga, ON) and soaked briefly in 2X SSC (0.3 M sodium chloride and 30 mM trisodium citrate, pH 7.0). The membrane and mesh stack were rolled into a tight spiral and inserted into a hybridization bottle. Prewarmed (42 $^{\circ}$ C) prehybridization buffer solution was added to the bottle with a minimum volume of 125 μ L/cm² of membrane. The prehybridization solution contained:

SSC	6X
Denhardt's solution	5X
20% SDS	0.5% (v/v)

Deionized formamide	50% (v/v)
0.1% DEPC-treated water	5.5% (v/v)
Denatured sonicated salmon sperm DNA (Pharmacia)	100 µg/mL

Denhardt's solution consisted of 5g Ficoll (Type 400, Pharmacia), 5g polyvinylpyrrolidone (Sigma) and 5g bovine serum albumin (Fraction V, Sigma) dissolved in water to a total volume of 500 mL. The filters were incubated and constantly rotated for 4 hours at 42 °C in a Tek Star JR hybridization oven (Bio/Can Scientific).

2.5.6 Hybridization with the ³²P-labeled DNA probe:

Following prehybridization, RNA was hybridized with either a PTHrP or cyclophilin [α -³²P]dCTP labeled DNA probe at a specific activity of approximately 1.0×10^6 cpm/mL hybridization solution. Hybridization was performed in the same solution used for prehybridization. Immediately prior to use, probe DNA was denatured by boiling for 5 minutes and was then cooled on ice. The probe was added to the hybridization solution and hybridization was carried out for 24 to 48 hours at 42 °C.

2.5.7 Washing of the blots:

Following hybridization, the blots were successively washed as follows:

1 X SSC, 1% SDS for 15 minutes at room temperature (RT)

0.5 X SSC, 0.5% SDS for 15 minutes at RT

0.5 X SSC, 0.5% SDS for 15 minutes at RT

0.1 X SSC, 0.1% SDS twice for 15 minutes at RT

0.1 X SSC, 0.1% SDS for 30 minutes at 55 °C.

Membranes were then autoradiographed as described in the following section.

2.5.8 Autoradiography:

After washing, the blot was sealed in a plastic bag and exposed to Kodak X-OMAT x-ray film (Picker International, Montreal, QC) in the presence of two intensifying screens at -70 °C. The exposed film was developed in a Kodak X-OMAT film developer.

2.5.9 Densitometric Analysis:

Quantitation of the signal was achieved by performing spot densitometry using Alpha Ease Software (Canberra Packard Canada, Montreal, QC). Intensity densitometric values were determined for the radioactivity on the autoradiographs. Quantitation was completed within the linear range of densitometric response to the radioactivity on the blot.

2.5.10 Reprobing Northern Blots:

The [α - 32 P]-labeled DNA probe was stripped from the Northern blot prior to reprobing the membrane with other [α - 32 P]-labeled probes. To remove the old probe, a boiling solution of 0.1% SDS (w/v) was poured onto the blot and allowed to cool at room temperature. The blot was then rinsed in 2X SSC (0.3 M sodium chloride, 30 mM Trisodium citrate, pH 7.0) and a Geiger Counter (Model 44-9, Ludlum Measurements Inc., Sweetwater, TX) was used to ascertain removal of the previous probe.

2.6 RADIOIMMUNOASSAY (RIA) OF IMMUNOREACTIVE PTHrP (iPTHrP)

2.6.1 Collection of samples:

Conditioned culture medium, 1.5 ml/well in 6-well cluster plates, was removed from the wells at timed intervals. Aliquots of 500 μ L were frozen immediately and stored at -70 $^{\circ}$ C for subsequent measurement of PTHrP by radioimmunoassay (RIA). Cells were trypsinized and a 200 μ L sample of the trypsinized cell mixture was counted in a Coulter Counter to determine cell number. Before radioimmunoassay, the samples of conditioned medium were evaporated to dryness in a Speedvac (Model SVC-100H, Savant Instruments Inc., Farmingdale, NY) and were subsequently reconstituted and assayed (section 2.6.6) using PTHrP(1-34) as a standard.

2.6.2 Aliquoting of Standards:

Sodium acetate (0.01M) was added to 0.2 mg of human PTHrP(1-34) to a final volume of 2 mL. This solution (also known as the iodination peptide solution) was then placed into disposable borosilicate culture tubes (12 x 75 mm, Fisher Scientific) and dried in a Speedvac. These aliquots were diluted to a final concentration of 25 ng/ μ L by the addition of 0.2M phosphate buffer (57.7 mL of NaH_2PO_4 added to 42.3 mL of Na_2HPO_4 , pH 7.0) and were stored at -70°C .

2.6.3 Iodination Peptide:

Sodium acetate (0.01M) was added to 0.2 mg of human PTHrP(1-34) Tyr^0 to a final volume of 2 mL. This solution was placed into culture tubes and dried in a Speedvac. The aliquots were diluted to a final concentration of 0.1 $\mu\text{g}/\mu\text{L}$ by the addition of 0.01 M sodium acetate, with 2 μg of hPTHrP(1-34) Tyr^0 per tube, and stored at -70°C .

2.6.4 Iodination of PTHrP(1-34) Tyr^0 : Preparation of Tracer:

The iodination peptide was thawed and dissolved in 100 μL of 0.2 M Phosphate buffer (pH 7.0). While vortexing, 1 mCi of ^{125}I was added to this solution. Iodination was initiated by the addition of 20 μL of freshly prepared Chloramine T (1.25 mg/mL in 0.2 M phosphate

buffer, pH 7.0) and vortexed for exactly 25 seconds. Iodination was terminated by the addition of 50 μL of freshly prepared of sodium metabisulfite (2.5 mg/mL in 0.2 M phosphate buffer, pH 7.0).

A trichloroacetic acid (TCA) test was performed to determine the amount of incorporated ^{125}I . Outdated blood bank plasma (OBBP; 200 μL), and tracer (2 μL) were added to a test tube. Next, 200 μL of a 40% TCA solution was added to the test tube while vortexing. The tube was then centrifuged at 1800g for 15 minutes. Supernatant and precipitate were separated and both were counted in a gamma counter (Model LKB-Wallac Gammamaster 1277, Turku, Finland). ^{125}I incorporation was calculated as follows:

$$\% \text{ incorporation} = \frac{\text{cpm (precipitate)}}{\text{cpm (precipitate + supernatant)}} \times 100$$

If the % incorporation was <30, the iodination procedure was repeated. Otherwise, the tracer was purified on a P-10 column to prepare for the assay.

2.6.5 Preparation of P-10 Column:

Unincorporated ^{125}I was separated by passage through a Sephadex G-50 column as described by Maniatis and Fritsch (1987) with minor modifications. Sephadex G-50 slurry was equilibrated in DEPC water and poured into a 12 mL sterile disposable pipette (Fisher Scientific) containing a pyrex fibreglass plug at the bottom. A piece of polyethylene tubing

(Becton Dickinson, Parsippany, NJ) was placed at the tip of the 10 mL pipette and was connected to a fraction collector (Model LKB Bromma 7000 Ultrac, Sweden). The column was initially washed with barbital buffer (0.05 M sodium barbital, 0.05% thimerosal). Next, the column was eluted with Barb S/50 buffer (barbital buffer containing OBBP (2%, v/v)). The tracer was placed on the column and eluted with Barb S/50. The eluant was collected in 10 drop fractions. All tubes were counted in the gamma counter and cpm values were graphed relative to the tube number. The first peak of radioactivity was indicative of the tracer whereas the second peak represented free unincorporated iodine. The peak tracer tube(s) were stored at -20 °C until further use.

2.6.6 Assay of Immunoreactive PTHrP (iPTHrP):

PTHrP secretion was measured using a five day radioimmunoassay (RIA). The standard curve for the RIA was set up in the following manner (Table 2.1):

Table 2.1: Experimental set up for RIA standard curve.

Tube #	Samples	OBBP	PTHrP Standard	Barb S/50	PTHrP(1-34) Antibody 1:8000	Tracer (10,000 cpm/tube)
1,2,3	Blank	300 μ L	-	100 μ L	-	100 μ L
4,5,6	Reference	300 μ L	-	-	100 μ L	100 μ L
7,8,9	Total	-	-	1400 μ L	-	100 μ L
10-31	Standards	-	300 μ L	-	100 μ L	100 μ L
	Samples	300 μ L	-	-	100 μ L	100 μ L

On day 1, the standard curve was set up as a duplicate set of 10 serial dilutions of PTHrP standard, ranging from 25ng to 0.025 ng hPTHrP(1-34) per tube. Total, Reference and Blank tubes were used to determine specific and nonspecific binding of the antibody to the known standard concentrations of PTHrP. The antibody was a rabbit polyclonal hPTHrP(1-34) antibody and was diluted to a final concentration of 1:8000. OBBP and antibody were added to sample tubes. All tubes were vortexed and stored at 4 °C for 2 days.

On day 3, a dilution of 10,000-12,000 cpm of ^{125}I -labelled PTHrP(1-34)Tyr⁰ in 100 μL of Barb S/50 was added to all assay tubes. The tubes were vortexed and incubated at 4 °C for 2 additional days.

On day 5, bound and free ^{125}I -labelled PTHrP(1-34)Tyr⁰ were separated. A mixture of 3% alkaline charcoal and 0.3% dextran in barbital buffer was stirred for 30 minutes prior to use. One mL was added to each assay tube except Total tubes (7,8,9). The tubes were vortexed, incubated for 30 minutes at 4 °C and then centrifuged for 15 minutes at 3000 rpm. The supernatant was decanted, supernatant tubes were corked and the radioactivity was counted in gamma-counter. Bound ^{125}I -labelled PTHrP(1-34)Tyr⁰ remained in the supernatant, whereas free ^{125}I -labelled PTHrP(1-34)Tyr⁰ remained in the pellet. Specific and nonspecific binding were calculated as follows:

$$\text{Specific Binding (\%)} = \frac{\text{Reference}}{\text{Total} - \text{Blank}} \times 100$$

$$\text{Nonspecific Binding (\%)} = \frac{\text{Blank}}{\text{Total} - \text{Blank}} \times 100$$

Specific binding >20% and nonspecific binding of <7% must have been achieved in order for the standard curve to be used to calculate sample results. Sample results were expressed as nanogram equivalents of hPTHrP(1-34)/10⁶ cells. The detection limit of the assay was 0.1

ng eq hPTHrP(1-34).

2.6.7 Statistical analysis:

All results of the RIA were expressed as the \pm Standard Error of the Mean (SEM) of the replicate determinations and statistical comparisons based on the Student's *t* test (two-tailed). This type of analysis was used as it is the standard statistical method used in testing for statistical significance in RIA in this field of research (Henderson *J et al*, 1991; Kaiser SM *et al*, 1992; Kremer R *et al*, 1996). A probability value of <0.01 was considered to be significant.

CHAPTER 3: RESULTS

Previous studies have demonstrated that PTHrP is produced by many histological types of cancer cells as well as being identified in a number of normal adult and fetal tissues. More specifically, in our laboratory, the regulation of PTHrP has been examined in response to known (EGF, dexamethasone and 1,25-dihydroxyvitamin D₃) and potential tissue-specific (progesterone and 17 β -estradiol) regulators in an ectocervical cell line immortalized with HPV-16 (HEC-16) (Rahimtula M and Kaiser SM, submitted). This system is particularly interesting in view of the role of PTHrP in the regulation of cell growth and differentiation through its autocrine and paracrine actions. The ectocervical epithelium is unique in that it undergoes a cyclic change in differentiation during the menstrual cycle. Thus, in ectocervical cells, tissue levels of PTHrP and thus the growth and differentiation of these cells may be regulated during the menstrual cycle by estrogen and progesterone.

In this project, the human squamous cervical carcinoma cell line, CaSki, was used as a model of late stage oncogenesis of the cervix. The purpose of the experiments was to determine the regulation of PTHrP in these cervical carcinoma cells in comparison to immortalized ectocervical cells as well as to the regulation in other tissues. Northern analysis was used to assess the effects of various factors on PTHrP mRNA expression and a hPTHrP(1-34) radioimmunoassay was used to determine the effects on PTHrP secretion in these cervical

carcinoma cells.

3.1 NORTHERN ANALYSIS

3.1.1 Dose Response Experiments:

Initially, the regulation of PTHrP gene expression in response to varying concentrations of known (EGF, dexamethasone and 1,25-dihydroxyvitamin D₃) and potential tissue-specific regulators (progesterone and 17 β -estradiol) was examined. These dose-response experiments allowed the assessment of whether the substance under investigation induced a change in PTHrP mRNA expression and, if so, the concentration at which the observed response was maximal.

Initially, experiments were carried out in DMEM plus 10% FCS. However, varying the dose of EGF, progesterone and 17 β -estradiol produced maximal stimulation of the PTHrP transcript even at the lowest dose examined, with no further increase in PTHrP mRNA detectable at higher dose (data not shown). Therefore, further experiments with these factors were performed in growth medium with no added FCS. However, FCS was used in experiments studying the effects of dexamethasone and 1,25-dihydroxyvitamin D₃.

Cells were challenged at 40% and 50% for the time course and dose response experiments,

respectively. Through experimentation it was determined that the greatest response to FCS (with respect to PTHrP expression) was seen at this level of confluence (data not shown).

3.1.1.1 Known Regulators: EGF, Dexamethasone and 1,25-Dihydroxyvitamin D₃:

EGF has been shown to upregulate PTHrP in other tissues as well as in established ectocervical cells. Figure 3.1A shows that in CaSki cells, EGF similarly produced a dose-dependent increase in PTHrP mRNA levels 24 hours after stimulation, with a maximal increase (normalized to cyclophilin) at 20 ng/mL. Figure 3.1B is a graphical representation of PTHrP mRNA levels, expressed as a percentage of the basal level. At 20ng/mL of EGF, there is nearly a 4.5-fold increase in PTHrP mRNA levels, compared to basal. A further increase in the dose of EGF did not produce an additional increase in PTHrP mRNA. Figures 3.2A and 3.3A indicate that, at 24 hours after treatment, both dexamethasone and 1,25-dihydroxyvitamin D₃ produced a dose-dependent inhibition of PTHrP mRNA, with maximal effects (normalized to cyclophilin) observed at 10⁻⁸ M. Figures 3.2B and 3.3B represent PTHrP mRNA levels (normalized to cyclophilin) and demonstrate a 50% reduction in PTHrP mRNA levels, expressed as a percentage of control levels, for both dexamethasone and 1,25-dihydroxyvitamin D₃. Inhibition of PTHrP did not increase further with higher doses of either dexamethasone or 1,25-dihydroxyvitamin D₃.

3.1.1.2 Potential Tissue Specific Regulators: Progesterone and 17 β -Estradiol:

17 β -Estradiol, in the absence of FCS, produced an increase in PTHrP mRNA expression (normalized to cyclophilin) at 24 hours after treatment (Figure 3.4A). A 3.5-fold increase in PTHrP mRNA, compared to basal, was observed at 10⁻⁸M and is represented graphically in Figure 3.4B. Progesterone did not produce a notable change in PTHrP mRNA expression (normalized to cyclophilin) (Figures 3.5A and B) at 24 hours. It is important to note that PTHrP expression appears to be inconsistent with that of other basal cells. The increase seen in PTHrP expression is attributed to the fact that there was a longer exposure time.

3.1.2 Time Course Experiments

3.1.2.1 Effect of culture conditions on PTHrP mRNA expression:

In order to further evaluate the effects of the various factors on PTHrP gene expression, it was important to determine the effect of culture conditions, particularly the presence or absence of serum in the culture medium. In the absence of serum, (Figure 3.6A) there was an increase in PTHrP mRNA expression (normalized to cyclophilin) but maximal levels were not obtained until 4 hours after stimulation. Figure 3.6B shows that at this point there was a 10-15% increase in PTHrP mRNA levels (compared to that of time zero; basal). At 24 hours, PTHrP mRNA expression fell below basal levels and continued to decline thereafter. In the presence of FCS (10%), an early increase in PTHrP mRNA levels was observed but

effects were only slight and were maximal at 2 hours, as indicated in Figure 3.7A. Figure 3.7B is a graphical representation of PTHrP mRNA levels, expressed as a percentage of time zero (control). A 25% increase in PTHrP mRNA expression was seen at 2 hours. However, following this, a decline in PTHrP mRNA levels occurred, with a return to control levels by 24 hours. I concluded that CaSki cells did express PTHrP mRNA in the absence of other factors. The addition of serum to the media caused a further increase in the amount of PTHrP mRNA expressed by the cells.

3.1.2.2 Effects of EGF, Dexamethasone and 1,25-Dihydroxyvitamin D₃ on PTHrP mRNA expression:

These experiments were completed to determine if the known regulators of PTHrP would affect PTHrP production in the CaSki cells over the time course of the experiment. EGF (20 ng/mL) produced an early stimulation of PTHrP mRNA expression with an increase observed at the first time point of 30 minutes (Figure 3.8A). PTHrP mRNA levels (normalized to cyclophilin) were maximal at 2 hours. The graphical representation of this time course experiment (Figure 3.8B) shows that there is a maximum increase in PTHrP mRNA expression of 4.5-fold, relative to time zero (basal). PTHrP mRNA levels decreased slowly thereafter, but even at 72 hours, expression had not fallen to basal levels. At 10^{-8} M, both dexamethasone (Figure 3.9A) and 1,25-dihydroxyvitamin D₃ (Figure 3.10A) produced initial increases in PTHrP mRNA levels, but effects were only slight and were maximal at 2

and 4 hours, respectively. By 24 hours after stimulation, both dexamethasone and 1,25-dihydroxyvitamin D₃ produced an inhibition of PTHrP mRNA levels, relative to time zero. This persisted to the last time point of 72 hours, and is indicated graphically in Figures 3.9B and 3.10B, respectively.

3.1.2.3 Effects of 17 β -Estradiol and Progesterone on PTHrP mRNA expression:

These experiments were carried out in order to determine if the potential tissue-specific regulators would have an effect of PTHrP mRNA expression over the time course of the experiment. The effects of 17 β -estradiol (10⁻⁸ M) were assessed at timed intervals, and like EGF, increases in PTHrP mRNA expression (normalized to cyclophilin) were observed at the earliest time point of 30 minutes after stimulation (Figure 3.11A). Maximal stimulation of about 4-fold was observed at 6 hours, which was less than the increase observed with EGF. By 72 hours, levels of PTHrP mRNA had decreased, but still remained elevated about 2-fold above the basal level (Figure 3.11B).

Progesterone (10⁻⁸ M) also produced an early increase in PTHrP mRNA expression (normalized to cyclophilin) with maximal stimulation at 1 hour (Figure 3.12A). At this time, PTHrP levels were about 75% above the basal level. At 24 hours, PTHrP mRNA expression had returned to basal levels (Figure 3.12B). At 48 hours, PTHrP mRNA levels had decreased

below basal levels.

3.2 RADIOIMMUNOASSAY

3.2.1 Dose Response Experiments:

3.2.1.1 PTHrP secretion in response to known regulators: EGF, Dexamethasone and 1,25-Dihydroxyvitamin D₃:

These experiments were completed in order to determine if varying doses of the known regulators had an effect on iPTHrP and if so, at what concentration were these effects maximal. At 24 hours, EGF produced a significant increase in iPTHrP levels with maximum secretion obtained using 20 ng/mL (Figure 3.13). Further increases in the concentration of EGF did not produce a further increase in iPTHrP secretion. Both dexamethasone and 1,25-dihydroxyvitamin D₃ produced significant decreases in iPTHrP secretion with maximal inhibition observed at 10⁻⁸ M (Figures 3.14 and 3.15, respectively). Further increases in the concentration of either dexamethasone or 1,25-dihydroxyvitamin D₃ did not result in further decreases in iPTHrP secretion. These results did confirm that the known regulators did indeed affect PTHrP secretion in the CaSki cells and the dose at which the maximal response was observed was determined for these regulators.

3.2.1.2 PTHrP secretion in response to tissue-specific regulators: Progesterone and 17 β -Estradiol:

To determine if the tissue specific regulators had an effect on iPTHrP, dose response experiments were carried out using the two hormones and iPTHrP was measured. At 24 hours, 17 β -estradiol produced a significant increase in iPTHrP secretion with maximal levels observed at 10^{-8} M (Figure 3.16). Further increases in the concentration of 17 β -estradiol did not result in a further increase in iPTHrP secretion. The amount of iPTHrP released into conditioned medium (corrected for cell number) was less than that observed in response to EGF.

At 24 hours, progesterone did not produce a significant change in iPTHrP secretion, compared to basal levels, within the range of doses used (10^{-12} to 10^{-6} M; Figure 3.17). These results demonstrated that only 17 β -estradiol affected PTHrP secretion in the CaSki cells under the conditions tested.

3.2.2 Time dependent effects on iPTHrP secretion:

3.2.2.1 Effect of culture conditions on iPTHrP secretion:

To determine the characteristics of PTHrP secretion in CaSki cells, we first measured iPTHrP in conditioned medium removed at timed intervals from cells maintained under basal

conditions (Figure 3.18). That is, experimental results were obtained from cells maintained in DMEM alone. iPTHrP secretion did not increase until 48 hours, relative to the level at time zero. Addition of FCS (10%) to the cultures elicited a time dependent augmentation in iPTHrP that greatly exceeded the increase observed in cells cultured under basal conditions (Figure 3.19). A statistically significant increase in iPTHrP was measured at 24 hours and levels of iPTHrP secretion were higher than those demonstrated under basal conditions (Figure 3.18).

3.2.2.2 Time Course experiments of known regulators of PTHrP: EGF, Dexamethasone and 1,25-Dihydroxyvitamin D₃:

These experiments were completed to determine the characteristics of PTHrP secretion in response to the known regulators. The effect of 20ng/mL EGF on iPTHrP secretion was examined at timed intervals in the absence of FCS (Figure 3.20). At 24 hours, a peak in iPTHrP was observed with approximately a 3-fold increase in secretion at this time, compared to time zero. Levels continued to increase until the last time point of 72 hours. In contrast, both dexamethasone and 1,25-dihydroxyvitamin D₃ produced a decreased iPTHrP secretion and by 24 hours, levels had decreased by about 50% relative to levels observed at time zero (in the presence of 10% FCS; Figures 3.21 and 3.22, respectively).

3.2.2.3 Time Course experiments of regulators of PTHrP: 17 β -estradiol and progesterone:

To determine the characteristics of PTHrP secretion, CaSki cells were treated with the tissue-specific regulators. The effects of 17 β -estradiol (10^{-8} M) were also assessed at timed intervals, and like EGF, increases in iPTHrP secretion were observed. At 24 hours, there was a 3-fold increase in secretion relative to time zero (Figure 3.23). iPTHrP continued to increase until the last time point of 72 hours.

Progesterone (10^{-8} M) did not cause a significant change in iPTHrP secretion during the time course (Figure 3.24). There was an increase in iPTHrP secretion, however, the results were slight and were not significant, compared to time zero.

3.3 CASKI VERSUS HEC-16

My experimental results from CaSki cells were compared to those of HEC-16 cells. Experimental work was carried out on the HEC-16 cells by SM Kaiser and M Rahimtula previous to my arrival into the laboratory. Experimental results from the HEC-16 cells have been submitted for publication at the time of the writing of this thesis (Rahimtula M and Kaiser SM, submitted).

3.3.1 Effects of EGF, Dexamethasone, 1,25-Dihydroxyvitamin D₃, Progesterone and 17 β -Estradiol on PTHrP mRNA expression:

EGF (20 ng/mL) and 17 β -estradiol both increased PTHrP mRNA expression with about a 1.5-fold greater increase observed in the CaSki cells than in HEC-16 cells for both treatments (Table 3.1). An inhibition of PTHrP mRNA was present in both cell lines after treatment with 1,25-dihydroxyvitamin D₃ (10^{-8} M) with about a 50% decrease in PTHrP mRNA 24 hours after treatment. Dexamethasone (10^{-8} M) produced a 50% decrease in PTHrP mRNA in CaSki cells however, in HEC-16 cells no decrease was seen at 24 hours. However, a decrease was observed in HEC-16 cells at 48 hours (Rahimtula M and Kaiser SM, submitted). In CaSki cells, progesterone (10^{-8} M) did not produce a significant change at 24 hours in PTHrP mRNA but it produced a 2.5-fold greater increase in PTHrP mRNA in HEC-16 cells.

3.3.2 Effects of EGF, Dexamethasone, 1,25-Dihydroxyvitamin D₃, Progesterone and 17 β -Estradiol on iPTHrP secretion at 24 hours after treatment:

Twenty-four hours after EGF (20 ng/mL) treatment, iPTHrP secretion was 0.862 ng eq/ 10^6 cells in CaSki and 17 ng eq/ 10^6 cells in HEC-16 (Table 3.2). That is, there was a 20-fold greater increase in iPTHrP secretion in HEC-16 cells compared to CaSki. Treatment with 1,25-dihydroxyvitamin D₃ resulted in iPTHrP secretion of 0.12 and 6 ng eq/ 10^6 cells in CaSki

and HEC-16 cells, respectively. This corresponded to a 50-fold greater increase in iPTHrP in HEC-16 cells compared to CaSki cells. Incubation of the cells with dexamethasone resulted in iPTHrP levels of 0.16 and 3 ng eq/10⁶ cells in the CaSki and HEC-16 cells, respectively. Again, iPTHrP secretion by HEC-16 cells exceeded that of CaSki cells, this time by nearly 20-fold. Treatment of the cells with 17 β -estradiol resulted in iPTHrP secretion of 0.538 and 17 ng eq/10⁶ cells for CaSki and HEC-16 cells, respectively, which roughly translates to a 30-fold greater secretion in HEC-16 cells. Progesterone stimulation resulted in iPTHrP levels of 0.22 and 20 ng eq/10⁶ cells in CaSki and HEC-16 cells, respectively, or nearly a 100-fold greater secretion in HEC-16 cells.

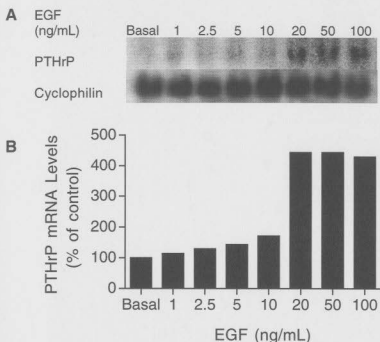


Figure 3.1: Dose-dependent effects of EGF on PTHrP mRNA

expression. CaSki cells were plated at 2.4×10^5 cells/well in 6-well cluster plates and grown to 50% confluence, followed by a 24 hour period of serum deprivation.

CaSki cells were then exposed to DMEM alone (basal) or in DMEM plus the indicated concentration of EGF for 24 hours. Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with varying concentrations of EGF. The filters were hybridized with ^{32}P -labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry and changes in PTHrP mRNA expression (normalized to cyclophilin) after EGF treatment were expressed as a percentage of control cells receiving DMEM alone.

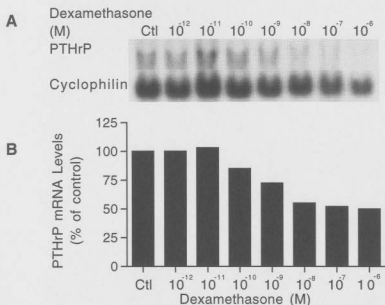


Figure 3.2: Dose-dependent effects of dexamethasone on PTHrP mRNA

expression . CaSki cells were plated at 2.4×10^5 cells/well in 6-well cluster plates and grown to 50% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM (in the presence of 10% FCS; control) or in DMEM (in the presence of 10%FCS) plus the indicated concentration of dexamethasone for 24 hours. Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with varying concentrations of dexamethasone. The filters were hybridized with 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry and changes in PTHrP mRNA expression (normalized to cyclophilin) after dexamethasone treatment were expressed as a percentage of control cells receiving DMEM + 10% FCS.

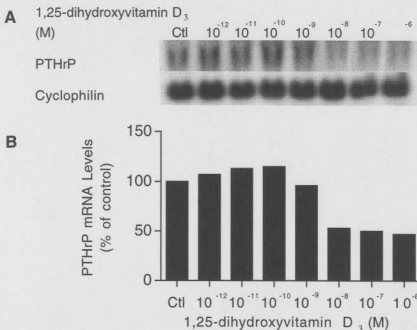


Figure 3.3: Dose-dependent effects of 1,25-dihydroxyvitamin D₃ on PTHrP mRNA expression. CaSki cells were plated at 2.4×10^5 cells/well in 6-well cluster plates and grown to 50% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM (in the presence of 10% FCS; control) or in DMEM (in the presence of 10% FCS) plus the indicated concentration of 1,25-dihydroxyvitamin D₃ for 24 hours. Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with varying concentrations of 1,25-dihydroxyvitamin D₃. The filters were hybridized with ³²P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry and changes in PTHrP mRNA expression (normalized to cyclophilin) after 1,25-dihydroxyvitamin D₃ treatment were expressed as a percentage of control cells receiving DMEM + 10% FCS.

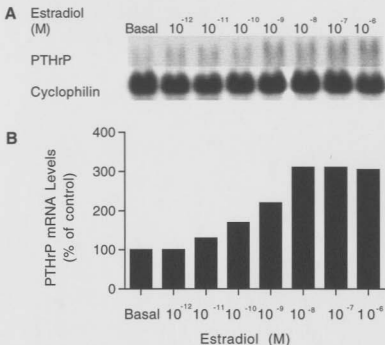


Figure 3.4: Dose-dependent effects of estradiol on PTHrP mRNA

expression. CaSki cells were plated at 2.4×10^5 cells/well in 6-well cluster plates and grown to 50% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM alone (basal) or in DMEM plus the indicated concentration of estradiol for 24 hours. Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with varying concentrations of estradiol. The filters were hybridized with ^{32}P -labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry and changes in PTHrP mRNA expression (normalized to cyclophilin) after estradiol treatment were expressed as a percentage of control cells receiving DMEM alone.

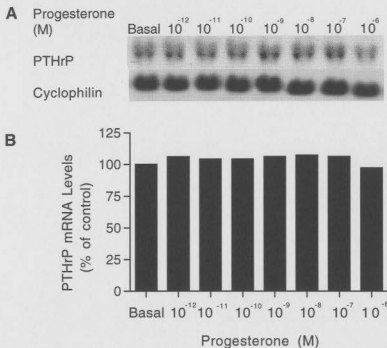


Figure 3.5: Dose-dependent effects of progesterone on PTHrP mRNA expression. CaSki cells were plated at 2.4×10^5 cells/well in 6-well cluster plates and grown to 50% confluence, followed by a 24 hour period of serum deprivation.

CaSki cells were then exposed to DMEM alone (basal) or in DMEM plus the indicated concentration of progesterone for 24 hours. Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with varying concentrations of progesterone. The filters were hybridized with 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry and changes in PTHrP mRNA expression (normalized to cyclophilin) after progesterone treatment were expressed as a percentage of control cells receiving DMEM alone.

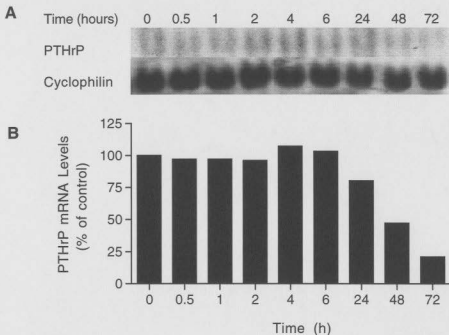


Figure 3.6: Time course of PTHrP mRNA expression under basal conditions. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM alone. CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells under basal conditions at indicated time intervals. The filters were hybridized with a 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after basal treatment were expressed as a percentage of control cells at time zero.

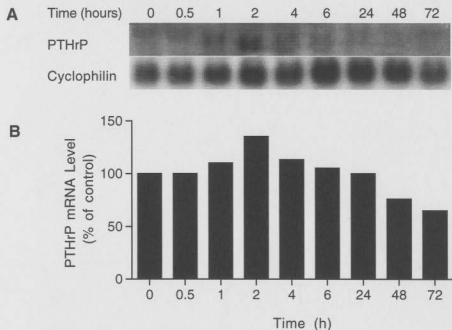


Figure 3.7: Time course of FCS mediated effect on PTHrP mRNA

expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation.

CaSki cells were then exposed to DMEM alone or DMEM plus 10% FCS. CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with 10% FCS at indicated time intervals. The filters were hybridized with a 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after 10% FCS treatment were expressed as a percentage of control cells receiving DMEM alone.

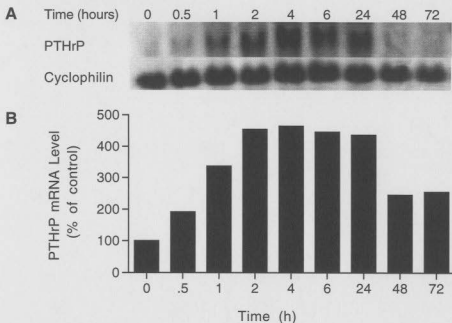


Figure 3.8: Time course of EGF mediated effect on PTHrP mRNA expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM alone or DMEM plus EGF (20 ng/mL). CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with EGF at indicated time intervals. The filters were hybridized with a ^{32}P -labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after EGF treatment were expressed as a percentage of control cells receiving DMEM alone.

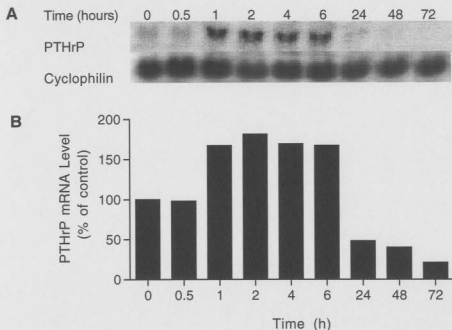


Figure 3.9: Time course of dexamethasone mediated effect on PTHrP mRNA expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM alone (in the presence of 10% FCS) or in DMEM (in the presence of 10% FCS) plus dexamethasone (10^{-8} M). CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with dexamethasone at indicated time intervals. The filters were hybridized with a 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after dexamethasone treatment were expressed as a percentage of control cells receiving DMEM alone.

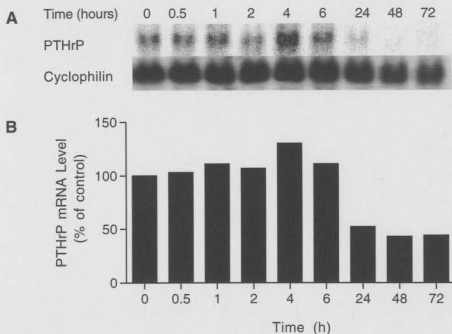


Figure 3.10: Time course of 1,25-dihydroxyvitamin D₃ mediated effect on PTHrP mRNA expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM (in the presence of 10% FCS) or in DMEM (in the presence of FCS) plus 1,25-dihydroxyvitamin D₃ (10^{-8} M). CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with 1,25 dihydroxyvitamin D₃ at indicated time intervals. The filters were hybridized with a ³²P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after 1,25-dihydroxyvitamin D₃ treatment were expressed as a percentage of control cells receiving DMEM alone.

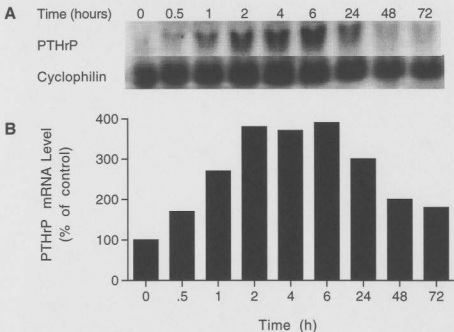


Figure 3.11: Time course of estradiol mediated effect on PTHrP mRNA expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates

and grown to 40% confluence, followed by a 24 hour period of serum deprivation.

CaSki cells were then exposed to DMEM alone or DMEM plus estradiol (10^{-8} M).

CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with estradiol at indicated time intervals. The filters were hybridized with a 32 P-labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after estradiol treatment were expressed as a percentage of control cells receiving DMEM alone.

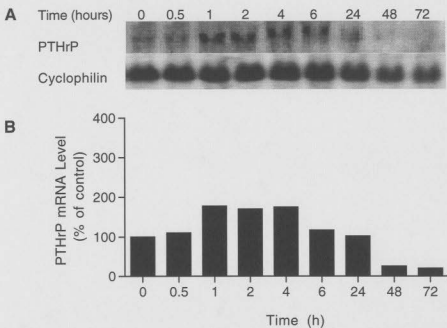


Figure 3.12: Time course of progesterone mediated effect on PTHrP mRNA expression. CaSki cells were plated at 1.5×10^5 cells/well in 6-well cluster plates and grown to 40% confluence, followed by a 24 hour period of serum deprivation. CaSki cells were then exposed to DMEM alone or DMEM plus progesterone (10^{-8} M). CaSki cells were removed at indicated time intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". Experiments were repeated in triplicate.

A) Northern blot analysis of PTHrP mRNA expression after treating the cells with progesterone at indicated time intervals. The filters were hybridized with a ^{32}P -labelled cyclophilin probe as a control for RNA loading.

B) The intensities of the autoradiograph signals were quantified by spot densitometry. Changes in PTHrP mRNA expression (normalized to cyclophilin) after progesterone treatment were expressed as a percentage of control cells receiving DMEM alone.

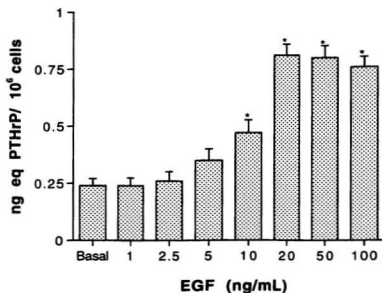


Figure 3.13: Changes in immunoreactive (i) PTHrP levels in response to varying doses of EGF. After 24 hour period of growth factor deprivation (basal), CaSki cells at 50% confluence were treated with DMEM alone (basal) or with DMEM plus the indicated concentration of EGF for 24 hours. Conditioned medium was removed at 24 hours and was assayed for iPTHrP as described in "Materials and Methods". Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$)

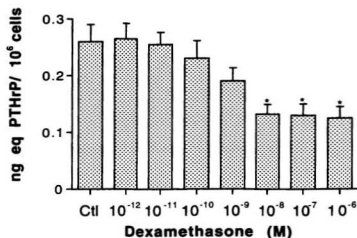


Figure 3.14: Changes in immunoreactive (i) PTHrP levels in response to varying doses of dexamethasone. After 24 hour period of growth factor deprivation, CaSki cells at 50% confluence were treated with dexamethasone (in the presence of 10% FCS; control) or with DMEM (in the presence of 10% FCS) plus the indicated concentration of dexamethasone for 24 hours. Conditioned medium was removed at 24 hours and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$)

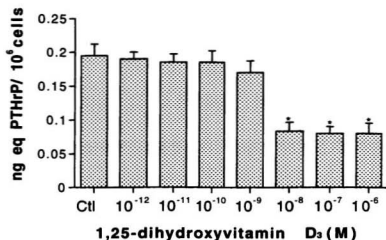


Figure 3.15: Changes in immunoreactive (i) PTHrP levels in response to varying doses of 1,25-dihydroxyvitamin D₃. After 24 hour period of growth factor deprivation, CaSki cells at 50% confluence were treated with DMEM (in the presence of 10% FCS; control) or with DMEM (in the presence of 10% FCS) plus the indicated concentration of 1,25-dihydroxyvitamin D₃ for 24. Conditioned medium was removed at 24 hours and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$)

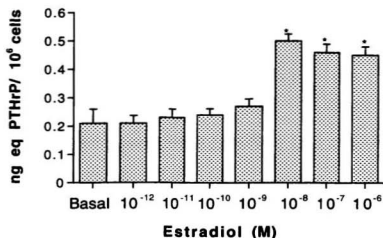


Figure 3.16: Changes in immunoreactive (i) PTHrP levels in response to varying doses of 17 β -estradiol. After 24 hour period of growth factor deprivation, CaSki cells at 50% confluence were treated with DMEM alone (basal) or with DMEM plus the indicated concentration of 17 β -estradiol for 24 hours. Conditioned medium was removed at 24 hours and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$)

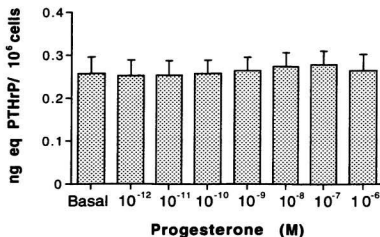


Figure 3.17: Changes in immunoreactive (i) PTHrP levels in response to varying doses of progesterone. After 24 hour period of growth factor deprivation, CaSki cells at 50% confluence were treated with DMEM alone (basal) or with DMEM plus the indicated concentration of progesterone for 24 hours. Conditioned medium was removed at 24 hours and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$)

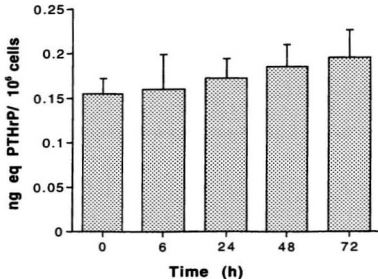


Figure 3.18: Time course of immunoreactive (i) PTHrP under basal conditions. After 24 hours of serum free conditions, fresh medium (DMEM alone) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$).

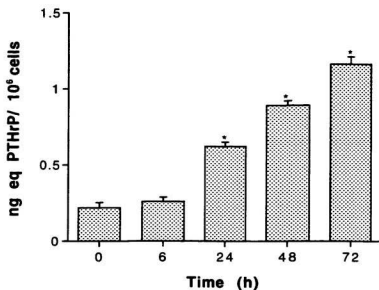


Figure 3.19: Time course of immunoreactive (i) PTHrP in response to 10% FCS. After 24 hours of serum free conditions, fresh medium (DMEM + 10% FCS) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods". Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$).

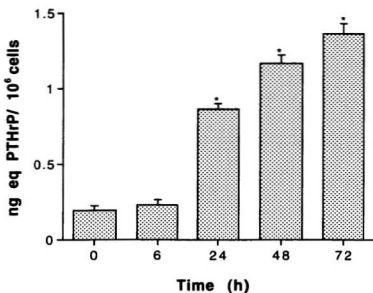


Figure 3.20: Time course of immunoreactive (i) PTHrP in response to EGF. After 24 hours of serum free conditions, fresh medium (DMEM alone) plus EGF (20 ng/mL) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$).

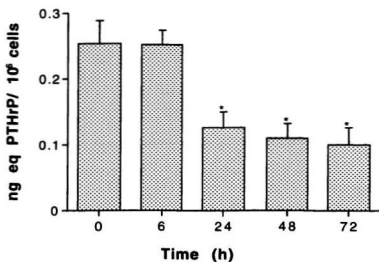


Figure 3.21: Time course of immunoreactive (i) PTHrP in response to dexamethasone. After 24 hours of serum free conditions, fresh medium (DMEM + 10% FCS) plus dexamethasone (10^{-8} M) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10^6 cells. *significant difference from control (Student's t-test, $p < 0.01$).

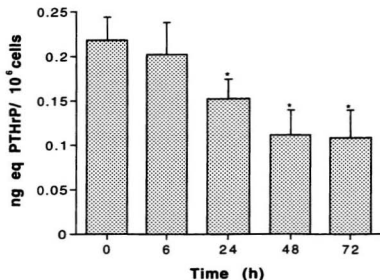


Figure 3.22: Time course of immunoreactive (i) PTHrP in response to 1,25-dihydroxyvitamin D₃. After 24 hours of serum free conditions, fresh medium (DMEM + 10% FCS) plus 1,25-dihydroxyvitamin D₃ (10⁻⁸M) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10⁶ cells. *significant difference from control (Student's t-test, $p < 0.01$).

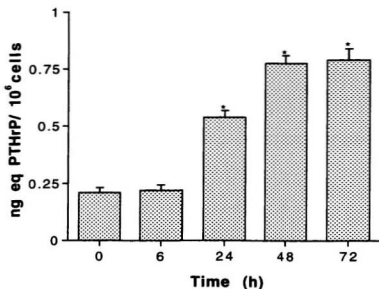


Figure 3.23: Time course of immunoreactive (i) PTHrP in response to 17 β -estradiol. After 24 hours of serum free conditions, fresh medium (DMEM alone) plus 17 β -estradiol (10^{-8} M) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10^6 cells. *significant difference from control (Student's t-test, $p < 0.01$).

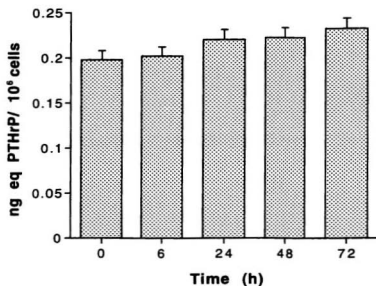


Figure 3.24: Time course of immunoreactive (i) PTHrP in response to progesterone. After 24 hours of serum free conditions, fresh medium (DMEM alone) plus progesterone (10^{-8} M) was added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods".

Results are corrected for cell number and are representative of replicate (at least triplicate) experiments. Each bar represents the mean \pm SEM of replicate determinations expressed as ng eq of hPTHrP(1-34)/ 10^6 cells. *significant difference from control (Student's t-test, $p < 0.01$).

Table 3.1: Effects of EGF, dexamethasone, 1,25-dihydroxyvitamin D₃, 17 β -estradiol and progesterone on PTHrP mRNA expression in HEC-16 and CaSki cells at 24 hours.

After 24 hours of growth factor deprivation and serum free conditions, fresh medium (KGM: HEC-16 cells; DMEM: CaSki cells) plus the specified concentrations of the hormones/factors indicated were added to the cells. Cells were removed at timed intervals (0-72 hours). Total cellular RNA was prepared as described in "Materials and Methods". PTHrP mRNA was analyzed by Northern blot analysis. The filters were hybridized with a ³²P-labelled cyclophilin probe as a control for RNA loading. The intensities of the autoradiograph were quantified by spot densitometry. Changes in PTHrP mRNA expression after treatment with the specified hormones/factors were normalized to control cells receiving medium alone. Twenty-four hour time period results were taken from both the HEC-16 and CaSki cells and compared. * expressed as % of Control

Treatment (dosage)	HEC-16 mRNA expression*	CaSki mRNA expression*
EGF (20 ng/mL)	288	450
Dexamethasone (10 ⁻⁸ M)	100	43
1,25-Dihydroxyvitamin D ₃ (10 ⁻⁸ M)	48	45
17 β -Estradiol (10 ⁻⁸ M)	164	300
Progesterone (10 ⁻⁸ M)	333	92

Table 3.2: Effects of EGF, dexamethasone, 1,25-dihydroxyvitamin D₃, 17 β -estradiol and progesterone on iPTHrP secretion in HEC-16 and CaSki cells at 24 hours.

After 24 hours of growth factor deprivation and serum free conditions, fresh medium (KGM: HEC-16 cells; DMEM: CaSki cells) plus the specified concentrations of the hormones/factors indicated were added to the cells. Conditioned medium was removed at timed intervals and was assayed for iPTHrP as described in "Materials and Methods". Twenty-four hour time period results were taken from both the HEC-16 and CaSki cells and compared.

Results are corrected for cell number and are representative of three separate experiments.

Each bar represents the mean \pm SEM of triplicate determinations expressed as ng eq of hPTHrP(1-34)/10⁶ cells.

Treatment (dosage)	HEC-16 iPTHrP secretion \pm SEM (ng eq/10 ⁶ cells)	CaSki iPTHrP secretion \pm SEM (ng eq/10 ⁶ cells)
EGF (20 ng/mL)	17 \pm 1.5	0.86 \pm 0.03
Dexamethasone (10 ⁻⁸ M)	3 \pm 0.3	0.16 \pm 0.03
1,25-dihydroxyvitamin D ₃ (10 ⁻⁸ M)	6 \pm 0.4	0.12 \pm 0.02
17 β -estradiol (10 ⁻⁸ M)	17 \pm 1.4	0.54 \pm 0.04
Progesterone (10 ⁻⁸ M)	20 \pm 2	0.22 \pm 0.03

CHAPTER 4: DISCUSSION

4.1 SERUM

Fetal calf serum contains many components including proteins (such as hemoglobin and bilirubin), ions (such as sodium, potassium, chloride, calcium and phosphate) and hormones (including estradiol, progesterone, testosterone, insulin and growth hormone) (Appendix A).

Serum is a positive regulator which rapidly enhances PTHrP mRNA expression and concomitantly increases PTHrP secretion in cultured normal human keratinocytes (Kremer R *et al*, 1991). Studies in normal human keratinocytes have shown that induction of PTHrP gene expression by serum is controlled by elements lying 1.2 kb 5' to the cap site (Kremer R *et al*, 1991). *Cis*-acting elements which are responsive to serum are known as serum response elements (SREs). The SRE is a well-characterized 22-nucleotide region of dyad symmetry that confers serum inducibility onto the c-fos gene (Treisman R, 1990). Comparative sequence analysis and promoter function studies have shown that several other immediate-early genes, including zif268 and egr-1, contain fos-like SREs in their 5' flanking sequences and require these SREs for induction in response to serum (Christy B and Nathans D, 1989; Qureshi SA *et al*, 1991). However, the c-fos gene does not stand as a prototype for all immediate-early response genes which are induced by serum. For example, no fos-like regulatory elements have been found within several kilobases of the 5' or 3' flanking sequences or within the coding sequences of the JE gene, but the JE gene is induced by serum (Freter RR *et al*, 1992).

My experiments have demonstrated that in CaSki cells, serum induction of PTHrP mRNA expression was rapid, with an increase detectable within 30 minutes and maximal at 2 hours. At 2 hours, there was a 1.5-fold increase in PTHrP mRNA compared to zero time point. The timing of the response was similar to effects observed in GH₄C₁ pituitary cells (Holt EH *et al*, 1994). Serum rapidly enhanced PTHrP mRNA expression and subsequently also increased PTHrP secretion.

The presence of cis-acting regions responsive to growth factors and serum within the PTHrP promoter region using transient transfection in normal human keratinocytes in culture has previously been reported (Kremer R *et al*, 1991). These studies have shown that serum induction of the PTHrP gene was controlled by SREs. Despite this, sequencing of 950 bp upstream of the cap site did **not** reveal a consensus sequence for an SRE of the c-fos type (Karaplis A *et al*, 1990). This indicates either that the SRE is different from that of the c-fos gene or, alternatively, that it lies further upstream of the cap site.

4.2 EGF

Epidermal growth factor (EGF) is a mitogen which stimulates the growth of many mesenchymal and epithelial cell lines in culture (Carpenter G and Cohen S, 1979; Carpenter G, 1987). EGF induces a pleiotypic response, characterized by increases in the transport of low molecular weight compounds, glycolytic activity, nucleic acid and protein synthesis,

and phosphorylation of membrane-associated and intracellular proteins (Carpenter G and Cohen S, 1979; Fox CF *et al*, 1982; Sarup JC *et al*, 1988). Among the many growth factors that have been reported, EGF is one of the polypeptides implicated in the growth of malignant cells (Lelle RJ *et al*, 1993).

It has been reported that EGF is present in many tissues and plays an important role not only through its function as a potent mitogen in a variety of cells, but also as a regulator or modulator of hormone secretion in some endocrine tissues (Taga M *et al*, 1995). In oncogenesis, the growth regulating network of EGF and other growth factors may be involved in neoplastic cellular proliferation (Lelle RJ *et al*, 1993).

The membrane receptor for EGF is a well-characterized 170 kDa glycoprotein (Cohen SJ, 1982). EGF receptor (EGFR) has an external domain as the EGF binding region, an internal domain containing a tyrosine-specific kinase and a transmembrane domain (Cohen SJ, 1982).

When EGF binds to its receptor, it activates protein kinase, which phosphorylates various cellular proteins, as well as EGFR itself (Alsat E *et al*, 1993). Autophosphorylation of EGFR is necessary in order to mediate the physiological actions of EGF and reflects the presence of biologically active receptors in the cell (Alsat E *et al*, 1993).

Cervical, ovarian and endometrial cancer have been found to exhibit strong EGFR expression

(Battaglia F *et al*, 1989; Bauknecht T *et al*, 1989). These findings support the idea that the enhanced expression of EGFR stimulates the proliferative capacity of tumours, which is clinically visible by its more aggressive behaviour (Bauknecht T *et al*, 1989). The rate of transcription of the EGFR gene is regulated by EGF itself, leading to an increase in EGFR mRNA (Earp HS *et al*, 1988), as well as by other factors and hormones such as PTH and PTHrP (Alsat E *et al*, 1993). Thus, EGF may play an important role in the growth promotion of these neoplastic cells (Lelle RJ *et al*, 1993).

In CaSki cells, we have demonstrated an increase in PTHrP expression and secretion with EGF treatment. In dose-response and time course experiments, there was a maximal 4.5-fold increase in PTHrP mRNA expression at 20 ng/mL at 24 hours. The time course experiments also revealed that the maximal 4.5-fold increase in PTHrP mRNA expression was achieved at 2 hours and maintained this level of production for up to 24 hours, compared to zero time point. There was a similar increase in iPTHrP secretion with a 4-fold increase in iPTHrP secretion at 24 hours. This is presumably a direct result from the increase in PTHrP mRNA expression.

The results from my experiments are similar to those of other investigators. EGF has been reported to cause an increase in PTHrP in a variety of different cells including a canine squamous carcinoma cell line (Grone A *et al*, 1998), in human prostatic epithelial cells

(Cramer SD *et al*, 1996) and in human keratinocytes (Allinson ET *et al*, 1992). EGF also produced a significant increase in PTHrP production in HEC-16 cells (Rahimtula M and Kaiser SM, submitted) and in gynecic squamous carcinoma cells (Tait DL *et al*, 1994). Hence, CaSki cells like many other cells demonstrate an observable increase in PTHrP production in response to EGF.

It has been suggested that the mechanism whereby EGF regulates PTHrP expression is mediated via an interaction of the peptide with EGFR. It is also known that CaSki cells overexpress EGFR (Singer GA *et al*, 1998). This could account for the substantial increase in PTHrP in response to EGF. That is, the EGF ligand may work via EGFR to increase PTHrP levels. I cannot firmly conclude however, that EGF regulates the rate of PTHrP gene transcription, since mRNA levels may be enhanced by other process. That is, there could be an increase in gene translation or mRNA stability. This could be determined by performing nuclear runoff assays or cycloheximide treatment, respectively, on the cells. In one study, EGF treatment of human keratinocytes increased transcriptional activity of the P2 promoter of the PTHrP gene, suggesting the presence of EGF response elements in that region of the promoter (Heath JK *et al*, 1995). This has not been confirmed since EGF response elements have **not** been found in the PTHrP gene to date.

4.3 STEROID HORMONES

Steroid hormones are synthesized from cholesterol and interact with the steroid/nuclear receptor superfamily which share common functional domains (Evans RM, 1988; Mangelsdorf DJ *et al*, 1995). These domains are responsible for properties such as ligand binding, dimerization, DNA binding and transactivation (Glass CK, 1994). Ligand binding is typically followed by dimerization with subsequent binding of the ligand-receptor complex to specific DNA sequences possessing a high level of dyad symmetry. Such binding sites are termed "hormone-response elements" (HREs) (Beato M, 1989) and contain perfect or imperfect palindromic or directly repeating half-sites that are normally five to six nucleotides in length. It is through these sequences that steroid hormone receptors exert their regulatory effects on specific genes (Freedman LP and Luisi BF, 1993).

4.3.1 Dexamethasone and 1,25-Dihydroxyvitamin D₃:

Dexamethasone and 1,25-dihydroxyvitamin D₃ are steroid hormones and have been shown to modulate PTHrP gene expression in normal (Kremer R *et al*, 1991) as well as malignant (Ikeda KC *et al*, 1989) cells.

4.3.1.1 1,25-Dihydroxyvitamin D₃:

The active form of vitamin D, 1,25-dihydroxyvitamin D₃, functions via interaction with a nuclear receptor (vitamin D receptor [VDR]) in a manner analogous to other members of the

steroid-thyroid hormone superfamily (DeLuca HF *et al*, 1998). Vitamin D receptors are widely distributed (Lan NC *et al*, 1984; Reichel H *et al*, 1989).

Vitamin D response elements (VDREs) are small segments of DNA that represent the binding domain for the vitamin D receptor. These elements mediate the responsiveness of a gene to 1,25-dihydroxyvitamin D₃ and result in transcriptional activation or repression of that gene. The VDRE consists of a direct repeat of two hexanucleotide half-sites separated by a three-nucleotide spacer (Ozono K *et al*, 1991). The first example of a negative VDRE (nVDRE) was identified in the promoter region of the human PTH gene and mediates the suppression of the PTH gene by 1,25-dihydroxyvitamin D₃ (Demay MB *et al*, 1992). However, it differs from a classical VDRE in that it has only one motif (Demay MB *et al*, 1992); the element contains only one of the two hexameric DNA sequences that forms the core sequence of the consensus DNA sequence (VDRE). It has been suggested that the VDRE sequence and the presence of either one or two hexameric sequence motifs may play a role in determining whether there is an increase or decrease in transcriptional activity of the individual gene (Darwish H and DeLuca HF, 1993).

The present studies have indicated that 1,25-dihydroxyvitamin D₃ produced a down-regulation of PTHrP mRNA expression in both a dose- and time-dependent manner. At 24 hours, PTHrP mRNA levels had fallen to 50% of zero time point level. However, there was

an initial increase in mRNA at 4 hours. Similarly, a decrease in PTHrP secretion was observed. Down-regulation of PTHrP mRNA expression and PTHrP secretion could be explained by the presence of a nVDRE. The PTHrP gene contains a DNA sequence which is homologous to the nVDRE of human PTH (Nishishita T *et al*, 1998). Kremer R *et al* (1996a) have localized this nVDRE to a region between -1.2 and -0.3 kb upstream of the transcriptional start site. This evidence strongly suggests that the nVDRE results in inhibition of PTHrP expression and secretion in CaSki cells. The mechanism whereby 1,25-dihydroxyvitamin D₃ weakens the binding between the nVDRE and VDR, which results in a decrease in transcriptional and consequently translational activity of a gene, is unclear and is the subject of current investigation.

Darwish and DeLuca (1999) have recently reported a putative transcription factor that binds to a site adjacent to the nVDRE in the promoter region of the hPTH gene. Transfection experiments have determined that this factor is required for the high transcriptional activity of the hPTH gene. This high activity is significantly suppressed by 1,25-dihydroxyvitamin D₃. This factor seems to be expressed in several cell types and may be expressed in the CaSki cells. It is possible that this transcription factor, or an equivalent factor that acts in a manner similar to it, may explain the mechanism by which PTHrP is suppressed in the presence of 1,25-dihydroxyvitamin D₃.

It has also been suggested the parent substrate $1\alpha 25-(\text{OH})_2\text{D}_3$, may produce a major stable metabolite called $1\alpha 25-(\text{OH})_2\text{-3-epi-D}_3$. This has been found to suppress PTH secretion by 50% in cultured bovine parathyroid cells (Brown AJ *et al*, 1999). This metabolite may contribute to the prolonged suppressive effect of $1\alpha 25-(\text{OH})_2\text{D}_3$ on PTH gene transcription. This may also help explain the suppressive effect of $1\alpha 25-(\text{OH})_2\text{D}_3$ on PTHrP gene expression

4.3.1.2 Dexamethasone:

Dexamethasone is a synthetic glucocorticoid, commonly referred to as a glucocorticoid agonist. It is thought to function in a manner similar to $1,25\text{-dihydroxyvitamin D}_3$ and other members of the steroid-thyroid hormone superfamily (Beato M, 1989). Like vitamin D receptors, glucocorticoid receptors are widely distributed (Lan NC *et al*, 1984; Reichel H *et al*, 1989). Functional receptors have been demonstrated in human uterus (Giannopoulos G *et al*, 1983).

In our studies, dexamethasone produced a biphasic response. A dose- and time-dependent decrease in PTHrP mRNA expression was observed with a maximal decrease of 50% of time zero level at 24 hours. However, there was an increase in PTHrP mRNA expression at 1 hour which persisted to 6 hours after treatment. Similarly, a 50% decrease in iPTHrP

secretion was observed at 24 hours. Glucocorticoids have previously been reported to profoundly down-regulate PTHrP expression (Lu C *et al*, 1989; Glatz JA *et al*, 1994) and results of the current studies support these findings.

Therefore, dexamethasone has been established as a negative regulator of PTHrP in the cervical squamous carcinomacell line, CaSki, with effects that are dose-dependent and rapid. The action of dexamethasone was shown to be steroid-specific as exposure of the cells to 17β -estradiol, for example, caused an increase in both PTHrP expression and secretion. It is possible that the effects of dexamethasone are mediated via classical receptor response elements in the 5' regulatory region of the PTHrP gene. Glucocorticoid response elements (GREs), like the VRDEs, are small segments of DNA that represent the binding domain for the glucocorticoid hormones. The GRE has been proposed to be a 15-mer consensus sequence (Beato M, 1989). It is anticipated that one or more so-called negative glucocorticoid response elements (nGREs) will be found in the 5' flanking region of the PTHrP gene. nGREs are less well defined than positive GREs (Beato M, 1989). Such elements have **not** been found in the 2 kb of DNA 5' of the putative up-stream promoter of the PTHrP gene or by transfection techniques (Lu C *et al*, 1989). It is possible that the consensus sequence for a nGRE is very different from the typical GRE sequence or it may be located further 5' or 3' to the region of the PTHrP gene examined. Therefore, the

mechanism by which dexamethasone decreases PTHrP mRNA expression is unknown and is the subject of current investigation.

In summary, both 1,25-dihydroxyvitamin D₃ and dexamethasone produced a down-regulation of PTHrP mRNA expression and iPTHrP secretion. This is similar to results seen in other investigations. Glatz JA *et al* (1994) found that dexamethasone decreased PTHrP expression in a squamous cancer cell line. Dvir R *et al* (1995) found that PTHrP decreased PTHrP secretion by 75% in amniotic fluid cells. Nishishita T *et al* (1998) and Falzon M and Zong J (1998) found that 1,25-dihydroxyvitamin D₃ decreased gene expression of PTHrP. My results confirm that dexamethasone and 1,25-dihydroxyvitamin D₃ have an effect on CaSki cells and down-regulate PTHrP expression and secretion. Since it is known that both dexamethasone and 1,25-dihydroxyvitamin D₃ can down-regulate PTHrP and that PTHrP is involved in autocrine/paracrine functions then it may be possible to use this information to alter the growth of certain cells using these steroid hormones with respect to the effect they have on PTHrP production. Further work must be completed in the area in order to determine how PTHrP affects the growth of a particular cell type.

4.3.2 Sex steroids: Progesterone and 17 β -Estradiol:

The sequence elements recognized by different steroid receptors constitute a family of identical or closely related sequences. As previously mentioned, the consensus GRE is a 15 bp palindromic sequence (Beato M, 1989). This sequence cannot be discriminated from the progesterone response element (PRE) sequence. The question of how glucocorticoid and progesterone receptors can generate distinct patterns of gene expression despite similar, if not identical, DNA sequence recognition properties is a central question in steroid hormone biology.

The minimal functional estrogen response element (ERE) consensus sequence is a 13 bp perfect palindrome. The half-site of the ERE is identical to the half-site of the thyroid hormone response element (TRE) (Glass CK *et al*, 1988). It, too, shares a few essential nucleotides with the GRE (Beato M, 1989). No evidence exists that the ERE palindromecan interact with steroid hormone receptors other than the ER. However, a few single mutations within the ERE are sufficient enough to convert it to a GRE (Klock G *et al*, 1987). This suggests that there are only minor differences in the DNA recognition mechanisms of the various hormone receptors.

Human progesterone receptors are expressed as two major isoforms: PRA and PRB. PRA

and PRB are products of a single gene, but each transcript is individually regulated by unique promoter sequences upstream from two different transcriptional start sites (Kastner P *et al.*, 1990). It has become quite clear that PRA and PRB are not functionally identical. PRB is a strong transcriptional activator on many more PRE containing promoters than PRA (Wen DX *et al.*, 1994). These differences are cell-specific but a few exceptions do exist whereby the transcriptional activity of PRA can be significantly greater than that of PRB (Tora L *et al.*, 1993).

Like progesterone, receptors for 17 β -estradiol are expressed as two major isoforms: ER α and ER β . ER β also has several mRNA variants including ER β 1 and ER β 2 (Peterson DN *et al.*, 1998). Ligand binding induces a conformational change in ER and subsequent DNA binding to specific EREs present in target genes (Ogawa S *et al.*, 1998). ER α and ER β are homologous and have a similar affinity for 17 β -estradiol (Kuiper GG *et al.*, 1997). The DNA binding domain (DBD) of ER β is highly homologous with that of ER α , implying that both ER α and ER β share the same DNA response element (Ogawa S *et al.*, 1998). However, there is only a 55% amino acid identity in the ligand-binding domain of the two isoforms (Mosselman S *et al.*, 1996). Known ligands, including estrogen metabolites and partial agonists/antagonists such as tamoxifen, do not discriminate between ER α and ER β (Kuiper GG *et al.*, 1997). Tamoxifen is a nonsteroidal antiestrogen which acts predominantly as an

estrogen agonist rather than as an antagonist (Macinga D *et al*, 1995). It may act as an agonist in the presence of EREs in the promoter region of the target gene. It may activate the ER differently than if it were activated for example, by 17 β -estradiol. ER α and ER β each have distinct, yet overlapping patterns of expression (Kuiper GG *et al*, 1997). The physiological roles of ER α and ER β are currently under investigation and this will undoubtedly increase our understanding of the mechanisms of action of medically and environmentally important estrogen-like compounds.

In my experiments with CaSki cells, a significant dose-dependent change in PTHrP mRNA levels in response to progesterone treatment was not observed within the range of doses used at 24 hours. Time course experiments were carried out with progesterone using a concentration of 10⁻⁸ M which is the concentration that had produced maximal responses both in our cervical system and in other cell types. There was a time-dependent increase in PTHrP mRNA in response to progesterone. Rapid induction of PTHrP mRNA occurred in response to progesterone with a maximal 2-fold increase observed at 1 hour. At 4 hours, PTHrP mRNA began to decline and had fallen to basal levels (time zero) by 24 hours. This could explain the lack of response at 24 hours in the initial mRNA dose response experiments. That is, PTHrP mRNA expression had increased earlier and subsequently returned to basal levels at 24 hours when the cells were harvested and RNA extracted in the

dose-response studies. There was a dose-dependent increase in PTHrP mRNA in response to 17 β -estradiol as well as a time-dependent increase in PTHrP mRNA expression. Rapid induction of PTHrP mRNA occurred in response to 17 β -estradiol, with a maximal response of 4-fold at 6 hours. PTHrP mRNA began to decline by 24 hours and continued to decrease after both progesterone and 17 β -estradiol stimulation. At 48 hours the cells had reached confluence. At this time there was a significant decrease in PTHrP mRNA expression. This decrease in PTHrP mRNA at cellular confluence is observed in many monolayer cell lines (Merryman JI *et al*, 1993; Werkmeister JR *et al*, 1998).

The timing of the response to 17 β -estradiol is similar to observations in rat uterus *in vivo* (Thiede MA *et al*, 1991) and in GH₄C₁ rat pituitary cells (Holt EH *et al*, 1994). The CaSki cell line was originally isolated and established in continuous culture in 1977. Hence, these cells are being examined at a very high passage number. It has been noted in other normal and established cell lines that PTHrP expression and secretion decreased in high passage relative to low passage number cells (Kremer R *et al*, 1996b). Henderson J *et al* (1991) also found that in response to serum stimulation in both established (HPK1A) and malignant (HPK1A-*ras*) cells, dose-dependent increases were greater in HPK1A than in HPK1A-*ras*. The mechanism underlying these decreased levels of PTHrP expression and secretion upon repeated cell passage is unknown but could be a consequence of autocrine or intracrine

growth control mechanisms of non- or lower/lesser PTHrP-producing cells having a selective growth advantage over higher PTHrP-producing cells. This may explain why these malignant cells produce less PTHrP than HEC-16 immortalized cells.

It is not known whether the effects of estrogen on PTHrP mRNA in CaSki cells are mediated by increased gene transcription or increased mRNA stability. However, Holt EH *et al* (1994) treated GH₄C₁ rat pituitary cells with estrogen and found that there was an increase in PTHrP mRNA expression. They then performed nuclear runoff transcriptional assays to determine if the increase in expression was due to an increase in transcription. They found a transcriptional response that was rapid and transient, peaking at the earliest time point examined. This confirmed that the increase in PTHrP mRNA expression was indeed due to an increase in transcription. Although the mechanism has not yet been elucidated for CaSki cells it is quite possible that CaSki cells responded to estrogen, as did the GH₄C₁ cells, with an increase in transcription. It is unclear whether 17 β -estradiol regulates PTHrP production directly or indirectly. In one study, it was suggested that estradiol might modulate the nuclear nVDR indirectly by increasing circulating concentrations of 1,25-dihydroxyvitamin D₃ (Duncan WE *et al*, 1991), subsequently resulting in a decrease in PTHrP mRNA expression. However, this cannot explain the observed increase in PTHrP mRNA expression in response to 17 β -estradiol treatment in CaSki cells. 17 β -Estradiol has been shown to

increase EGF receptor (EGFR) levels in various tissues (Mukku VR and Stancel GM, 1985; Gardner RM *et al*, 1989). For example, when 17β -estradiol was administered to female rats, it produced increases in EGFR levels which were suggested to be mediated by a transcriptional mechanism (Mukku VR and Stancel GM, 1985). In HEC-16 cells, the effect of 17β -estradiol on EGFR mRNA levels has been examined (Gogal L, 1998). An increase in EGFR mRNA would suggest that estrogen may act in a similar fashion to EGF to increase PTHrP mRNA expression. In HEC-16 cells, 17β -estradiol was indeed found to increase EGFR mRNA levels (Gogal L, 1998). These results support the possibility that 17β -estradiol-stimulated changes in EGFR number could stimulate the synthesis of PTHrP. An ERE has **not** been found in the promoter region of the PTHrP gene. This further supports the possibility that 17β -estradiol may act via EGFR at a transcriptional level to modulate PTHrP mRNA expression in HEC-16, as well as in CaSki cells.

Progesterone has not previously been shown to produce an increase in PTHrP expression in other tissues. For example, in both rat uterus *in vivo* (Thiede MA *et al*, 1991) and in rat Leydig cell tumour H-500 cells (Liu B *et al*, 1993), there was a decrease in PTHrP mRNA expression after treatment with progesterone. In cultured human endometrial cells, progestins also produced a decrease in PTHrP production (Casey ML *et al*, 1993). However, in HEC-16 cells (Rahimtula M and Kaiser SM, submitted), progesterone was

observed to increase PTHrP mRNA levels in both a dose- and time-dependent manner. Although a change was not observed in dose-response studies, a significant increase in PTHrP mRNA was seen in time course studies in response to progesterone. An increase in EGFR may explain the change in PTHrP mRNA in response to progesterone. The effect of progesterone on EGFR has been studied both *in vitro* and *in vivo* with variable results. Progesterone did produce an increase in EGFR numbers in human breast cancer cells (Lange CA *et al*, 1998). In the human uterus, progesterone modified endometrial EGFR with a decrease in endometrial EGFR numbers in the secretory phase of the menstrual cycle, in response to progesterone levels (Jasonni VM *et al*, 1991). In the established and malignant cells we have studied, potential changes in EGFR mRNA in response to progesterone, have not been examined. Evidence of a PRE has not been found to date in the promoter region of the PTHrP gene. This suggests that progesterone, like estrogen, may increase EGFR at a transcriptional level to produce an increase in PTHrP mRNA expression in these cells. It is also plausible that a completely separate mechanism exists whereby the expression of PTHrP mRNA in CaSki cells is altered.

Recent studies have reported that malignant transformation of sex-steroid-dependent tissues is often associated with a loss of expression of sex steroid hormone receptors (Kanai M *et al*, 1997). Malignant transformation of the squamous epithelium of the uterine cervix may similarly result in aberrant expression of steroid hormone receptors. While the presence of the estrogen receptor has been reported in all samples of normal cervical tissue (Soutter WP and Leake RA, 1987), several investigators have measured ER and PR levels in cervical cancer. The expression of PR and ER in cervical tissues has been controversial in that a variable proportion of PR and ER exists in different samples of cervical tissue (Ciocca DR *et al*, 1989; Konishi I *et al*, 1991; Robertson DI *et al*, 1993). For example, using immunohistochemical (Vargas-Roig LM *et al*, 1993) and ligand-binding assays (Hahnel R *et al* 1979), very low or undetectable levels of ER and PR were reported in one study of cervical cancer. Others have reported somewhat higher levels (Ford LC *et al*, 1983; Gao YL *et al*, 1983). The differences among studies may be due to differences in methods of tissue collection, storage conditions, assay techniques and perhaps in patient populations. We have not tested CaSki cells ourselves to ascertain if they do indeed possess PR and/or ER. In one study using CaSki cells, three assays were negative for both receptors (Grenman S *et al*, 1988). However, it is not stated which methods or which conditions were used to test for the presence or absence of these receptors. Further investigation using immunoblot analysis has confirmed that CaSki cells do possess the high-affinity 56 kDa estrogen receptor (Macinga D *et al*, 1995). Given that estrogen produced increases in PTHrP mRNA, and that

these responses may be mediated via ER, it is probable that ER numbers were sufficient to bring about the changes in response to the 17 β -estradiol. In one study using ectocervical cells, estrogen receptor levels were greatly diminished in cells positive for the HPV genome (Macinga D *et al*, 1995), thus suggesting a selective advantage that may exist for malignant cervical cells with nonfunctional ER pathways. Although a separate mechanism may exist for progesterone's action on CaSki cells, is also possible that PRs were present and their numbers may have been sufficient, as confirmed in the present studies, to bring about a change in response to progesterone.

17 β -Estradiol also produced an increase in iPTHrP secretion. iPTHrP production increased nearly 2-fold compared to basal (time zero). This increase in PTHrP secretion was likely due to the upregulation of PTHrP mRNA levels. However, there was no significant increase in iPTHrP secretion after progesterone treatment in both dose- and time-dependent experiments. The question arises as to how progesterone could produce an increase in PTHrP mRNA but fail to elicit a significant increase in iPTHrP secretion. The possibility exists that a PRE consensus sequence may not exist, or one with a similar function, or that progesterone may act through a different response element all together within the gene. Nevertheless, given that an increase in PTHrP mRNA was indeed observed in response to progesterone, the question remains as to why iPTHrP secretion was not affected. It is

known that the level of iPTHrP secretion was not less than the detection limit of the RIA.

A possible explanation for the lack of response is that the increase in PTHrP produced in response to progesterone is rapidly degraded, either intracellularly or extracellularly, to a nonimmunoreactive product. This is unlikely given that other factors similarly increased the secretion of iPTHrP which could be detected by the RIA. It may also be that intracellular PTHrP is being produced within the CaSki cells but is simply not being secreted. It is even possible that alternate promoters exist and are utilized in the presence of progesterone and not in the presence of any other hormones/growth factors used in experimentation. It remains unclear as to why there was a lack of iPTHrP secretion. There may be other levels of control including post-transcriptional regulation or post-translational processing that prevents the secretion of iPTHrP. Further investigation would be necessary to determine the reason for this finding.

These data suggest that, in cervical squamous carcinomas, the physiological actions of These data suggest that, in cervical squamous carcinomas, the physiological actions of estrogen may be mediated, at least in part, by PTHrP. Further studies to explore changes in PTHrP mRNA expression and protein levels in cultures of the cervical squamous carcinoma cells, CaSki, would be helpful to better understand the mechanism whereby 17 β -estradiol and progesterone produce their effects.

4.4 CASKI VERSUS HEC-16

Like HEC-16 cells, both EGF, progesterone and 17β -estradiol increased PTHrP mRNA in CaSki cells whereas $1,25$ -dihydroxyvitamin D_3 decreased its expression. Dexamethasone also decreased PTHrP mRNA expression in the Ca Ski cells but at 24 hours no response was apparent in the HEC-16 cells. Dexamethasone did decrease PTHrP mRNA expression in HEC-16 cells, but not until 48 hours (Rahimtula M and Kaiser SM, submitted). Thus, it is evident that CaSki cells have a pattern of response of PTHrP mRNA similar to that of HEC-16 cells.

There were differences between CaSki and HEC-16 cells however, in the changes in PTHrP mRNA in response to stimulation with the various factors. For example, EGF produced about a 1.5-fold greater increase in PTHrP mRNA in CaSki cells than in HEC-16 cells, whereas progesterone treatment caused a 2.5-fold greater increase in PTHrP mRNA in HEC-16 cells than in CaSki cells. PTHrP has previously been noted to respond differently in different cells. For example, calcium caused an increase in PTHrP expression in cultured Leydig cells (Rizzoli R *et al*, 1989) however, it decreased its expression in a rat parathyroid cell line (Zajac JD *et al*, 1989). It is also known that PTHrP responds differently under different culture conditions. For example, Henderson J *et al* (1991) demonstrated that varying the calcium concentration in the HPK1A cell line caused significant differences in PTHrP

mRNA expression. CaSki cells were maintained in DMEM plus 10% FCS and HEC-16 cells were maintained in Keratinocyte Growth Medium (KGM). Constituents of the two media differ and this may explain the difference in PTHrP mRNA expression between the two cell lines.

Patterns of iPTHrP secretion were similar in CaSki cells and in HEC-16 cells. That is, EGF and 17 β -estradiol both increased iPTHrP secretion, whereas dexamethasone and 1,25-dihydroxyvitamin D₃ both decreased secretion at 24 hours. Progesterone stimulation produced a significant increase in iPTHrP secretion in HEC-16 cells at 24 hours. Progesterone did not significantly change iPTHrP secretion in CaSki cells. Further investigation would be necessary to determine the reason for this difference in secretion between the two cell types. Nonetheless, these results demonstrated that the malignant CaSki cells secreted significantly less iPTHrP than did established HEC-16 cells. There was an obvious dysregulation of PTHrP in the malignant cell line.

My data suggests that increased PTHrP gene expression contributes to an increase in iPTHrP secretion. However, compared to immortalized cells, the level of iPTHrP secretion in CaSki cells has dramatically decreased. Reasons for the decrease in iPTHrP are not known but, considering the possibility of PTHrP's involvement in cell growth and differentiation, the

decrease in PTHrP secretion may provide a selective advantage to these malignant cells with respect to their growth and differentiation. As HHM is characterized by an increase in circulating levels of iPTHrP within the body, this decrease in iPTHrP secretion by malignant cells may explain why gynecologic malignancies are not commonly associated with HHM.

4.5 FUTURE DIRECTIONS

It would be informative to investigate the effects of an estrogen antagonist, such as tamoxifen or raloxifene, on PTHrP expression and secretion. It is anticipated that tamoxifen would decrease PTHrP production. We anticipate these results as 17 β -estradiol increased PTHrP mRNA expression and secretion in CaSki cells and tamoxifen is an anti-estrogen. Dose- and time-dependent experiments could be performed to examine the effect of tamoxifen on PTHrP expression in cervical squamous carcinoma cells. It is possible that tamoxifen could reverse or antagonize the effects of estrogen in these cells and thus provide a new approach to the management of HHM or more interestingly, the growth of the tumour cells.

It would also be informative to treat CaSki cells with cycloheximide and to perform nuclear runoff assays after treating the cells with various hormones and factors (in particular, EGF and 17 β -estradiol as these two factors produced the largest increases in PTHrP expression in CaSki cells). This would determine whether the increase in mRNA expression is due to

an increase in mRNA stability, an increase in transcription or both.

Another possible study would be to assess the effect of PTHrP on the growth rate of CaSki cells. Neutralizing antibody would be added to the culture medium during time course experiments and cell numbers would be assessed at timed intervals. These results would be compared to cell numbers obtained in current experiments performed without the addition of a neutralizing antibody. This relatively simple experiment would allow us to conclude whether or not PTHrP has an effect on the growth of these cells.

Immunocytochemistry could be performed to confirm the presence or absence of PTHrP protein in these ectocervical squamous carcinoma cells. The pattern of staining (for example, cytoplasmic and/or nucleolar staining) would yield useful information with respect to functions of PTHrP, other than those classically mediated via interaction with its G-protein coupled receptor.

4.6 CONCLUSIONS

The cervical squamous carcinoma cells used in these studies had been infected with HPV-16, the virus most commonly associated with cervical carcinoma. Treatment of these cells with various hormones and factors affected the expression of PTHrP mRNA and iPTHrP secretion. Not only did the previously studied regulators (EGF, dexamethasone and 1,25-

dihydroxyvitamin D₃) have a significant influence on PTHrP expression and secretion, but the tissue-specific regulators, estrogen and progesterone, also produced the similar effect. Whereas cervical carcinomas are not conventionally considered to be steroid-hormone responsive, these changes in response to estrogen and progesterone suggest a potential for modulation. This could be assessed in our model using tamoxifen, other anti-estrogens or anti-progestins. Although these cervical carcinoma cells did not have a significant response in iPTHrP secretion in response to progesterone, the possibility remains that modulation of PTHrP secretion might occur in other, progesterone receptor-positive cells. The specific effects of PTHrP would have to be further analyzed through further experimentation to determine PTHrP's specific effects of cervical cancer. Although further investigation is necessary, it is possible that PTHrP may act as an intracrine growth regulator within these cervical cells.

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APPENDIX A

Biochemical Component	Units	Fetal Calf Serum	
		mean	range
Total protein	g/dL	6.9	(6.1 - 7.5)
PH	units	7.6	(7.3 - 8.1)
Osmolarity	mOsm/kg	292	(273 - 310)
Glucose	mg/dL	105	(90 - 118)
Hemoglobin	mg/dL	15.2	(10.2 - 23.2)
Bilirubin	mg/dL	0.2	(0.1 - 0.4)
Uric Acid	mg/dL	0.9	(0.8 - 1.0)
Urea Nitrogen	mg/dL	10.0	(6 - 13)
Creatinine	mg/dL	1.1	(0.7 - 1.6)
Sodium	meq/L	137	(134 - 142)
Potassium	meq/L	5.5	(4.9 - 6.0)
Calcium (total)	ng/dL	9.9	(9.7 - 10.0)
Chloride	meq/dL	106.7	(99 - 112)
Phosphorous (inorganic)	mg/dL	7.6	(6.8 - 8.4)
Iron (total)	µg/dL	74	(53 - 93)
Albumin	g/dL	3.6	(3.4 - 3.8)
Globulin (total)	g/dL	3.3	(2.6 - 3.7)
Alkaline Phosphatase	U/L	188	(125 - 295)
GG-Transpeptidase	U/L	53.3	(27 - 79)
SGOT	U/L	83.3	(64 - 94)
Lactate dehydrogenase	U/L	1067	(975 - 1220)
Cholesterol	mg/dL	78.3	(95 - 121)
Low Density Lipoprotein	mg/dL	56.7	(51 - 72)
High Density Lipoprotein	mg/dL	36.7	(41 - 63)
Triglycerides	mg/dL	16.3	(15 - 17)
Growth Hormone	ng/mL	26	(14 - 35)
Insulin	µIU/mL	5.4	(4.2 - 6.8)
Estradiol	ng/mL	32.9	(22.2 - 39.2)
Progesterone	ng/mL	1.65	(0.88 - 2.20)
Testosterone	ng/mL	1.05	(0.79 - 1.19)
T4 (Thyroxine)	µg/mL	9.2	(7.8 - 10.0)
T3	ng/mL	1.9	(1.5 - 2.5)



