

UNIVERSITY OF SOUTHAMPTON

The role of epidermal growth factor and parathyroid hormone related peptide (1-34) in three choriocarcinoma cell lines as a model for implantation of human trophoblast.

by

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ABSTRACT

UNIVERSITY OF SOUTHAMPTON
FACULTY OF SCIENCE
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Doctor of Philosophy

THE ROLE OF EPIDERMAL GROWTH FACTOR AND PARATHYROID HORMONE RELATED PEPTIDE (1-34) IN THREE CHORIOCARCINOMA CELL LINES AS A MODEL FOR IMPLANTATION OF HUMAN TROPHOBLAST.

By Kathleen Ann Bradford.

There is increasing interest in the early stages of pregnancy, in particular growth factors controlling embryonic trophoblast invasion since they dictate whether a reproductive cycle will be successful and have been implicated in abnormal placental growth and function in pathological pregnancies. The human placenta comprises a milieu of growth factors and I investigated Epidermal Growth Factor (EGF) and its receptor family since EGFR and its homologue erbB-2 are abundant in placental membranes showing divergent expression throughout placental growth and differentiation. An in vitro model was established using three choriocarcinoma cell lines of human trophoblastic origin, JAr, Jeg-3 and BeWo to determine the effects of EGF upon proliferation. The JAr cell line responded significantly to EGF at a range of doses (0.1ng/ml-20ng/ml) with a maximal increase in proliferation of 90% at 5ng/ml. Similarly, on addition of 5ng/ml BeWo growth was significantly increased by 20% but the Jeg-3 cell line did not show any proliferative response to EGF under my culture conditions. Receptor binding studies showed that following a 2h incubation at pH 7.6 and 4°C ^{125}I -EGF bound to all three cell lines with two affinities - a high affinity binding site and a low affinity binding site. In the JAr cell line, the dissociation constants for the high and low binding sites were $3.0 \pm 0.72 \times 10^{10} \text{M}^{-1}$ and $5.5 \pm 1.4 \times 10^9 \text{M}^{-1}$, respectively and 19,874 \pm 799 and 37,318 \pm 1,435 receptors per cell. BeWo and Jeg-3 cell lines displayed similar affinities - for BeWo ^{125}I -EGF bound with Kds of $2.2 \pm 1.65 \times 10^9 \text{M}^{-1}$ (3,601 \pm 572 receptors/cell) and $17.0 \pm 1 \times 10^9 \text{M}^{-1}$ (8,091 \pm 484 receptors/cell) for the high and low affinity sites respectively. Similarly, in Jeg-3 two dissociation constants of $2.7 \pm 1.43 \times 10^9 \text{M}^{-1}$ and $18.2 \pm 1.19 \times 10^9 \text{M}^{-1}$ with 5,994 \pm 1,321 and 11,752 \pm 1,904 receptors per cell, for both high and low binding sites. There are then differences between the effects of EGF upon each cell line, as seen in the proliferation studies, and the ligand binding studies suggest that this could be due to varying receptor number or incongruity in EGF/EGFR association. Parathyroid Hormone related Peptide is another growth factor found in the human placenta and is purported to regulate EGFR expression. I investigated the effect of PTHrP(1-34) upon the JAr cell line and discovered that PTHrP(1-34) up-regulates the expression of the EGFR. Conversely, PTH (1-34) down-regulated EGFR expression. Competitive ligand-binding studies between PTH(1-34) and PTHrP (1-34) in SaOS-2 Cells suggested the peptides were binding to the same receptor whereas in JAr cell lines PTH(1-34) did not displace PTHrP(1-34) suggesting that a novel PTHrP receptor exists in the JAr choriocarcinoma cell line. Clearly there is strong evidence supporting a role for these peptides in the control of trophoblast proliferation and/or differentiation in vitro but whether this is mimicked in vivo remains unknown.

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ABBREVIATIONS

1°	Primary
2°	Secondary
³ H	Tritium
¹²⁵ I	Iodine isotope 125
A	Adenine
A	Amps
aa	Amino acid
ABC-AP	Avidin-Biotin complex alkaline phosphatase
APS	Ammonium persulphate
APSS	Alkaline phosphatase substrate solution
AR	Amphiregulin
ARIA	Acetylcholine receptor inducing factor
ATP	Adenosine triphosphate
B	Bound
B/T	Bound to total added ratio
BL	Blastocyst
BM	Basement membrane
BSA	Bovine serum albumin
C	Centigrade
C-terminal	Carboxy terminal
Ca	Calcium
cAMP	cyclic adenosine-3',5'-monophosphate
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curie
CO ₂	Carbon dioxide
cpm	Counts per minute
CV	Crystal violet
CVDE	Crystal violet dye elution
CVi	Cytotrophoblastic Villi
cys	Cysteine
Da	Daltons
DAG	Diacylglycerol
DMEM	Dulbecco's minimal essential medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid.
dpm	Disintegrations per minute
E ₂	Estradiol
EC	Extracellular
EDTA	Ethylene-bis(B-amino-ethyl ether) N,N'-tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's Minimal Essential Medium
FCS	Foetal calf serum
GA	Growth Arrested
GDP	Guanosine diphosphate
GGF	Glial growth Factor
GMF	Glass microfibre
gp	Glycoprotein
Grb	Growth Factor receptor bound protein
GTP	Guanosine triphosphate

HB-EGF	Heparin Binding Epidermal Growth Factor
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
hEGF	Human Epidermal Growth Factor
hPL	Human placental lactogen
HRG	Heregulin
IC	Intracellular
Ig	Immunoglobulin
IHC	Immunohistochemistry
IP	Inositol -1-phosphate
IP2	Inositol-1,4,-bisphosphate
IP3	Inositol-1,4,5-triphosphate
Kd	Dissociation constant
MAP kinase	Mitogen activated protein kinase
Mg	Magnesium
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
MW	Molecular weight
NAF	Neu activating factor
NDF	Neu differentiation factor
NH2-terminal	Amino terminal
NSB	Non-Specific Binding
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Phosphatidylinositol
PI3 kinase	Phosphoinositide 3-kinase
PIP	Phosphatidylinositol - 4, 5,- phosphate
PIP2	Phosphatidylinositol-1,4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC γ	Phospholipase C Gamma
PSA	Penicillin, streptomycin and amphotericin antimycotic antibiotic
PTH	Parathyroid Hormone
PTH/PTHrPR	Parathyroid hormone/Parathyroid hormone related peptide receptor
PTHrP	Parathyroid hormone related peptide
RNA	Ribonucleic acid
rpm	revolutions per minute
RPTK	Receptor protein tyrosine kinase
RTK	Receptor Tyrosine Kinase
SB	Specific Binding
SDS	Sodium dodecyl sulphate
SFCS	Stripped foetal calf serum
SH2	Src homology 2
SH3	Src homology 3
Sos	Son of sevenless
Src	Rous sarcoma virus
ST	Syncytiotrophoblast
T	Thymidine
T	Total Added
TB	Total Binding
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TGF α	Transforming growth factor alpha
TMSR	Transmembrane spanning region

TNF α	Tumor Necrosis Factor alpha
TNF α R	Tumor Necrosis Factor alpha receptor
TTBS	Tween-20-Tris buffered saline
UBV	Uterine Blood Vessel
UE	Uterine Epithelium
UG	Uterine Gland
uv	Ultraviolet
VGF	Vaccinia Growth Factor

CHAPTER ONE

“Sexual reproduction in the ocean necessitates only the combination of gametes, followed by absorption of nutrients and oxygen from the surrounding watery medium. As life moved from the sea to the land, reproductive strategies required compensation for the loss of this aquatic environment. For the mammals, and scattered other animals, the solution to this problem was the development of the placenta.....”

Harvey Kliman, 1994

INTRODUCTION.

A staggering 85% of human conceptions fail to make it to full term thus it seems that for various reasons pregnancy fails between implantation and term. The majority of miscarriages (>60%) occur in the first trimester, due either to genetic defects or a failure of placental development. Implantation of the conceptus is a key event in human reproduction and a breakdown in normal events may contribute to our apparent failure to reproduce but unfortunately trophoblast invasion is highly inaccessible for study. At the moment there is no animal species known that shows a similar pattern of trophoblast invasion as the human, therefore no comparable animal model is available to study in vivo or to provide tissue for in vitro studies.

In this study, I have utilised the fact that choriocarcinoma cells in culture (JAR, Jeg-3 and BeWo) provide a model for studying trophoblast cells in vitro.

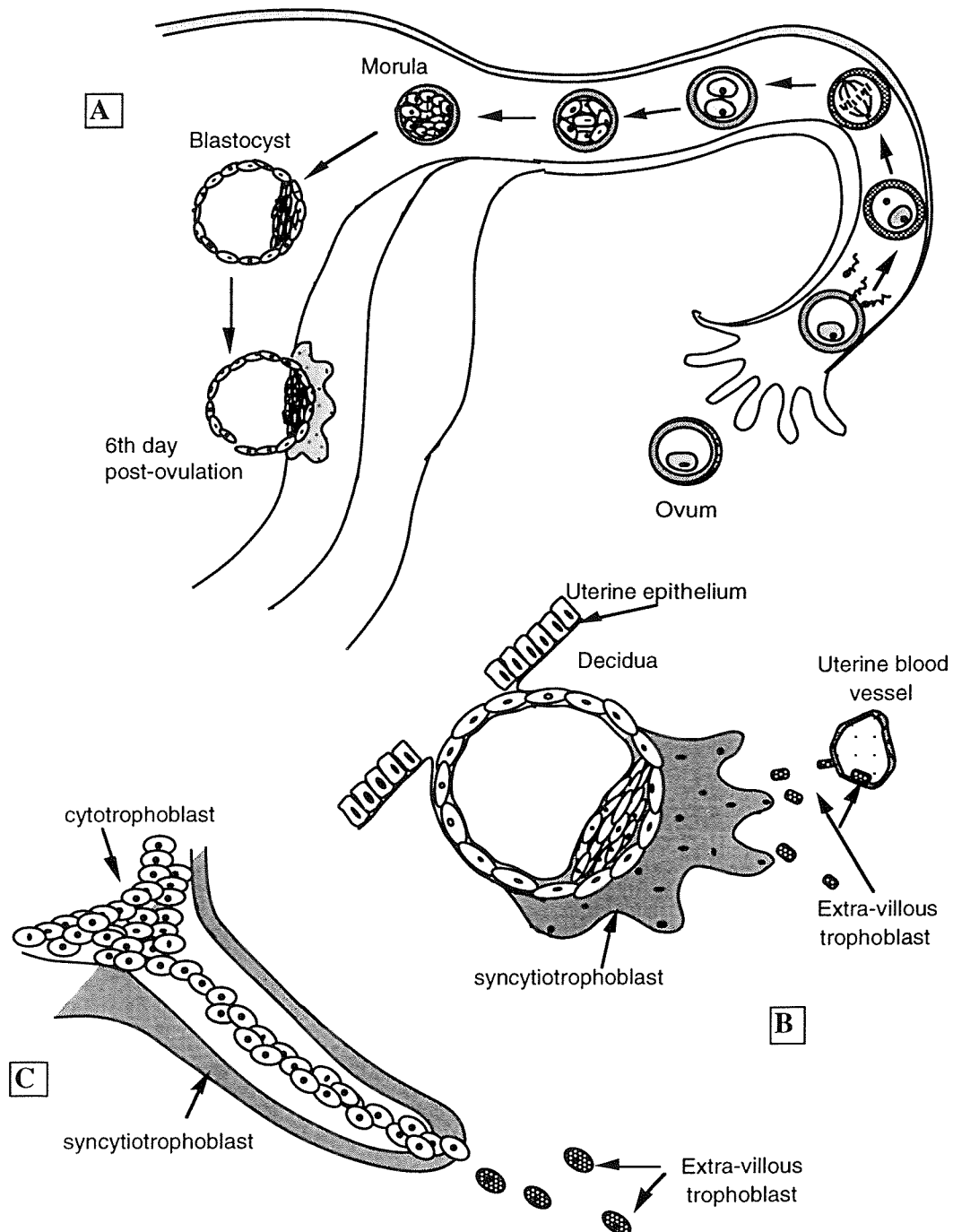
Many factors have been implicated in trophoblast proliferation and this thesis concentrates particularly on the role of Epidermal Growth Factor (EGF) and its receptor (EGFR), Parathyroid Hormone (PTH) and Parathyroid Hormone related Peptide (PTHrP).

To understand fully the contribution of these “growth factors” to trophoblast invasion and subsequent development, the bulk of this thesis is devoted to the characterisation of the growth factor receptors for EGF and the binding of PTH and PTHrP in the choriocarcinoma cells. The proliferative effect of EGF upon the cell-lines was investigated in detail and Chapter three highlights the differing properties of this potent mitogen. In Chapter four, the precise nature of the EGFR was investigated and this provided the basis of the work in Chapter five whereby

the effects of PTH and PTHrP upon EGF receptor expression were looked at. Finally, in Chapter six, I undertook a study of the PTH/PTHrP receptors since work in this area is relatively new and constantly being reviewed and up-dated.

I hope that the research outlined in this thesis will provide some insight into the precise nature of three growth factors present in the placenta but what became apparent whilst undertaking this research was the fact that we still remain very much in the dark as to the remarkable series of events which physically connect the mammalian embryo to the mother.

Diagram 1.1. A schematic overview of implantation.



- A. Apposition, adhesion and penetration of the blastocyst.
 B. Embedded blastocyst.
 C. Primary villus showing the three different trophoblast phenotypes.

1.1. Implantation.

Implantation is the process by which the conceptus (the fertilised egg and all its derivatives) is joined to the uterus. Following fertilisation the egg is transferred into the uterus where it becomes implanted in the endometrium. Cell division has already resulted in the formation of a solid clump of cells - the morula - and days after fertilisation, fluid begins to accumulate between cells of the morula and the blastocyst is formed. The blastocyst is a hollow sphere which has shed its non cellular outer coat, the zona pellucida, and comprises a polarised embryoblast and an outer layer of trophoblast (Diag. 1.2).

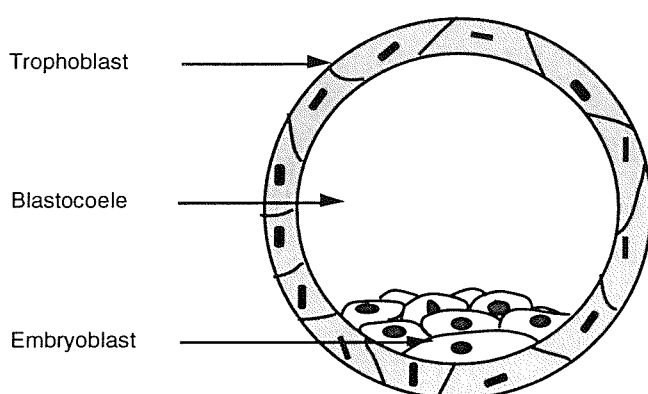


Diagram 1.2. Cartoon representation of a day 7 blastocyst (zona pellucida is not shown).

The central cavity or blastocoele is asymmetrical due to the presence of the Inner Cell Mass (ICM) at the embryonic pole. The trophoblast constitutes a continuous peripheral layer, whose role is to isolate the embryonic component and to induce the expansion of the blastocoele.

The embryoblast is destined to form the embryo whereas the peripheral trophoblast's role is to contact maternal tissue, invade it and form a placenta and other extraembryonic structures. Four-to five days after ovulation the blastocyst arrives in the uterus and remains in the lumen of the uterus for a further two days before it implants.

Six days after fertilisation the blastocyst begins to attach to the uterine mucosa but this is not simply a process of mechanical adhesion; the embryo and the endometrium interact with one another to determine the specific site of implantation and the degree of invasion.

The process of implantation can be divided into three phases, apposition, adhesion and penetration (Diagram 1.1). Apposition involves the correct orientation of the blastocyst but does not appear to involve any connection between the two

interfaces as simple washing will dislodge the blastocyst from the uterus (Bischof *et al*, 1992).

Adhesion involves complex cell-cell interactions mediated by adhesion molecules. Penetration involves breaching of the uterine epithelium by thin folds of trophoblast which extend through endometrial epithelial cells towards the basement membrane. The basement membrane is degraded and the trophoblast cells are able to invade the underlying endometrial stroma and maternal vessels and begin to establish a haemochorial placenta.

1.2. Trophoblast.

In the human, trophoblast attaches to the uterine wall by penetrating the endometrial stroma and the invasion process is led by a group of stem cells called cytotrophoblasts. These progenitor cytotrophoblast cells fuse to form a syncytial layer or syncytiotrophoblast which is primarily responsible for invasion in the early stages of pregnancy. There is differentiation into a peripheral layer of primitive syncytiotrophoblast and an inner layer of cytotrophoblast which starts proliferating to form the precursors of the primary villi. Later, mesoderm grows into these villi forming the secondary and tertiary villi.

Cytotrophoblastic cell columns extend through the peripheral syncytium, fanning out and creating a cytotrophoblastic shell. In the early stages of implantation, the erosion of maternal tissues lies mainly with the syncytial layer but cytotrophoblastic shell formation causes an increase in the implantation area due to the proliferative activity of the cytotrophoblasts. By eight weeks this proliferation is confined mainly to the cytotrophoblast present at the tips of the anchoring villi which make contact with the basal decidua and the villus tips become plugged with cytotrophoblast.

These columnar cytotrophoblast cells extend into the decidua and intermediate trophoblast cells begin to migrate individually into the maternal tissue. The intermediate or extravillous trophoblast cells (EVT) are the most highly invasive and migratory type of trophoblast and are visible by 4 weeks of gestation. They are responsible for penetrating the maternal blood vessels and thus allowing blood into the lacunae in the syncytiotrophoblastic mass, to form a primitive blood supply to the fetus. Thus, cytotrophoblast invasion not only anchors the fetus to

the mother but also creates large diameter, low resistance vessels that carry blood to the floating villi at the maternal-fetal interface (Bass *et al*, 1994)

From 10 weeks of gestation onwards, the number of extravillous trophoblasts declines and large multinuclear trophoblastic giant cells are observed. The exact function of these cells is not known but it has been suggested that they represent the ultimate stage of extravillous cytotrophoblast differentiation because they portray no invasive characteristics. At term, the giant cells are the only recognisable cells of trophoblastic origin. (Diagram 1.3.)

1.3. The role of EGFR in the trophoblast.

The Epidermal growth factor receptor (EGFR) is found in large amounts in the placenta (Bulmer *et al*, 1989; Lai and Guyda 1984) and binding of EGF to its receptor stimulates both human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) production (Maruo *et al*, 1987). Hoshina *et al* (1982) supposed that the secretion of these hormones represents subsequent levels of trophoblast differentiation.

Culture of first trimester trophoblasts shows that EGF and TGF α (Transforming Growth Factor α) stimulate their proliferation and differentiation (Genbachev *et al*, 1993). EGF stimulates the proliferation of both villous and columnar cytotrophoblast and also causes the migration of pre-existing and newly differentiated extravillous trophoblast. EGF induces differentiation of trophoblast cells and increases in vitro differentiation from cytotrophoblast to syncytiotrophoblast during the first four days in culture (Bass *et al*, 1994).

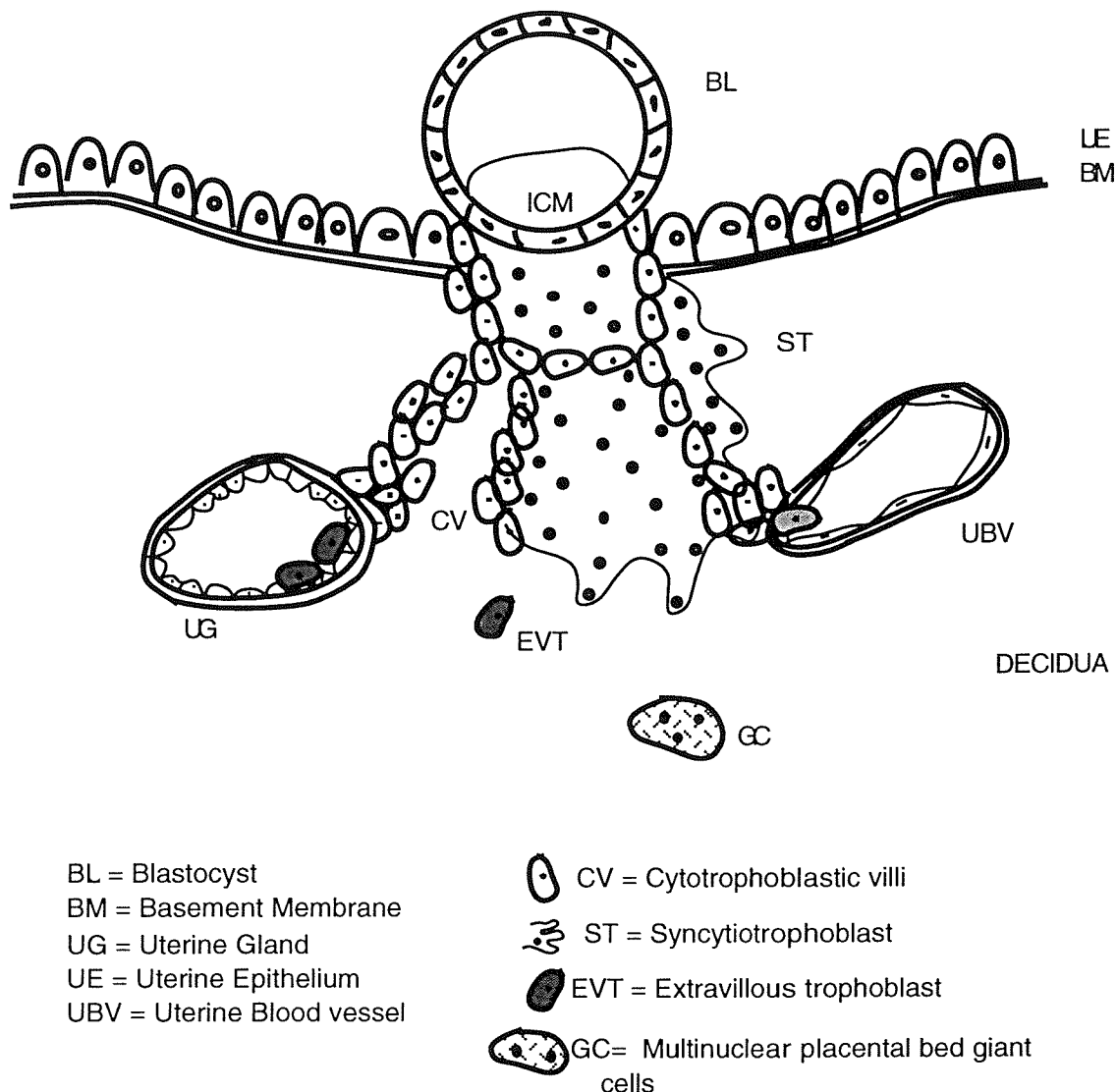


Diagram 1.3. Schematic representation of the human blastocyst at 12 days after ovulation.

The blastocyst has positioned itself correctly in the 'implantation window' and the trophoblast has penetrated the maternal uterine epithelium. The different trophoblast phenotypes are indicated in the human placental bed in their different stages of differentiation - the proliferative cytotrophoblast, the hormone producing syncytiotrophoblast, the migratory and invasive extravillous trophoblast and ultimately the multinucleated giant cells.

Immunohistochemistry has shown that the EGFR and its homologue erbB-2 (section 1.6) are expressed along the plasma membranes of placental trophoblast and of cell islands and cell columns but in a stage and differentiation-specific pattern. EGFR expression is predominant in the proliferative trophoblast and, with advancing pregnancy, confined to the differentiated villous trophoblast, whereas the erbB-2 gene product is expressed at advanced stages of trophoblast differentiation. This divergent expression suggests that the EGFR is involved in proliferation of trophoblast and in contrast erbB-2 plays a role in differentiation

(Muhlhauser *et al*, 1993). The balance of proliferation and differentiation ultimately determine the structure and function of the developing placenta and so it seems that EGFR and erbB-2 are vital components of this process.

Placental trophoblast cell growth has been compared with tumour cell growth, in as much that:

- i) Metabolism in the placenta is largely governed by rapid demands for growth;
- ii) No stimulus is required for cell division as replication is autonomous beyond the needs of tissue replacement;
- iii) The trophoblast cells invade at the site of implantation and infiltrate blood vessels;
- iv) Proteases are produced during invasion and migration of trophoblast cells;
- v) There are biochemical similarities between placental cells and tumour cells - histaminase activity, plasminogen activator activity and the production of alkaline phosphatase isoenzymes, and
- vi) the placenta induces an adaptive circulatory response in the local maternal tissues (Hoekstra and Herscheid, 1985)

However, unlike malignant tumours, normal placental growth is under strict control with the correct degree of cell invasion, migration, proliferation and differentiation all being carefully balanced. If EGFR and erbB-2 expression plays a part in this control mechanism then the degree of expression of one or both receptors may elicit different biological responses and hence dictate placental growth. Bearing this in mind, the importance of studying this particular growth factor and its receptor becomes paramount, not only in placental development, but also in the understanding of malignant neoplastic growth.

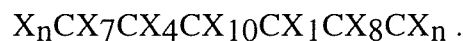
1.4. Epidermal growth factor and its related peptides.

EGF was first identified in mouse submaxillary gland extract from its ability to induce premature eyelid opening in neonatal mice (Cohen, 1962). It has many diverse biological functions and is found in numerous tissues.

hEGF is synthesised as a membrane anchored 1207 aa precursor which is biologically active and can function in a juxtacrine fashion. cDNA analysis showed that the precursor not only contains the EGF aa sequence but also eight EGF-like units. The mature 53 aa peptide is derived from its precursor by proteolytic cleavage. The gene for EGF has been mapped to chromosome 4.

EGF is a member of the epidermal growth factor family which also includes TGF α (Derynck, 1986), amphiregulin (AR) (Shoyab *et al*, 1989), heparin-binding EGF (Higashiyama *et al*, 1991), β -cellulin (Shing *et al*, 1993), vaccinia growth factor (VGF) (Brown *et al*, 1985) and neu differentiation factor/heregulin/glia growth factor (NDF/HRG/GGF) (Holmes *et al*, 1992; Marchionni *et al*, 1993; Wen *et al*, 1992). The function of this family is the mitogenic stimulation and differentiation of epidermal tissues (and others) (Higashiyama and Taniguchi, 1994).

These proteins all contain an aa sequence with 6 cys residues in a specific spacing pattern:



hTGF α was isolated from the conditioned medium of some human tumour cells by its ability to bind to EGFR (Marquardt and Todaro, 1982). It contains 50 aa and has 40% homology with EGF and its gene has been mapped to chromosome 2 (Diag 1.4.). It exerts its effects by binding to the EGFR (Reynolds *et al*, 1981) and in chickens it does so with a greater affinity for the receptor than EGF itself (Lax *et al*, 1988; Onagbesan *et al*, 1995). It has been shown to enhance proliferation of first trimester trophoblast cells propagated in culture (Lysiak *et al*, 1993).

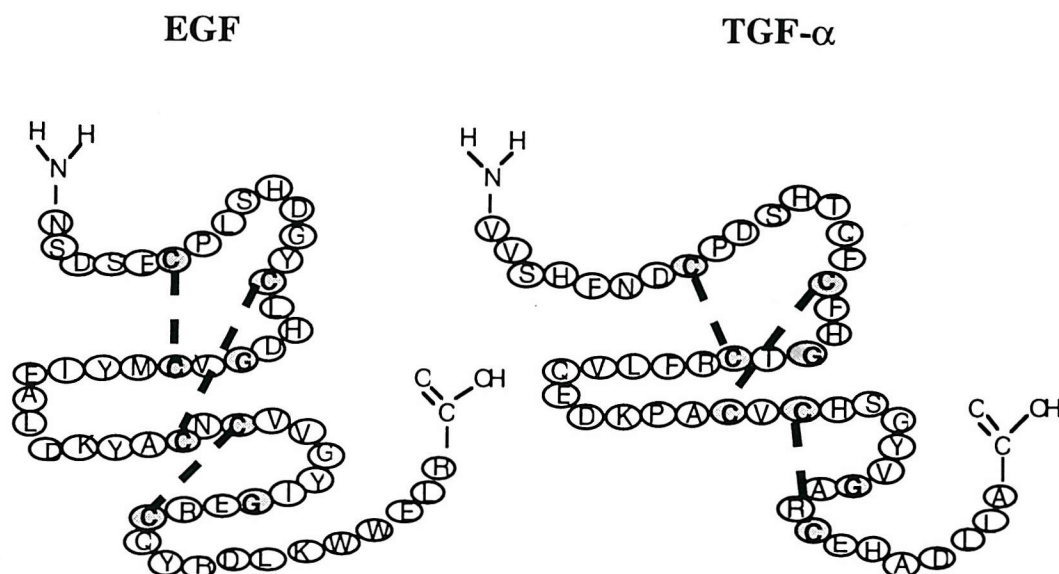


Diagram 1.4. Cartoon Representation of Two Members of the EGF Peptide Family.

Cysteine bridges are indicated by dashed lines. Shaded circles represent conserved residues between the two peptides.

(Adapted from Prigent and Lemoine, 1992).

hAmphiregulin (AR) was originally purified from MCF-7 breast tumour cell-line conditioned medium (Shoyab *et al*, 1988). It binds the EGFR with low affinity yet still activates the tyrosine kinase (Johnson, G. *et al*, 1993a). It has been shown to have both stimulatory and inhibitory effects upon cells (Shoyab *et al*, 1988; Cook *et al*, 1991a; Johnson *et al*, 1991, 1992, 1993b; Normanno *et al*, 1993, 1994). Plowman and Green first identified AR mRNA in the human placenta (1990) and it has subsequently been reported to be a paracrine/juxtacrine growth stimulatory molecule in cytotrophoblasts early in gestation (Lysiak *et al*, 1995).

Heparin-binding EGF (HB-EGF) is a single polypeptide consisting of 86 amino acids and is O-glycosylated at two threonine domains. It has two distinguishable domains - a heparin binding domain and an EGF-like domain (Thompson *et al*, 1994). The binding of HB-EGF to the EGF receptor is dependent upon the association of its heparin-binding domain with cell-surface heparan sulphate proteoglycan (Higashiyama and Tanaguchi, 1994). EGF, TGF α , HB-EGF and

amphiregulin are found in large amounts in uterine tissue in the peri-implantation period (Cross *et al*, 1994)

1.5. EGF Receptor.

The EGF receptor is the best characterised of the Type I growth factor receptors and serves as a paradigm for the other members of this family. It is a 170 kDa glycoprotein with an intrinsic tyrosine specific protein kinase which is activated on binding of EGF or TGF α . (Hunter *et al*, 1984)

The EGFR precursor is a single polypeptide chain of 1210 amino acids and cleavage of a 24 aa signal sequence results in the mature 1186 aa, membrane spanning, receptor molecule (Prigent and Lemoine, 1992^a).

The EGFR can be divided into several domains as indicated in diagram 1.4:

i) Extracellular domain - This comprises 621 amino acids and includes the amino (NH₂) terminal region. It is crucial in ligand binding and must maintain correct folding for high affinity ligand binding ($K_d=10^{-10}$). 12 N-linked glycosylation sites are present of which 10-11 are occupied by oligosaccharide chains thus accounting for the high carbohydrate content of this region. There are two cysteine rich regions of about 170 aa each (positions 132-313 and 446-612) containing 51 cysteine residues between them (roughly 10% of the domain). Their exact role is unclear, yet the formation of disulphide bridges is most likely, although the disulphide content is not known. They may help form a 'lobed' structure that facilitates EGF binding (Hunter *et al*, 1984), and may also confer rigidity on the receptor and make it resistant to proteases. Alternatively they may provide a scaffold for a mediation of receptor-receptor interaction leading to subsequent dimerisation.

ii) Transmembrane domain - This is a hydrophobic 26 aa alpha helix which shows little conservation with other receptor-tyrosine kinases. It has several basic amino acids on the cytoplasmic side (13 residues) and a proline residue at the extracellular interface which is homologous with many Transmembrane Spanning Regions (TMSRs) and may be instrumental in anchoring the protein to the lipid bilayer (Yarden and Ullrich, 1988).

iii) Juxtamembrane domain - This region separates the TMSR from the highly conserved tyrosine kinase domain and is 41-50 amino acids long. It contains the stop transfer sequence for protein targeting. There is a protein kinase C phosphorylation site within the sequence at Threonine 654 suggesting a negative

feedback mechanism in signal modulation but its true function is not yet known (Hunter *et al*, 1984).

iv) Cytoplasmic domain - 542 amino acids make up the intracellular region which is responsible for generating signal transduction. It can be subdivided into 2 regions:

a) Tyrosine kinase domain - Tyrosine kinase activation is the primary effector in signal transduction in group 1 growth factor receptors and hence there is high sequence homology between the EGFR and other tyrosine kinase receptors. At aa 694 there is a span of 25 aa which is considered to be the catalytic domain (Hunter *et al*, 1984). In this region lies a lysine residue at 721 which is involved in ATP binding through the formation of a salt bridge via an oxygen atom on the beta phosphate of ATP. This residue is crucial for enzyme activity as are several other sequences in this region including a Gly-X-Gly-X-X-Gly consensus sequence at the amino terminal end of the catalytic region.

b) Carboxy-terminal tail - This region is very hydrophilic and rich in small amino acids enabling flexibility of the polypeptide chain which could allow the C-terminal to fold over the tyrosine kinase domain thus modulating or interacting with receptor activation. The last 240 aa of the C-terminal contain the sites for EGF mediated tyrosine(Y) phosphorylation at positions 1068, 1148, 1086 and 1173 with Y1173 being the major site of autophosphorylation (Carpenter, 1987). In summary, the EGFR contains an extracellular ligand-binding site connected by a transmembrane spanning region to an intracellular domain possessing intrinsic protein tyrosine kinase activity.

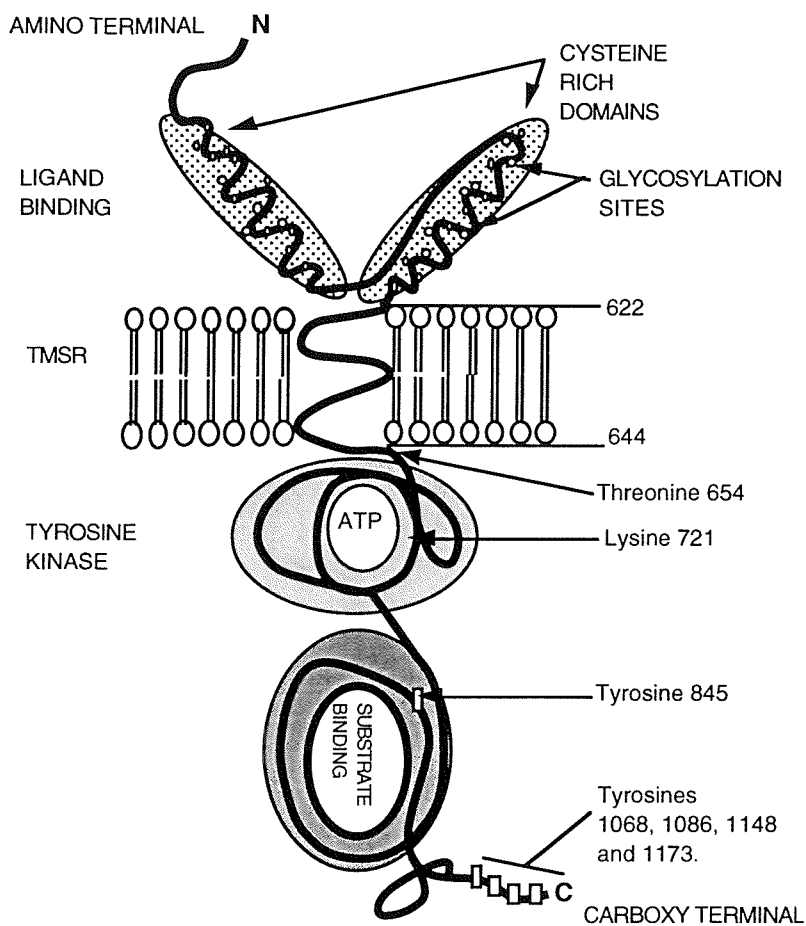


Diagram 1.5. The Epidermal Growth Factor Receptor (not to scale).

This diagram (modified from Carpenter, 1987) shows the separate domains of the EGFR. The extracellular domain is heavily glycosylated and comprises two cysteine rich domains essential in ligand binding. The threonine residue at position 654 is important in receptor modulation by protein kinase C and the lysine residue at 721 is implicated in ATP binding. The tyrosine residue at 845 is important in autophosphorylation whereas phosphorylation of those indicated at 1068, 1086, 1148, 1173 are responsible for

initiating subsequent cytoplasmic events.

1.6. ErbB-2.

The erbB-2 gene product was originally isolated from a chemically induced rat neuroblastoma and called neu (Shih *et al*, 1981). The equivalent human gene has now been isolated and called HER2/c-neu or cerbB-2 and bears a considerable homology with the EGFR (Schechter *et al*, 1984).

The gene product or erbB-2 is composed of 1255 aa as its precursor with an N-terminal signal sequence of 20aa. The mature protein is heavily glycosylated and has a molecular weight of 185 kDa. Its sequence is colinear to EGFR except for an additional 40 aa near its C-terminus. The extracellular domain of erbB-2 has two clusters of cysteine residues which have the same spacing as EGFR but this region displays only 43% homology with the EC domain of the EGFR, thus the two proteins are distinct and in fact, are encoded by genes on different chromosomes (EGFR on chromosome 7, and cerbB-2 on chromosome 17). The TMSRs have no signal sequence homology (Peles *et al*, 1993).

erbB-2 has tyrosine kinase activity and its cytoplasmic sequence has a TK domain with 82% homology to EGFR as shown in Diagram 1.6.

The mutation which activates the c-neu (c-erbB-2) to become onc-neu has been defined. A single nucleotide transversion mutation (from T to A) converts a valine residue to a glutamic acid at position 664 in the TMSR about 5 aa inside the Extracellular (EC) face of the plasma cell membrane (Bargmann and Weinberg, 1988). This transformation results in elevated tyrosine kinase activity and an accelerated rate of turnover (Yarden, 1990) of erbB-2. How this is achieved is unknown yet several theories have been put forward. Firstly, that the alteration of a sharp bend in this transmembrane region to an alpha helix, following mutation, encourages receptor aggregation (Brandt-Rauf *et al*, 1990). Alternatively the glutamic acid forms hydrogen bonds that stabilise the dimers (Sternberg and Gullick, 1989).

Truncation of the extracellular domain and amplification/overexpression of erbB-2 also confer the transformed phenotype (Bargmann and Weinberg, 1988; Di Fiore *et al*, 1987).

Several peptides were originally proposed as ligands for erbB-2, including HRG (heregulin), NDF (Neu differentiation factor), gp30, gp 75, NAF (Neu activating factor), ARIA (acetylcholine receptor inducing factor), GGF (Glial growth factor) (Lupu *et al*, 1990; Lupu *et al*, 1992; Holmes *et al*, 1992; Dobashi *et al*, 1991; Tarakhovsky *et al*, 1991) and these are now collectively known as the Neuregulins. The failure to find a specific ligand for erbB-2 raised the possibility that it might be an atypical receptor-like molecule that functions without a ligand (Lonardo *et al*, 1990). However, as discussed later, it is currently thought that erbB-2 plays a crucial role in receptor heterodimerisation with the other members of the EGFR family.

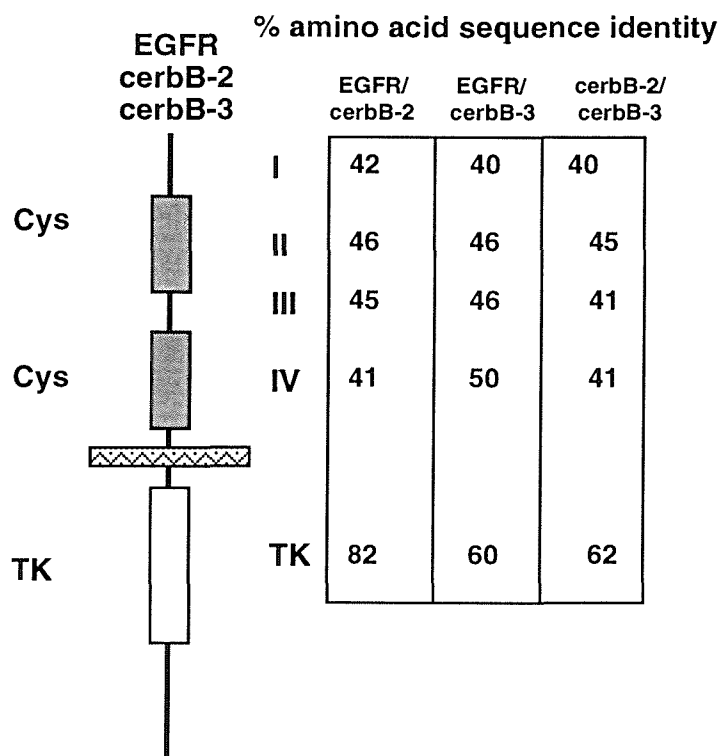


Diagram 1.6.
Amino acid
sequence homology
between members
of the Type 1
RPTK family.
(Reproduced from
Prigent and
Lemoine, 1992)

1.7. ErbB-3, ErbB-4.

erbB-3 is 44% identical with erbB-1 and erbB-2 in the extracellular domain and it also has a highly homologous tyrosine kinase domain (60%), but its C-terminal domain and a 29 aa residue domain C-terminal to the ATP binding site are markedly different (Plowman *et al*, 1993). The EC domain has 50 cysteine residues, 47 of which occur in two clusters - like those seen in the EGFR. However, erbB-3 lacks several residues which are highly conserved in tyrosine kinases e.g. the aspartate residue which has been proposed to be the catalytic base in the PKA catalytic subunit is an asparagine in erbB-3 (Van de Geer *et al*, 1994). The pattern of expression of erbB-3 is similar to erbB-2 and it is transcribed in term human placenta (Prigent and Lemoine, 1992).

erbB-4 is maximally expressed in brain, heart, kidney and is also present in parathyroid, cerebellum, pituitary, spleen, testis and breast; also in a variety of mammary adenocarcinomas and neuroblastoma cell lines (Plowman *et al*, 1993). Based on catalytic domain similarity, erbB-4 is most like erbB-2 and contains all the structural features of the EGFR family receptors. Again, the extracellular domain has two cysteine rich regions, its TMSR has 73% homology with the

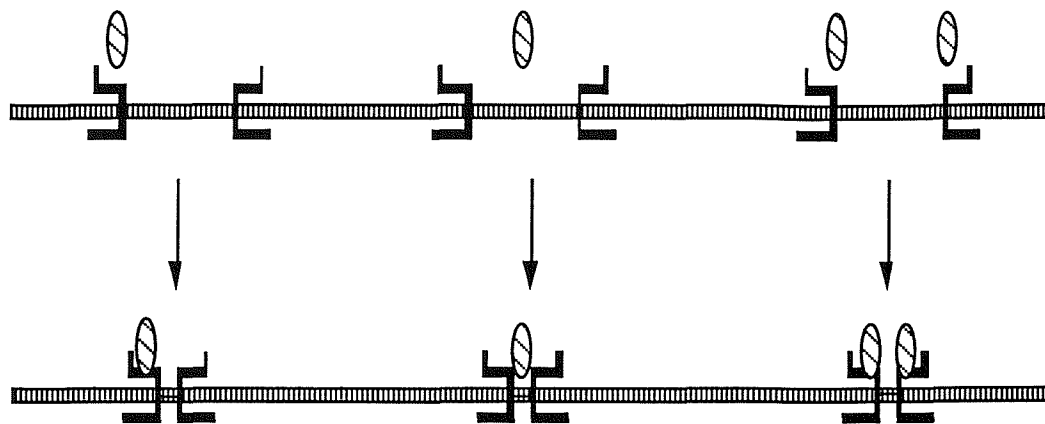
EGFR, and the catalytic domain is 79%, 77% and 63% identical to those of erbB-1, 2 and 3 respectively (Diagram 1.6.) (Peles *et al*, 1993).

1.8. Receptor dimerisation and activation.

It was originally thought that activation of the EGFR occurred via an intramolecular model i.e. binding of EGF to the extracellular domain conferred, through vertical dislocation of the hydrophobic TMSR, a conformational change in the cytoplasmic kinase region. However, the cluster of charged amino acids in the juxtamembrane region of the receptor could impose a high energy barrier and thus inhibit any conformational changes (Hunter and Cooper, 1984). Therefore other methods of activation were conceived.

As early as 1978, even before the receptor had been characterised at a molecular level, Schlessinger proposed that EGF and other growth factors could induce oligomerisation of their specific receptors which was important in transmembrane signalling (Schlessinger, 1978). More recently, the generation of dominant-negative mutants has provided further evidence that an intermolecular model is favoured. Cross-phosphorylation between a tyrosine-kinase negative mutant when expressed with wild-type EGFR again indicates that receptor dimerisation occurs (Honegger *et al*, 1990). This mutant was used in further experiments, in conjunction with truncated forms of the EGFR, and was shown to inhibit the mitogenic response elicited by wild-type EGFR through the formation of inactive mutant/wild-type heterodimers (Kashles *et al*, 1991).

So what exactly is oligomerisation and how does it occur? The pioneer of this work, Josef Schlessinger, suggests that inactive receptor monomers are in equilibrium with active receptor dimers and that ligand binding stabilises the active dimeric state (Schlessinger, 1988). The dimer may be composed of identical receptors i.e. a homodimer, or may be due to dimerisation between different receptors of the same family i.e. a heterodimer. Dimerisation is crucial for activation of intrinsic protein kinase activity and autophosphorylation, which will be dealt with in more detail later (Ullrich and Schlessinger, 1990).



Theory 1.
1 molecule of EGF
is sufficient for
dimerisation

Theory 2.
1 molecule of EGF binds
to both receptors
resulting in dimerisation

Theory 3.
2 molecules of EGF are
required to occupy both
receptors before
dimerisation can occur.

?

?

X

Diagram 1.7. Proposed models for Epidermal Growth Factor (EGF) induced EGFR dimerisation.

One hatched lozenge represents one molecule of EGF and the thick black lines represent EGFR located in the plasma membrane. Three supposed theories are outlined in the accompanying text. The question marks indicate potential mechanisms and the X represents an unlikely scenario.

The exact mechanism of ligand-induced receptor homo-hetero oligomerisation is unclear, but appears to involve the crosslinking of receptor molecules through their association with distinct binding sites on the ligand. There are three available options (refer to Diagram 1.7.):

1. One molecule of EGF binds to one receptor which attracts unoccupied receptors resulting in formation of a dimer. Spectroscopic studies have shown that binding of a single molecule of EGF to its receptor induces conformational changes which may stabilise the dimer (Greenfield 1989);

2. One molecule of EGF may be bivalent for two receptors. Bearing in mind that EGF is only 53 aa long and, as one will learn later, has the capacity to induce heterodimerisation between many species this method is unlikely since it

would require several receptor specific binding sites in a comparatively short ligand peptide sequence. However, recent calorimetric studies have shown that a single molecule of EGF can bind simultaneously to two receptors resulting in the dimerisation of the extracellular domain of EGFR (Lemmon and Schlessinger, 1994). Therefore, this model cannot be ruled out.

3. Two molecules of EGF binds to two receptors resulting in aggregation and formation of an oligomer. This is obviously not the case, since dominant-negative mutant studies have shown that truncated EGFR (lacking an extracellular ligand binding site) still retain the capacity to dimerise with wild type receptor and to be autophosphorylated (Honegger *et al*, 1990).

Detailed knowledge of the structure and stoichiometry of receptor-ligand interactions is required so that a precise mechanism can be elucidated, but what is certain is that the interaction of members of the EGFR family with one another forming combinations of homo/hetero-dimers opens up an array of different intracellular pathways leading to increased signal diversity. This goes some way towards explaining the pleiotropic effects of EGF and similar ligands in differing physiological systems.

As mentioned previously there are currently 4 members of the epidermal growth factor receptor family: EGFR itself (erbB-1) and the homologues erbB-2, erbB-3 and erbB-4 (and a potential erbB-5).

It is well documented that intermolecular phosphorylation of erbB-2 by EGFR occurs (Connelly and Stern, 1988) through formation of a complex (Spivak-Kroizman *et al*, 1992) following stimulation with EGF. The extracellular regions of the two receptors are sufficient for this to occur - Qian *et al* (1994) recently showed that a kinase-deficient mutant of erbB-2 can suppress normal EGFR signalling in a dominant-negative fashion supporting further the idea that heterodimerisation is more favourable than homodimerisation. EGF itself however, does not bind to erbB-2 and yet is capable of phosphorylating it via the EGFR i.e. EGF can activate erbB-2 in the absence of its own natural ligand. This conflicts with Lemmon's theory of the EGF molecule acting as a 'linker' (1994) and it remains unclear as to how EGF-driven heterodimerisation occurs.

The ligand for erbB-2 is not known. It was previously thought that heregulin acted specifically at erbB-2 but expression of erbB-2 alone does not result in phosphorylation in the presence of heregulin (Plowman *et al*, 1993). This anomaly led to the proposal that phosphorylation of erbB-2 arises through

heterodimerisation with another protein which would in fact be the true receptor for heregulin. EGFR does not bind heregulin and is therefore ruled out as a candidate.

Sliwkowski *et al* (1994) have shown that heregulin will bind to erbB-3 expressed alone in insect cells and can be chemically cross-linked to erbB-2. Concomitantly, erbB-4 expressed in a T-cell line (devoid of any other erbB receptors) can also be activated by heregulin, and again erbB-4 can be cross-linked chemically to erbB-2 (Carraway and Cantley, 1994). Thus both groups have proposed that activation of erbB-2 by heregulin is indirectly mediated through either erbB-3 or erbB-4 via heterodimerisation. The level of expression of erbB-3 or erbB-4 within a tissue will dictate the degree of activation of erbB-2 and could account for the different effects observed in breast and ovarian carcinomas where the level of erbB-2 expression is similar and yet the effects of heregulin are quite different.

The actions of EGF and heregulin upon the receptor tyrosine kinases is illustrated in Diagram 1.8.

Interestingly, oncogenic or intrinsically active erbB-2 (i.e. that which has an uncharged valine transformed to a charged glutamate) is able to form dimers in the absence of any ligand.

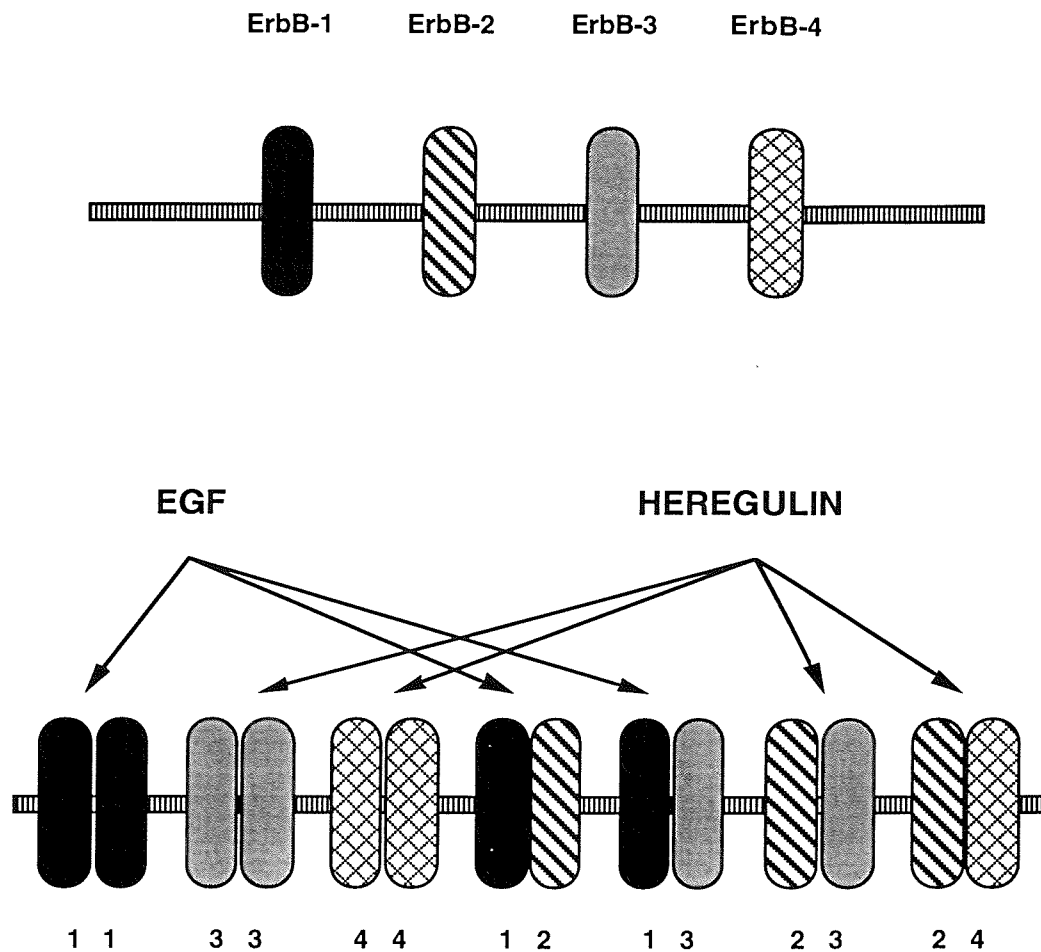


Diagram 1.8. Heterodimerisation between members of the type-I RPTK family.

Four members of the EGFR family are shown - EGFR (black); erbB-2 (stripes); erbB-3 (grey) and erbB-4 (checks) in possible homo/heterodimerised combinations following binding of either EGF or Heregulin

1.9. Signal transduction mechanisms.

RTK(receptor tyrosine kinase) dimerisation leads to receptor autophosphorylation at distinct sites outside the catalytic domain on the 4 tyrosine residues located near the carboxy terminal tail (Y1068, Y1148, Y1086 and Y1173) (Carpenter, 1987; Yarden and Schlessinger, 1987). (It is still not known how autophosphorylation occurs but it could be that the monomeric receptor has a low basal kinase activity which is sufficient to phosphorylate and activate the companion receptor following dimerisation (Heldin *et al*, 1995)). Transphosphorylation follows yielding a dimeric RPTK complex available to phosphorylate cytoplasmic substrates whose affinity for the dimer has been increased by the presence of the phosphate groups (Van de Geer *et al*, 1994). These phosphorylated tyrosines act as binding sites on the activated receptor for proteins that contain SH2 domains.

Src-homology 2 (SH2) domains are about 100 aa in length and are not required for kinase activity (Pawson and Bernstein, 1988). The N and C terminals of the domain are close together in a globular structure and this enables the domain to protrude from the rest of the protein and function independently to interact with binding sites on the EGFR (Booker *et al*, 1992) - one could envisage it almost as creating a pocket for the phosphorylating tyrosine on the EGFR (Cohen *et al*, 1995) The 3-6 amino acids C-terminal to the tyrosine residue are also important since different SH2-domain containing proteins have different preferences for this region (Pascal *et al*, 1994; Eck *et al*, 1993; Waksman *et al*, 1993) .

It is this ability of EGFRs to bind SH2-domain containing proteins that is the starting point in intracellular signal transduction (although it must be borne in mind that SH2-mediated stability is not essential for phosphorylation of all substrates in vivo (Glenney, 1992)).

There are many proteins that could be phosphorylated on tyrosine residues and hence participate in signal transduction following EGFR activation and several have been identified including, phosphoinositide 3-kinase (PI 3-kinase), Grb2/Sos, c-Src, phospholipase C gamma, GAP, MAP kinase, raf kinase and other kinases that lead to phosphorylation of ribosomal-6-kinase, growth factor receptors and transcription factors which in turn can alter gene transcription. Diagram 1.9. indicates a variety of the different intracellular substrates capable of being phosphorylated and hence activated by EGF and Table 1.1. shows some of the target proteins that contain SH2 and SH3 domains.

The binding of SH2 domain-containing proteins to a member of the EGFR family may have three effects:

1. The binding of the SH2-domain containing protein results in it being phosphorylated on its tyrosine residue(s) and hence being activated/inhibited e.g. PLC gamma (Kim *et al*, 1991).
2. Binding of the SH2-domain-containing protein to the EGFR may result in allosteric activation of itself e.g. PI3 Kinase's enzymatic activity increases 3-10 fold following binding (Glenney, 1992).
3. The SH2-domain-containing protein may act as an adapter as in the case of GRB2 (Growth factor receptor bound 2), which is not itself phosphorylated, but instead, through binding to the EGFR, attracts its associated partner SOS . SOS is a Ras guanine nucleotide release factor (GNRF) thus allowing the EGFR to indirectly activate the Ras pathway.

Enzymes	SH2	SH3
PLC γ 1 and 2	✓	✓
Ras GAP	✓	✓
Src family kinases	✓	✓
PI3 kinase p85/p110	✓	✓
Adapters		
GRB2	✓	✓

Table 1.1. Epidermal Growth Factor receptor substrates known to contain either a src homology two (SH2) or src homology three (SH3) domain.

SH3 domains are similar protein-protein interactive regions which are about 60 aa long and have been found in several proteins (Yu *et al*, 1994).

Interestingly, although the EGFR activates PI-3 kinase it does not have the corresponding SH2 binding domains of the regulatory p85 subunit of PI-3 kinase (Soltoff *et al*, 1994). However, erbB-3 does have such a site which has been shown to become phosphorylated in the presence of EGF (Carraway and Cantley,

1994). It could be that erbB-3 is a docking protein for substrates unable to locate the correct SH2 binding domain on the EGFR. Similarly, the association of erbB-2 with other members of its family may elicit different responses from the homodimers. Indeed, it has been shown that the erbB-1/erbB-2 heterodimer has a higher affinity for EGF than the erbB-1 homodimer (Qian *et al*, 1994).

Several Growth Factor Receptor Bound proteins (Grbs) have been cloned and given the nomenclature of Grb3 through to Grb10. Some of these are proteins which have previously been identified e.g. Grb6 is in fact PLC γ (Margolis, 1994). Grb7 is a 535aa protein isolated from the mouse embryo (Margolis *et al*, 1992) and unlike many SH2 domain proteins it has limited tissue distribution. Of relevance to this thesis is the fact that the Grb7 gene is amplified in breast tumours to the same extent as erbB-2 - this is supported by the overexpression of Grb7 in cell-lines that overexpress erbB-2 (Margolis, 1994; Stein *et al* 1994). In addition, not only is Grb7 amplified and overexpressed with erbB-2 but it is also bound to erbB-2.

Although the precise mechanism of action of Grb7 remains unknown, it is thought to contribute to the metastatic phenotype of tumour cells. It does not associate with Sos or initiate the MAP kinase pathway but it contains a GM domain similar to pleckstrin which is likely to be involved in protein/protein interactions. Pleckstrin domains (c100-110aa) are found in more than 60 intracellular proteins such as ras and thus may provide the answers to the second messenger cascade activated by Grb7 (Musachio *et al* , 1993; Mayer *et al*, 1993).

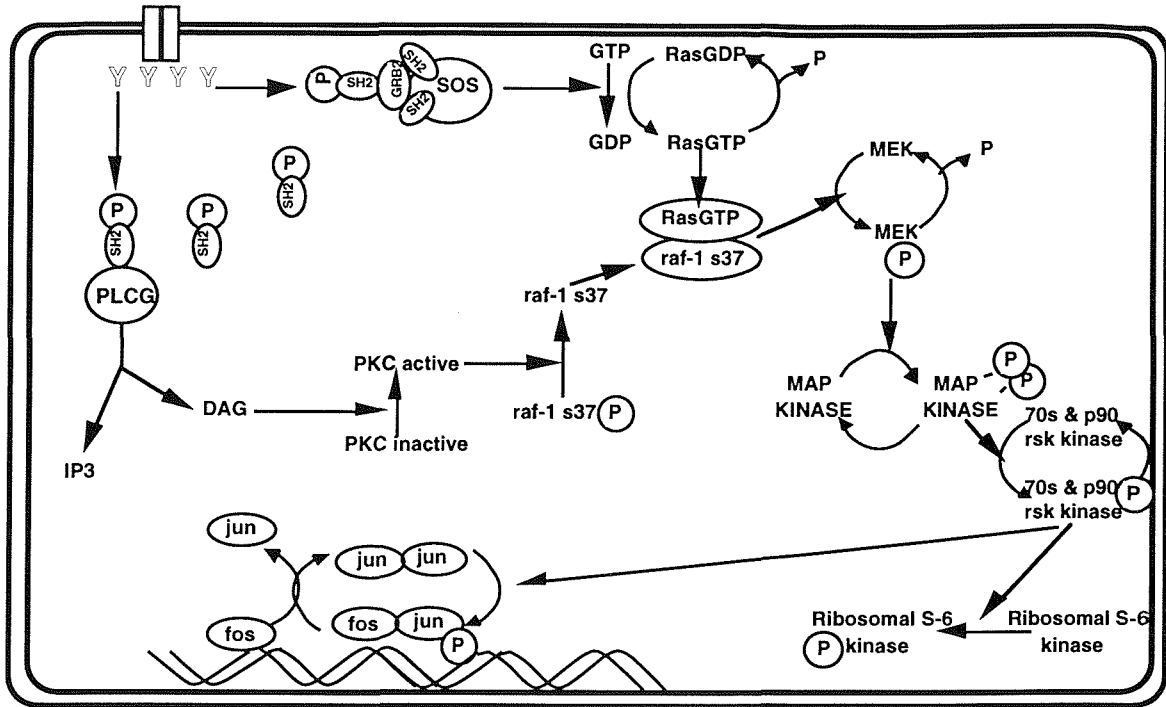


Diagram 1.9. A schematic representation of some of the available signalling pathways following EGFR activation.

The EGFR activated tyrosine residues are indicated (Y) which, following EGF binding and receptor dimerisation, confer phosphorylation upon corresponding SH2 domains leading to subsequent phosphorylation of substrates. Activation of the Phospholipase C, IP3 and DAG pathway are indicated ultimately resulting in transcription via the transcription factors jun and fos. Stimulation of the MAP kinase pathway by Ras is also shown whereby movement of Ras-GDP to the plasma membrane is required for association with the SOS-Grb2 complex. Not shown is the activation of PI3-kinase or the JAK/Stat pathways. EGFR are represented by white boxes and DNA is shown as two parallel squiggly lines at the bottom of the diagram. P-Phosphorylated residue; SH2-src homology 2 domain; GRB2-Growth Factor receptor bound protein 2; Sos-son of sevenless; GTP-Guanosine triphosphate; GDP-Guanosine bisphosphate; MAP kinase; PKC, IP3; DAG-diacylglycerol; PLCG-Phospholipase C gamma; PKC - protein kinase C

Therefore it is apparent that one of the results of heterodimerisation is the recruitment of a different set of SH2-domain containing proteins from that of homodimers alone. In addition, the association with a variety of Grbs and subsequently differing second messenger pathways provides ample opportunity for a wide-range of intracellular responses.

This opens up enormous potential for the same ligand, e.g. EGF, to elicit a multitude of responses which will be dependent upon the level of expression of erbB-1, erbB-2, erbB-3 and erbB-4 in a tissue.

The rate of transcription of the EGFR gene can be regulated by several factors (Clark, 1985) including PTHrP (1-34), which increases EGF binding to its receptor in cultured human trophoblast cells from early and term placenta (Alsat *et al*, 1991; Alsat *et al*, 1993). Little is known of the precise mechanism by which PTHrP interacts with EGF/EGFR responses to bring about proliferation, migration or differentiation during trophoblast invasion. It is this interaction that is of interest in my thesis. Therefore the next section of the introduction provides an insight into PTH, PTHrP and their receptor(s), with particular emphasis on the role of PTHrP in the placenta.

1.10. Parathyroid Hormone (PTH).

Parathyroid Hormone is the major regulator of calcium and phosphate homeostasis through its actions on bone and kidney. The PTH gene is a single copy gene located on the short arm of chromosome 11 (Mannens *et al*, 1987) and cDNA predicted amino acid sequences have been obtained from several species (Potts *et al*, 1982; Vasicek *et al*, 1983; Orloff *et al*, 1989). A large 115 amino acid precursor is enzymatically cleaved to produce an active peptide of 84 amino acids with the pro and prepro forms of PTH serving no, or very little, biological function (Habener *et al*, 1984).

Only the amino terminal 1-34 fragment is required, to evoke all classical functions of PTH (Kronenberg *et al*, 1993); however, a synthetic fragment of PTH, PTH (53-84) increases alkaline phosphatase activity in a rat cell line ROS 17/2.8 and PTH (39-84) and (53-84) stimulate the differentiation of osteoclast precursors into osteoclast-like cells (Murray *et al*, 1994; Nakamoto *et al*, 1993; Kaji *et al*, 1994).

PTH elicits its actions through binding to the extracellular domain of the Type 1 PTH receptor via association of residues 1-15 and 25-27. There is also a binding region near the C-terminus comprising residues 35-84. Preservation of the first two amino acids is essential for activation of the second messenger adenylate cyclase in ROS 17/2.8 (Gardella *et al*, 1991).

1.11. Parathyroid hormone related peptide (PTHrP).

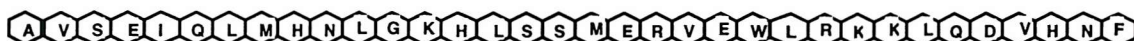
Parathyroid hormone related peptide - (PTHrP) was initially identified as the product of human tumours associated with humoral hypercalcaemia of malignancy (HMM) (Moseley *et al*, 1987) but it is now known to be expressed in a wide range of normal tissues (Halloran and Nissenson, 1992). In a review, in 1996, Philbrick pointed out that “the PTHrP gene is expressed in essentially every tissue and organ of the body at some point in fetal development or adult life” and this is supported by the fact that mutation or deletion of the PTHrP gene is lethal (Philbrick *et al*, 1996; Karaplis *et al*, 1994).

The first 13 amino acids of N-terminal PTHrP display high homology with those of PTH (40%) yet these peptides have little similarity in their primary sequence between 14-34 aa. However, synthetic peptides of PTH(14-34) and PTHrP (14-34), formed as hybrids, bind with similar affinity to the “classical” PTH receptor suggesting that the three-dimensional conformation of the two peptides is very similar despite their being dissimilar in primary structure.

Because of the similarity between PTH and the N-terminal of PTHrP they were originally considered to be products from the same gene but, in fact, the PTHrP gene is found on the short arm of chromosome 12 (Mangin *et al*, 1988.). The structure of the genes are consistent with their having arisen from a gene duplication (Mangin *et al*, 1989) and this is quite likely as the chromosomes themselves are thought to have arisen from a duplication process (Comings *et al*, 1972).

The PTHrP gene encodes three isoforms of 139, 141 and 173 residues and cDNA cloning predicted a mature protein comprising 141 aa (Rabbani *et al*, 1990). It is an unstable peptide with many proteolytic cleavage sites yielding several peptides and these processed secretory forms of the peptide predominate over intact unprocessed peptide.

PTHrP



PTH

Diagram 1.10. A comparison of the first 34 amino acids of human PTHrP and PTH. The histidine residue highlighted at position 5 is thought to be crucial in receptor recognition and the phenylalanine at position 23 is implicated in binding.

The precise role of PTHrP cannot be narrowed down to one function. It has been shown to play a role in transepithelial calcium transport in the distal nephron, mammary epithelium, placenta and the shell gland of the chicken. It is a potent smooth muscle relaxant and its mRNA can be induced by stretch and relaxation of smooth muscle in the uterus, urinary bladder, stomach and ileum. It has been shown to regulate growth, differentiation and development in every tissue in which these effects have been sought! It is particularly abundant in fetal organs - in the dermis, epidermis, mammary gland, pancreatic islet and the skeleton. It is also expressed in the central nervous system, neurones, parathyroid glands and adrenal medulla and its physiological role in these locations is entirely unknown (Philbrick *et al*, 1996).

PTHrP (1-34) promotes bone resorption (Kemp *et al*, 1987) but its potency and comparison with the effects of PTH (1-34) are reported differently by several groups (Kemp *et al*, 1987; Stewart *et al*, 1988; Fukayama *et al*, 1988). It has been investigated widely for its growth factor-like effects (Kaiser *et al*, 1992) and, in the kidney, it appears to be responsible for both growth and calcium transport!

PTHrP (107-139) is an inhibitor of osteoclastic bone resorption and has been named osteostatin by Fenton *et al* (1991).

Several workers have suggested that the central fragments of PTHrP are responsible for alternative responses, in particularly, placental calcium transport - a phenomenon which is not elicited by PTHrP 1-34 but by the mid molecular 36-84 peptide (Loveridge *et al*, 1988; Care *et al*, 1990).

1.12. PTH/PTHrP Receptors.

The classical type 1 PTH/PTHrP receptor is a member of the G-protein coupled receptor superfamily (Abou-Samra *et al*, 1992) and binds PTH and PTHrP with equal affinity (Halloran *et al*, 1992). This receptor is expressed in bone and kidney (Orloff *et al*, 1989; Abou-Samra *et al*, 1989^b; Caulfield *et al*, 1990).

Although the receptor displays the typical 7 TMSR (290 aa), with predominantly hydrophobic residues commonly found in members of this family, it bears only 10% homology with other members of the receptor superfamily. This homology is due, in part, to eight conserved cysteine residues in the extracellular domain. The three-dimensional structure of the PTH receptor and its topological positioning within the membrane are currently unknown although cartoons of suggested structures have been produced.

Three Type 1 PTH/PTHrP receptors have been cloned to date - from the rat, opossum and human. A large extracellular N- terminal region (200 aa) is thought to participate in ligand binding and displays high homology across species (91% between rat and human). The transmembrane core comprises 290 aa and the intracellular cytoplasmic tail consists of 110 amino acids. There are four potential sites for N-linked glycosylation and intramolecular disulphide bridges in the extracellular domain (Nissenson *et al*, 1993).

Interestingly, substitution of an Arg for Val at position 2 in the PTH peptide results in increased binding to the rat receptor with a reduction of adenylate cyclase activity whereas the converse was observed with the opossum receptor - a decrease in affinity yet a 3 times more potent agonist response (Gardella *et al*, 1991). Thus it seems that the synthetic peptide Arg2 PTH(1-34) can distinguish between two related PTH receptors. Gardella later employed this peptide to examine potential binding sites of PTH to its receptor . He suggested that residues in or near the transmembrane region of the PTH receptor contribute directly, or indirectly, to interactions with Arg2PTH(1-34) - in particular Val 371 and Leu 427 (Gardella *et al*, 1994). A single Serine residue at position 370 is responsible for activation of cAMP and no other residues appeared to be involved.

The signal transduction mechanisms employed following activation of the type 1 PTH/PTHrP Receptor are twofold: a) activation of the adenylate cyclase pathway

resulting in increased cAMP levels and b) activation of PLC γ resulting in increased IP3/DAG levels (Kronenberg *et al*, 1993; Jueppner *et al*, 1994).

Owing to the diverse properties of PTH and PTHrP in various tissues, there is a suggestion that other PTH/PTHrP receptors exist. For example, in lymphocytes there is a ligand induced increase in free calcium but no apparent effect upon cAMP levels (McCauley *et al*, 1992). In addition, a separate class of high affinity, low capacity receptors for amino terminal PTH has been identified in bone cells (Seitz *et al*, 1990). More convincingly, recent work on keratinocytes and squamous epithelial cells concluded that despite responding to PTH/PTHrP (with an elevation in free calcium) these cells did not express the classical PTH/PTHrP receptor (Orloff *et al*, 1995).

The potential PTH/PTHrP receptors can be divided into three categories:

- 1) An N-terminal PTHrP receptor that binds the PTH-like region of PTHrP - presumably the Type 1 PTH/PTHrP receptor.
- 2) Receptors for mid-region and carboxy-terminal PTHrP secretory forms which are possibly unrelated to the PTH/PTHrP receptor.
- 3) A PTH2 receptor which is selectively activated by PTH (1-34) and not PTHrP (1-34) (Behar *et al.*, 1996^a).

This latter, PTH2 receptor, is a G-protein linked member of the seven TMSR subfamily with 70% sequence homology to the classical Type 1 receptor. Binding of PTH (1-34) stimulated an increase cAMP activity and a rise in intracellular calcium levels with PTH (7-34) acting as a pure agonist. Despite the inability of PTHrP (1-34) to cause a response, PTHrP (7-34) acted as a weak agonist of the PTH2 receptor (Behar *et al.*, 1996^a).

So it seems that topological similarities observed with PTH/PTHrP binding to the Type 1 receptor are not relevant when the peptides are presented to the Type 2 receptor. A histidine residue at position 5 is the only nonconserved amino acid between the two peptides and could be the structurally disruptive element (Gardella *et al*, 1996). In this recent paper, Gardella and colleagues, show that Residue 5 (His in PTHrP and Ile in PTH) is the switch in determining signalling capability, and residue 23 (Phe in PTHrP and Trp in PTH) is implicated in determining binding affinity. By changing these residues they were able to alter the property of each peptide (Gardella, *et al* , 1996).

Other members of the G-protein linked family do exist in alternative spliced forms e.g. calcitonin receptor (Zolnierowicz *et al*, 1994) and therefore it seems plausible that the PTH/PTHrP Receptor may exist in multiple spliced variants - this is suggested by the multiple cDNAs of differing sizes shown by Orloff *et al* (1992). Clearly, there is a growing interest in this area of study and a more in-depth discussion of peptide/receptor interactions and the particular role of receptor regions is given in chapter six.

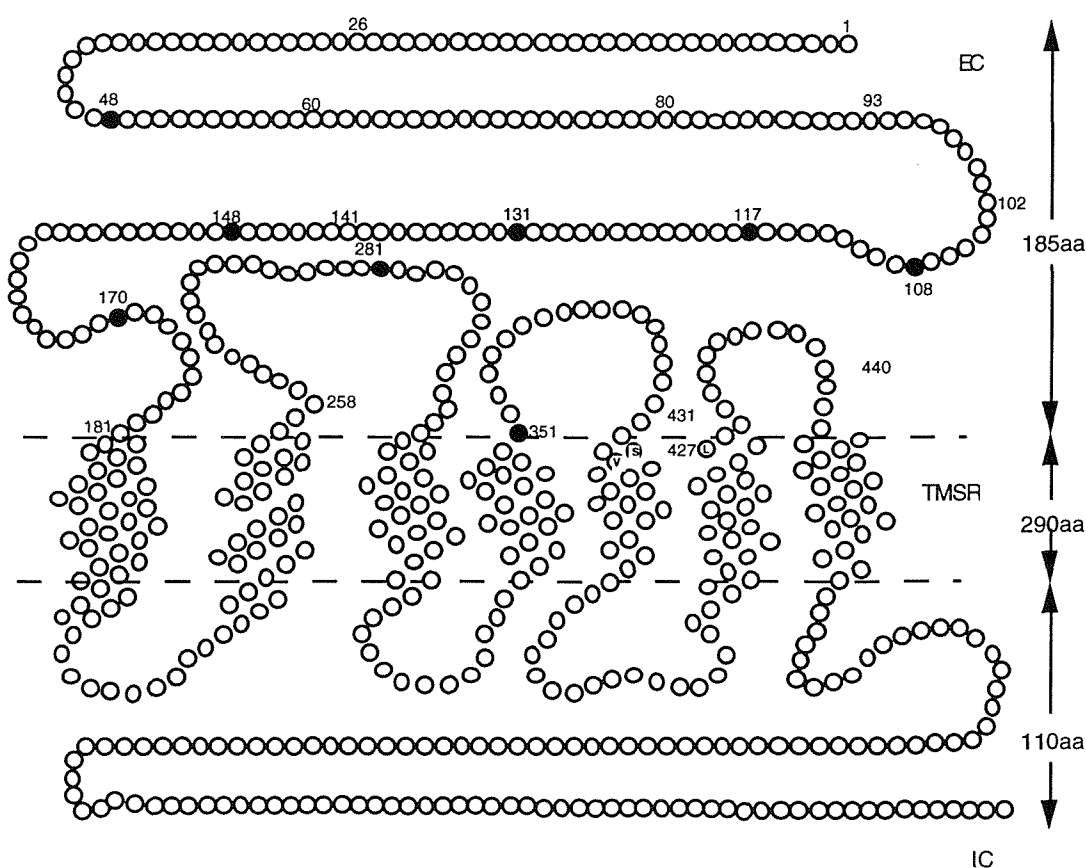


Diagram 1.11. Model of the Type 1 human PTH/PTHrP receptor.

The possible topology and orientation of the receptor in the cell membrane are shown. The residues that affected binding (Val 371, Leu 427) and signalling (Ser 370) properties are shown. Cysteine residues are indicated as dark circles. EC = Extracellular domain, TMSR = Transmembrane spanning region, IC = Intracellular domain. (Redrawn from Gardella *et al*, 1994).

1.13. The role of PTHrP in the placenta.

PTHrP is known to be a hormone of the feto-placental unit (Allgrove *et al*, 1985) and, since PTH is not present in the placenta (Selvanayagam *et al*, 1991), PTHrP has been implicated in the regulation of placental calcium transport. During pregnancy, the embryonic calcium supply comes solely from placental transfer of calcium from the maternal circulation (Braithewaite *et al*, 1972) and therefore this must be strictly regulated to ensure that the demands of the fetus upon the mother are met without disturbing the latter's calcium homeostasis.

The role of PTHrP in implantation is not known but positive immunostaining for PTHrP has been observed in human placental tissues as early as 6-8 weeks gestation (Hayman, 1993). No positive staining was found in the cytotrophoblast or syncytiotrophoblast but the intermediate cells in the placental villi and maternal decidua were positive. This suggests that in the early stages of pregnancy, PTHrP acts in a paracrine fashion to promote calcium transfer to the developing embryo although it does not preclude a role in other structures. So far, there has not been an in depth study into the level of expression of PTHrP throughout gestation in the human but work by Rodda *et al* in sheep indicates that PTHrP (1-34) is more abundant in early and mid-term placenta than full term (Rodda *et al*, 1988).

Early in gestation, a feto-maternal calcium gradient is established with fetal calcium concentrations being higher than maternal (Fisher *et al*, 1986, Rodda *et al*, 1988). Bearing in mind that PTH does not cross the placenta (neither does calcitonin (Garel, 1987)) PTHrP may be responsible for maintaining this gradient. Calcium can be transported in both directions across the placenta in humans but it is unidirectional in sheep (Garel, 1987).

Unfortunately, the majority of studies looking at calcium transport have been carried out in sheep and therefore cannot be applied too readily to the condition in humans. By using thyroparathyroidectomised fetal sheep, Care *et al* (1988) showed that PTH (1-34), PTHrP (1-34) and PTHrP (1-141) all increased fetal calcium concentrations following infusion into the fetal circulation yet the maternal calcium concentrations remained constant, indicating that the increased fetal calcium concentration must have come from fetal bone.

Subsequent work indicated that the fetal parathyroid glands secreted a factor responsible for placental calcium transport (Care *et al*, 1988) and this appeared to

be the mature form of PTHrP(1-141) as PTH and PTHrP(1-34) had no effect. Therefore mature PTHrP behaves independently from the bioactive region (1-34) and recent work by Care *et al* (1990) suggests that small mid-molecule synthetic fragments may stimulate placental calcium transport. How this is achieved is unclear. Calcium binding proteins and calcium pumps have both been identified in human placenta (Tuan, 1982) but in order to determine the precise role of PTHrP in modulating these, an in vitro model needs to be developed.

PTH receptors have been demonstrated in the human placental syncytiotrophoblast brush border and basal plasma membranes (Lafond *et al*, 1988) areas which are important in the transport of minerals between mother and fetus; but if PTH cannot cross the placenta, and is not produced by the fetus what use are specific PTH receptors? It seems very likely that there are unique receptors for PTHrP in the placenta which have not yet been identified.

1.14. Aims and Objectives of this project.

It is evident from the introduction that EGF plays an important role in the differentiation and endocrine functions of cultured trophoblast cells (Truman and Ford, 1986; Maruo *et al*, 1987; Morrish *et al*, 1987) and is expressed in high levels in the syncytiotrophoblast of human placenta (Rao *et al*, 1985). In addition, PTHrP has been localised by immunohistochemistry in human cytotrophoblast (Hellman *et al*, 1992) and first trimester placenta (Dunne *et al*, 1994).

The interactions between PTHrP and EGF in cultured human trophoblastic cells from early and term placenta have been investigated by Alsat *et al* (1991, 1993) confirming that responses to EGF and EGFR expression can be modulated by PTHrP in trophoblast.

In this project I wanted to look at several areas. Firstly, I investigated the proliferative effects of EGF on three choriocarcinoma cell-lines - BeWo, JAr and Jeg-3. I wanted to determine if there were any fundamental differences in the cell-lines' proliferation in response to EGF and to select which cell-line afforded the most appropriate model for proliferating first-trimester trophoblast.

Since EGF elicits its actions through association with the EGFR on the cell surface any difference in proliferation following stimulation with EGF would be affected

by the characteristics of the EGFR. It seemed logical, therefore, to look more closely at the EGFR in each choriocarcinoma cell-line with the intention of characterising by quantitative analysis the binding of EGF to its monomeric and dimeric receptor species. In addition, I aimed to quantify the number of EGF receptors present in each cell-line and to determine if differing proliferative responses could be accounted for by i) alterations in the affinity of EGF for its receptor or ii) differences in the number of EGFR available for activation.

PTHrP (1-34) is present in first trimester placenta (Dunne *et al*, 1994) and in human cytotrophoblast (Hellman *et al*, 1992) suggesting a paracrine or autocrine function. PTHrP (1-34) has previously been shown to upregulate EGFR expression in the mature placenta (Alsat *et al*, 1993). I wanted to see if PTHrP(1-34) also caused an increase in EGFR expression in the choriocarcinoma cell-line JAr. PTHrP (1-34) is more potent in a variety of cells than PTH (1-34) (Kemp *et al*, 1987) therefore I wanted to see if the effect of both peptides on interaction of EGF with its receptor EGFR were comparable. Furthermore, I investigated to what extent PTH/PTHrP (1-34) altered the affinity of EGF for its receptor or perhaps up/downregulated the number of EGFR expressed on the cell surface.

Finally, if the effects observed in response to PTH and PTHrP were different-how could this be accounted for? I undertook preliminary binding studies of PTH, PTHrP(1-34) and associated peptides in both JAr and SaOS-2 cell-lines. SaOS-2 cell-lines express a classic type I PTH/PTHrP receptor and would therefore serve as a good control. By studying the competitive displacement of ligands I wanted to see if the binding of PTH and PTHrP (1-34) were comparable thus indicating whether the peptides were interacting at similar or dissimilar receptor sites in the JAr cell-line. A colleague within the laboratory was studying the effects of PTH (1-34), PTHrP(1-34), PTHrP(1-86), PTHrP(7-34) on proliferation of the JAr cell-line therefore I decided to include the latter two peptides in the binding study. Previous work has demonstrated that PTH (1-34) and PTHrP (1-34),(1-86) and (7-34) elicit different effects e.g. Yamamoto *et al* (1997) found that secretion of vasopressin in the supraoptic nucleus was increased by PTHrP (1-34) whereas PTHrP (7-34) and PTH (1-34) had no effect on vasopressin secretion. I wanted to see if PTH (1-34), PTHrP(1-34), PTHrP(7-34) and PTHrP(1-86) were displaying similar binding characteristics in the JAr cell-line. If so, this might be due to the existence of a novel PTH/PTHrP receptor subtype. This would account for the differences observed in our laboratory in their effects on EGF induced proliferation and EGFR expression.

In summary, this work provides an insight into the role of EGF in the proliferation of BeWo, Jeg-3 and JAr cell-lines. It also investigates the interaction of PTHrP (1-34) and PTH(1-34) in regulating EGF-induced proliferation of the JAr choriocarcinoma cell-lines. The binding studies have enabled me to characterise the binding of EGF to its receptor in each cell-line, to investigate PTH(1-34) and PTHrP(1-34) modulation of this binding and to provide the basis for further work in our laboratory as to how the two discrete receptor systems for both EGF and PTHrP may interact to control proliferation in cells of trophoblastic origin. Finally, the competitive displacement of ^{125}I -PTHrP(1-34) by PTHrP(1-34) but not PTH(1-34) has provided evidence to suggest that a novel PTHrP receptor is present in the JAr choriocarcinoma cell-line.

CHAPTER TWO

MATERIALS AND METHODS.

2.1. Tissue Culture.

METHOD.

Choriocarcinoma cell lines were maintained in 25ml culture flasks in the appropriate medium supplemented with L-glutamine(2mM), PSA, Kanamycin and 10% FCS. hEGF was suspended in medium at a concentration of 200ng/ml. All media preparation and tissue culture was carried out in sterile conditions. Stock cultures of each cell line were subcultured once or twice a week. Cells were removed from the flask by brief trypsinisation (1-2mins) with 10% trypsin /EDTA in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hank's solution and subcultured into new flasks supplemented with 10% FCS. Cells were grown at 37°C in 5% CO_2 in air and the medium changed twice weekly.

For proliferation experiments, the cells were growth arrested in serum free medium, 24h prior to use, harvested from stock cultures as they neared confluence and recultured in 24 well plates at a suitable cell density, as ascertained by measuring the DNA content of a 100 μl aliquot of the suspended cells (described below), for 24,48 and 72 hours in phenol red-free media with 2.5% SFCS.

MEDIA PREPARATION.

Hank's balanced salts "modified"		
(Calcium chloride, magnesium sulphate free)		Sigma
Hank's balanced salts (phenol red free)		Sigma
Ham's F12		Imperial
RPMI 1640 (with or without phenol red)		Sigma
DMEM (with or without phenol red)		Sigma
Sodium bicarbonate (NaHCO_3)	MW. 84.01	Sigma
L-Glutamine	MW 146.1	Sigma
Analar water		Fisons
Antibiotic/antimycotic solution (PAS)		Sigma

Insulin, transferrin & selenium (ITS)	Sigma
Kanamycin	Imperial
Foetal calf serum (FCS)	Imperial
Sodium hydroxide (NaOH)	Sigma
Hydrochloric acid (HCl)	Sigma
Trypsin EDTA	Sigma
Human recombinant epidermal growth factor	Bachem

Stripped Fetal Calf Serum (SFCS):

The serum was “stripped” to remove any endogenous growth factors or steroids which may interfere with cell growth. Stripped Fetal calf serum (SFCS) was prepared by the addition of 5% (W/V) charcoal to Fetal Calf Serum (FCS). The charcoal/FCS mixture was stirred for 1 hour at room temperature, transferred to 50ml centrifuge tubes and spun at 500 rpm for 5 minutes. The supernatant was filtered, firstly through Whatman filter paper, and in turn through 0.2 µm Gelman filters for sterilisation. 10 ml aliquots were stored at -20° C until required.

2.2. DNA Assay.

The DNA assay employed is based on the principle that the fluorochrome Hoechst 33258 (bis-benzamide) fluoresces when it binds to DNA (Labarca and Paigen, 1980). The assay has been widely employed as a non-radioactive alternative to the classical ³H-thymidine assay (Araki and Sako, 1987), (Blaheta *et al*, 1991).

METHOD.

For plating out, 100ul aliquots of cell suspension were sonicated prior to assay and made up to a total volume of 1ml with DNA assay phosphate buffer, in triplicate. DNA standards (0.5, 1, 2, 4 & 8µg/ml) were prepared from a stock solution of calf thymus DNA (40µg/ml) and aliquoted out at 1ml. Bis-benzamide was made up as 0.2mg/ml in analar water and the stock solution diluted 100 fold prior to use. 1ml of diluted bis-benzamide was added to each standard and to the cell suspension and samples read on a spectrophotometer (excitation 342nm, emission 458nm). The DNA content of the cell suspension can subsequently be calculated by interpolating against the standard curve (figure 2.1.).

To measure the DNA content of the wells, the incubation medium was discarded and 200ul of 10% trypsin/EDTA (in DNA Assay buffer) was added to each well

and the plates left at room temperature for 1 hour. The trypsin lyses the cells thus releasing the contents (including the DNA) and the EDTA is a calcium chelater which not only potentiates the action of trypsin but also hinders cell adhesion and facilitates their removal from the plates. Plates were then frozen, which again aids cell detachment and assayed the following day. After thawing, plates were sonicated for five minutes or their contents transferred to glass tubes and resonicated. 200µl samples were removed from each well and the DNA contents assessed in the same way as previously described.

REAGENTS.

a) DNA assay buffer - (0.05M phosphate buffer, 2M NaCl, pH 7.4)

Na₂HPO₄ (50mM) was titrated with NaH₂PO₄ (50mM) to pH 7.4 followed by the addition of 2M NaCl. The pH was readjusted to 7.4 using NaOH and HCl. The high salt content of this buffer encourages unravelling of DNA from histones and thus facilitates binding of bis-benzamide to DNA.

b) Bis-benzamide

(2'-[4-Hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole)(C₂₅H₂₄N₆O₃·3HCl) - Bis stock solution was made up to a concentration of 0.2mg/ml in analar water. This stock was diluted 100 fold prior to use in DNA assay buffer.

c) Standards - 4mg of DNA was made up in 100ml of DNA assay buffer to give a final concentration of 40µg/ml. The solution was stirred overnight and diluted 5 fold to give the highest standard (8µg/ml) and this was serially diluted to give the rest of the standards.

Sodium di-hydrogen orthophosphate (NaH ₂ PO ₄)	Sigma
Disodium hydrogen phosphate (Na ₂ HPO ₄)	Sigma
Sodium Chloride (NaCl)	Sigma
Bis-benzamide	Sigma
Calf thymus DNA (for standards)	Sigma
Trypsin/EDTA	Sigma

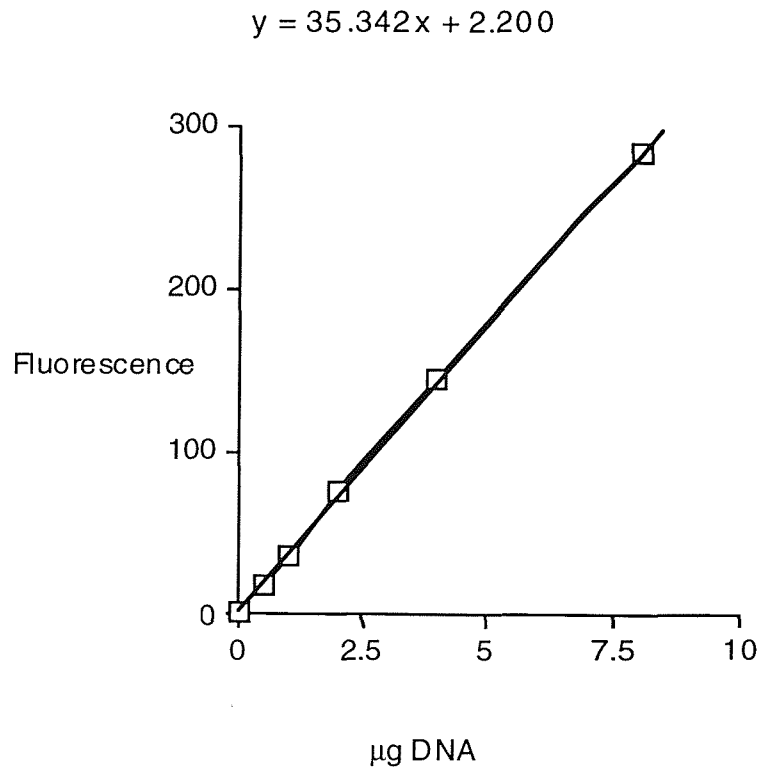


Figure 2.1. DNA Assay standard curve.

100µl of Calf thymus DNA at differing concentrations (0.5,1,2,4,8µg/ml) were prepared from a stock solution (40µg/ml) and made up to 1ml with DNA Assay Buffer. 1ml of a prepared bis-benzamide solution was added to each sample and the fluorescence of Hoechst 33258, on binding the DNA, measured on a spectrophotometer (excitation 342nm, emission 458nm). $y=35.342x + 2.2$ where 35.342 is the gradient of the slope (m) and 2.2 is the y axis intercept (c).

2.3. MTT Assay.

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (C₁₈ H₁₆ N₅ SBr) is a water soluble tetrazolium salt which is converted to an insoluble purple formazan product by the action of dehydrogenase enzymes (by cleavage of the tetrazolium ring). Active dehydrogenases present in mitochondria will elicit this change and this has been correlated with both cellular protein and cell count (Alley *et al.*, 1988). Therefore the assay has been widely employed as an indicator of cell number and viability (Mossman, 1983; Martin and Clynes, 1993; Carmichael *et al.*, 1987).

METHOD.

Following tissue culture, 100µl (5 mg/ml) MTT was added to each well and incubated for 4 hours at 37 °C in 5 % CO₂ in air. The medium was discarded and 250µl/well of solubilizing solution was added. Plates were taped and sealed in plastic bags to prevent evaporation or crystal formation and left overnight at room temperature (RT). 100 µl aliquots in duplicates were transferred to 96 well plates and optical density read at 570 nm using an Argus plate reader.

REAGENTS.

Solubilizer Solution:

20 % (w/w) SDS (Sodium Dodecyl Sulphate) was added gradually to 80 ml of 50% (w/w) of DMF (N',N Dimethylformamide) in water, with slight warming of the solution. 0.5 ml of 1.0 N HCl and 0.8 ml glacial acetic acid were added to the mixture and subsequently made up to 100 ml with DMF and stored at room temperature.

MTT	Sigma (M-2128)
SDS	ICN
Glacial acetic acid (CH ₃ COOH)	Fisons (2789)
HCl	BDH
DMF	Sigma (D-4254)

2.4. Crystal violet dye elution (CVDE).

Crystal Violet (CV) is a triphenyl methane dye which is purported to bind to the cell membrane and was initially used as an estimate of cytotoxicity (Flick and Gifford, 1984). However, Gillie *et al* (1986) employed the assay to directly measure cell number and it offers another non-radioactive method for assessing cell proliferation.

METHOD.

A 0.25 % (w/w) solution of CV was made up in analar water and stored at 4°C. After the removal of culture medium, 24 well plates were rinsed with 200µl PBS/well and stained with 200µl pre-filtered aqueous CV for 10 min at room temperature. Plates were rinsed twice with 500µl PBS, then allowed to dry at 37°C. When dry, 500 µl/well of 33% glacial acetic acid (w/w) was added to the contents of each well, and mixed thoroughly on a shaker. 100 µl samples were transferred to a 96 well plate in triplicate and read at 570 nm using an Argus plate reader.

MATERIALS.

Crystal Violet (CV)	Sigma (C-3886)
Glacial acetic acid	Fisons (2789)
Whatman filter papers	Whatman
Phosphate Buffer Saline (PBS):	
NaCl	(80g) Sigma
KCl	(2g) BDH
NaH ₂ PO ₄	(14.4g) Sigma
KH ₂ PO ₄	(24g) Sigma
Analar water	Fisons
NaOH	BDH
HCl	BDH

2.5. ³H-thymidine incorporation assay.

Thymidine is a nucleoside which is readily taken up by the cell and incorporated into the DNA backbone. Uptake of the radioactive tracer ³H-Thymidine can be measured using a beta counter and correlates to the amount of cell proliferation.

METHOD.

1.0 mCi/ml of stock ^3H -thymidine was diluted to 65 $\mu\text{Ci}/1$ with thymidine free medium (Gibco F12). Culture medium was discarded and replaced with thymidine-free DMEM. 50 $\mu\text{l}/\text{well}$ of tritiated thymidine was added, cells were incubated for 3 hours at 37°C in 5% CO_2 in air. At the end of incubation, the medium was removed, and cells were washed twice with warm phenol red free HANKS balanced salt. The cells were trypsinized with 500 $\mu\text{l}/\text{well}$ of trypsin/EDTA in Hanks ($\text{Ca}^{2+}/\text{Mg}^{2+}$ free), and a further 500 $\mu\text{l}/\text{well}$ of Hanks was added. The cells were then precipitated using 10% TCA, and left overnight at 4°C . The cells were then filtered on Whatman GFC filters and washed through twice with 5ml of 5% TCA, followed by 5ml methanol. The filters were placed in scintillation vials and left to dry. 3 ml Pharmacia/LKB Hi-safe scintillation fluid was added to each vial and the vials were counted using a beta counter.

MATERIALS.

^3H -Methyl Thymidine	ICN
DMEM/F12	GIBCO
LP3 Tubes	Denley
Trichloro Acetic acid(TCA)	Sigma(T-6399)
Methanol	Fisons
GF/C Filters	Whatman
Inserts	Denley
LKB Hisafe Scintillation Fluid	Pharmacia

2.6. Slot Blot Analysis.

Slot blot analysis of cellular antigen immobilised on nitro-cellulose.

METHOD.

Cell pellets kept at -70°C were thawed and re suspended in re suspension buffer (1-1.5mls) and heated to 80°C for 10 min. The resulting suspension was sonicated briefly and centrifuged at 800g to remove any cell debris. Protein assay

was carried out based on a combination of the Bradford and Lowry methods (Bradford 1976), (Lowry, 1951) (see section 2.7).

Nitro-cellulose paper was cut to fit a Hoeffler manifold and soaked in PBS with 0.5% Tween-20 for 1hr to reduce background. The nitro-cellulose was placed in the manifold and protein sample (0-50 μ g) was added to each well. Any bubbles present were popped and the wells washed through twice with PBS containing 0.5% Tween-20.

The nitro-cellulose was removed from the manifold and incubated with appropriate blocking peptide/sera for 1hr at room temperature. Any excess blocking peptide was removed by washing with 0.5% Tween-20 PBS for 3 x 30 min, shaking, at Room Temperature (RT).

The samples were exposed to primary antibody in 5-10mls 0.05% Tween-20 PBS overnight at 4°C and washed three times for 20 min each time with an excess of PBS-Tween (0.05%) to ensure removal of any unbound primary antibody. An alkaline phosphatase (AP) conjugated secondary antibody (Sigma) was diluted accordingly in 0.05 % Tween-20 PBS and the membrane incubated in 20-50ml for 1-2 hr. The blots were washed 3 x 20 min with PBS adjusted to pH 9.5 and developed in 5ml of AP substrate solution for such a time that bands were sufficiently dark. (For an outline of the detection system see Diagram 2.1). The filters were then washed in distilled water and photographed.

Endogenous alkaline phosphatase activity can be blocked using 100mM levamisol, however it did not appear to have any effect in my experiments!

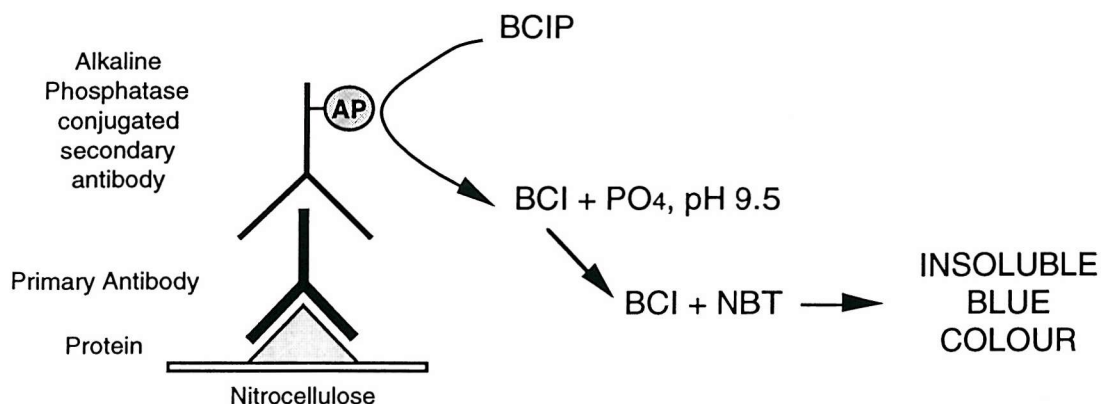


Diagram 2.1. Cartoon representing immunodetection of proteins using AP conjugated antibodies and BCIP/NBT substrate kit.

Protein is immobilised on the nitro-cellulose membrane and probed with a specific antibody raised to a corresponding epitope. A secondary - conjugated antibody is then added which recognises the primary antibody. The conjugated enzyme can be used to precipitate substrate solutions which result in a colour change. Non-specific binding sites can be blocked usually with serum from the same species as the secondary antibody or with 0.05% Tween 20 / 10% BSA.

REAGENTS.

PBS:	NaCl	80g
	KCl	2g
	Na ₂ HPO ₄ Anhydrous	14.4g
	KH ₂ PO ₄ Anhydrous	2.4g

Adjust to pH 7.4

0.5% Tween-20 - PBS:

Tween-20	0.5ml
PBS	100ml

0.05% Tween-20 PBS

Tween-20	75ml
PBS	1.5L

Levamisol	24mg
PBS	100ml

BCIP/NBT Buffered substrate tablet (B 5655):

BCIP/NBT (5 - bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets act as a precipitating substrate for the detection of alkaline phosphatase activity (Diag 2.1). Tablets stored at -20°C were allowed to reach room temperature before being dissolved and vortexed in 10 ml of distilled/deionised water. The substrate solution was poured onto the nitro-cellulose and allowed to react until sufficiently dark bands were obtained after which time the blots were rinsed in distilled water and photographed.

Re suspension buffer:

0.5844g 0.01M NaCl (MW 58.44) was weighed out and dissolved in 100ml distilled water and to this 0.01M Tris (MW 121.14-0.12g) was added (and pH to 7.6). Finally, to this 0.001M EDTA (MW 372.2 - 0.037g) was added to a final pH 8.0. The buffer was stored at 4°C . Immediately prior to use, 1µg/ml aprotinin and 100g/ml PMSF (stored in aliquots at -20°C) were added (sonicated prior to addition).

Antibodies:

erbB-1-Ab : 12E supplied by W. Gullick is a polyclonal antibody raised in rabbits to the hEGFR by injecting a synthetic peptide, 12E (DDTFLPVPEYINQS). It was used at a 1:300 dilution in PBS containing 10% Goat serum and 0.05% Tween-20.

erbB-2-Ab : 21N supplied by W. Gullick is a polyclonal antibody raised in rabbits to 1243-1255 aa (C-terminus) of the erbB-2 gene product. Used at 1:300 with 10% goat serum and 0.05% Tween-20.

Secondary antibodies :

Anti-rabbit IgG(Fc specific) (AP Conjugated) 1:10,000.

Anti-mouse AP conjugated 1:8,000 (Sigma)

Nitro-cellulose sheets (90.45 micron pore size)

Stratagen.

Tween-20 , aprotinin, and PMSF were purchased from Sigma.

2.7. Protein Assay.

METHOD.

Protein Standard (BSA) stock solution 10g/dL was diluted 100 fold in 0.85% NaCl to give a final stock concentration of 1mg/ml and standards prepared from this in the range 0-100µg/ml in NaCl as outlined below.

Table 2.1. Preparation of protein standards.

Tube No.	Dilute protein µl	NaCl µl	Protein (µg/ml)
1	0	200	0
2	5	195	2.5
3	10	190	5
4	20	180	10
5	50	150	25
6	60	140	30
7	80	120	40
8	100	100	50
9	150	50	75
10	200	0	100

Samples to be assayed were prepared at various dilutions and assayed in triplicate.

5ml of diluted Bradford reagent was added to each sample and to the standards and the reaction allowed to proceed for 5 minutes prior to measurement. The samples were not left longer than 30 min. Absorbance was read at 595nm on a Fluokon spectrophotometer by kind permission of Dr D. O'Connor. The protein concentration for each unknown was determined by interpolation with the protein standard curve using the Lowry computer programme (McPherson, 1985).

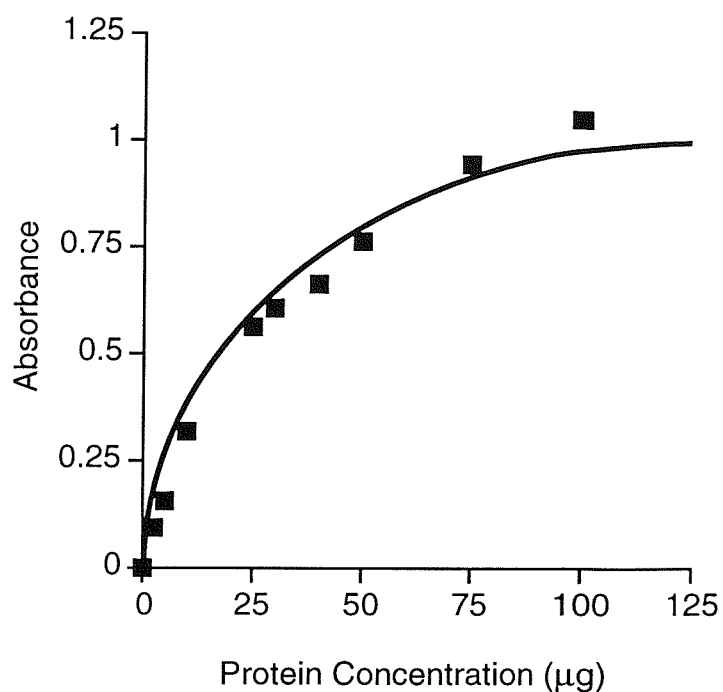


Figure 2.2. Representative Protein Assay Standard Curve.

Stock protein standards were prepared at 0, 2.5, 5, 10, 25, 30, 40, 50, 75, 100 µg/ml in 0.85% NaCl and incubated for 5 minutes with 5 mls Bradford reagent. Absorbance was measured at 595nm on a Fluokon spectrophotometer and plotted against protein concentration to generate a standard curve from which unknown protein sample concentrations could be extrapolated.

Table 2.2. Calculation of unknown protein sample concentrations by Lowry computer programme.

Tube No	Absorbance Replicates	Volume μ l		Assay concentration	Sample concentration (μ g/ml)
1	.0041	5		.0941632	18.83264
2	.0087	10		.201192	20.1192
3	.0176	20		.4244794	21.22397
				Protein Conc. =	20.06μg/ml

REAGENTS.

0.85% Sodium chloride solution
Bradford dye concentrate:

0.85g NaCl in 100ml analar H₂O
Coomassie brilliant blue 100mg
95% ethanol to 50ml
phosphoric acid 100ml
distilled water to 200ml

The Bradford dye was diluted 1:4 with distilled water and filtered through a Whatman filter paper No. 1 prior to use. The dye is stable for 6 months at 4°C.

Coomassie Blue G250
Phosphoric acid
Protein standard (BSA)

Sigma
Interchem
Sigma

2.8. Preparation of human placental membranes.

Membranes were isolated by a modification of the method employed by Hock and Hollenberg (1980).

METHOD.

Normal human placentae were obtained at term courtesy of The Delivery Suite, Princess Anne Hospital, Southampton. The placenta was kept on ice, the amnion was removed and the tissue chopped with scissors taking care to remove any large

blood vessels, fleeces and connective tissue. 100g wet wt of tissue was homogenised in 100ml of ice cold homogenisation buffer using a Jencons Homogeniser and the resulting homogenate filtered through two layers of cheesecloth. A sequence of high speed centrifugations, all at 4°C, followed - 600g (2400 rpm) for 10 min and the supernatant retained for a further spin at 10,000g (9250 rpm) for 30 min . The supernatant from this spin was adjusted to 0.1M NaCl and .02mM MgSO₄ and centrifuged at 48000g (20,000 rpm) for 40 min. The supernatant was then discarded and the remaining pellet washed by re suspension in 50mM tris HCl pH 7.6 and spun at 48000g (20,000 rpm) for 30 minutes. Finally, the supernatant was discarded and the pellet re-suspended in 50mM tris HCl (pH 7.6), aliquoted and stored at -70°C until required. Protein assay revealed the concentration of membrane present.

REAGENTS.

Homogenisation buffer (.25M sucrose buffered at pH 7.4 with 25mM Tris Cl):

For 100ml:

Sucrose	8.56g
Tris	0.3g

Water to 90ml, pH adjusted to 7.5 with HCl and volume adjusted to 100ml with distilled water.

Immediately prior to use, PMSF(1 aliquot -1ml=10mg) and Approtinin (2 aliquots - 50ul=50ug) were added (stored at -20°C before use).

1M NaCl: 5.8g NaCl in 100ml water

1M MgSO₄: 24.6g MgSO₄ (heptahydrate) in 100ml water

50mM TrisHCl: 6g Tris in 900ml water; pH adjusted to 7.5 with HCl and vol. made up to 1L with distilled water.

2.9. Iodination by the lactoperoxidase method.

METHOD.

Human recombinant EGF was iodinated using the lactoperoxidase method whereby the iodide is enzymatically oxidised. The method used was a modification of the original method developed by Marchalonis (1969) as outlined in the lactoperoxidase iodination kit purchased from ICN Flow.

G-25 Sephadex was hydrated overnight in distilled water (for every g 4-6ml distilled water was used). A small plug of siliconised glass wool was inserted into the nipple of two 2ml syringes and the columns filled with sephadex. They were then equilibrated using PBS and repeatedly centrifuged. In order to test that the columns were working i.e. desalting correctly, a 0.2ml sample of an aqueous solution of 0.1% water soluble phenol red and 0.1% dextran blue was centrifuged through the column. If the columns were correctly packed then the 0.2ml eluted following centrifugation would contain only dextran blue, with the phenol red being retained in the sephadex resin (Tuszynski 1980).

Prior to iodination 5-10µg of the protein to be labelled was dissolved in 25µl binding buffer and 10µg lactoperoxidase was dissolved in 25µl of distilled water and kept on ice. The peptide was added to the vial of radio-iodine (1mCi) using a Hamilton syringe, followed by 25µl lactoperoxidase and mixed. The reaction was initiated by addition of 5µl of 3% hydrogen peroxide. The oxidation was allowed to proceed for 10 min before addition of another 5µl aliquot of hydrogen peroxidase. This was repeated twice again so that in total 4 x 5µl aliquots were added to the vial at 10 min intervals. The reaction was stopped with 500µl termination buffer and the mixture applied to the sephadex columns (at 4°C). The desalted, radio-labelled peptide was eluted from the column and brought to 0.5%w/v with BSA. 100µl aliquots were stored at -20°C. The Specific Activity of the radio-labelled peptide was determined using self-displacement analysis.

REAGENTS.

Human recombinant EGF	Bachem
Human recombinant TGFα	Bachem
Sephadex G25 (fine)	Sigma
Dextran Blue	Pharmacia
Phenol Red	Sigma
Na ¹²⁵ I, carrier-free, 100mCi/ml in 0.01N NaOH	ICN
Lactoperoxidase	Sigma
BSA	Sigma
Hydrogen peroxide	Sigma

2.10. Iodination by the iodogen method.

An alternative iodination method was used to radiolabel PTHrP since the lactoperoxidase method was considered to be too oxidative and would increase the risk of damaging the peptide. Iodogen has been shown to provide a higher iodine incorporation with less damage to peptides than lactoperoxidase and chloramine T (Salacinski *et al*, 1981).

Siliconisation of glass vials:

Preparation of siliconised materials was conducted in a fume cupboard to minimise dust contamination and inhalation of solutions. 2 ml capped glass vials were immersed in 0.2 % dichloromethyl-silicone for 5 minutes, rinsed in toluene and left to drain for 2 min. The vials were then rinsed twice in ethanol, dried at 37°C in a dust free incubator and stored at -20°C until required.

Iodogen:

2.5 mg Iodogen (1, 3, 4, 6 tetrachloro-3 a, 6a-diphenyl glycouril) was dissolved in 25 ml methylene chloride, aliquoted into 500µl samples and the solvent evaporated off in the fume cupboard. The remaining dried powder was stored at -20 °C and remained stable for two months.

Terminating Buffer:

1.7 mg sucrose, 100 mg potassium iodide and 1.5 mg sodium metabisulphite were dissolved in 7 ml PBS (pH 7.4). 1 ml of stock bromophenol blue solution (1 mg/ml in PBS) was added, the volume adjusted to 10 ml with PBS and the final solution stored in 500 µl aliquots at -20 °C.

PTHrP peptide for iodination:

PTHrP 1-34 does not contain any tyrosine residues in its first 34 amino acids therefore a synthetic peptide was purchased containing a tyrosine residue incorporated at the C-terminal. (Tyr36)-PTHrP(1-36) was purchased from Bachem and supplied as a trifluoroacetate salt.

H-Ala-Val-Ser-Glu-His-Gln-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr-OH

Formula C₁₉₄H₃₀₃N₅₉O₅₃ MW 4309.9 g/mol

METHOD.

(Tyr36)-PTHrP (1-36) was dissolved in 0.01% acetic acid (to counteract the basic pH of the sodium iodide) in siliconised glass tubes at a concentration of 50µg/100µl and stored at -20°C until required.

G25 Sephadex beads were hydrated overnight in PBS at a bed volume of 1g/4-6ml and poured into a 25ml fractionation column. The column was equilibrated with PBS for 3 hours prior to use.

The iodination procedure was conducted in a fume cupboard in the designated radioactive isotope suite. 2mCi of ¹²⁵Iodine was added to the iodogen-prepared siliconised vial (capped with parafilm) using a Hamilton syringe, followed by immediate addition of the peptide. The mixture was agitated by aspiration 2-3 times and allowed to react for 15 minutes with gentle agitation every 5 minutes. 500µl of terminating buffer was added to the iodination mixture after the 15 minutes had elapsed.

5µl of the sample was removed, diluted with PBS and counted on a gamma counter to assess the total radiolabel present.

e.g.

5µl in 500µl PBS = 9,999,999.99 cpm

Resuspended 1:10 = 6,029,193 cpm

Resuspended 1:100 = 772,955 cpm

1 drop of bromophenol blue was added to the sample to enable visualisation in the column. The sample was applied to the Sephadex column and eluted with PBS at a flow rate of 5 drops/30 secs, which was roughly proportional to 500µl per minute, until 100 fractions had been collected. 50 µl samples were removed from each fraction, counted on an LKB Wallac gamma counter and the radioactivity plotted against fraction number to yield an elution profile (Fig 2.3). Two peaks were observed - a small initial peak which was assumed to contain the labelled peptide and a second larger peak representing the free iodine fraction. The fractions containing the peptide peak were pooled, aliquoted into 100µl samples (the radioactivity of which was noted) and stored in a lead-perspex box at -20°C in the designated radioactive freezer.

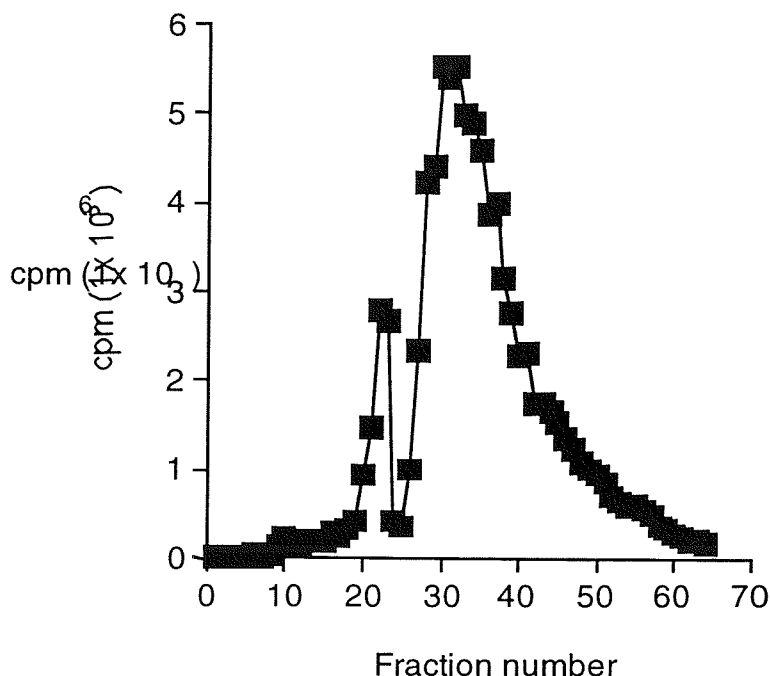


Figure 2.3. Elution profile of (Tyr) PTHrP 1-36 following iodination by the iodogen method.

Following iodination the radiolabelled sample was applied to a G-25 sephadex column and eluted with PBS at a rate of 500 μ l/min. The fractions were collected into LP4 tubes and 50 μ l of each aliquot removed for counting by an LKB gamma counter. Counts per minute (cpm) were plotted against fraction number to produce two peaks - a peptide peak and a free salt peak.

Since PTHrP contains only a single tyrosine residue it was not necessary to employ the self-displacement analysis method for determining specific activity as only a single species of radiolabelled peptide was generated. Several attempts were made to determine precisely the amount of peptide present. A G-25 column was run in parallel containing unlabelled peptide and the corresponding fractions assayed for protein concentration. Unfortunately, the protein assay was not sensitive enough to detect any “cold” peptide present. It was not feasible to conduct a protein assay on the radioactive peptide samples for safety reasons. Therefore, it was assumed that 100% of peptide was radiolabelled. This may have resulted in an over-estimation of 125 I-TyrPTHrP 1-36 but since the peptide was to be used in displacement experiments it was possible to obtain a more accurate determination of actual peptide concentration from the actual experiments. It is

generally considered wiser to over-estimate and thus guarantee displacement with higher concentrations of cold rather than under-estimate.

Calculation of Specific activity for radiolabelled PTHrP

In order to calculate the Specific Activity, one requires

- a) cpm of fraction of monoiodinated PTHrP
- b) gamma counter efficiency
- c) $1\mu\text{Ci} = 2,22 \times 10^6 \text{dpm}$
- d) PTHrP conc.

- 1) Calculate the cpm ^{125}I present in 5 ml stock fraction

$$\frac{994537}{5} \times 1000 = 199 \times 10^6$$

- 2) Convert to dpm

$$\frac{199 \times 10^6}{85} \times 100 = 234 \times 10^6 \text{ dpm/ml} = 105.4 \mu\text{Ci/ml}$$

$$\text{Specific Activity} = \frac{105.4 \mu\text{Ci/ml}}{50 \mu\text{g/ml}} = \underline{\underline{2.1 \mu\text{Ci}/\mu\text{g}}}$$

Mini gel analysis of iodination fractions

To ensure that the correct fractions were used for binding studies, the pooled fractions were subjected to min-gel SDS -PAGE. The procedure was identical to that outlined in section 2.14. 50 μl samples were loaded onto a 4% stacking /10% resolving gel and run at 40 mA for approximately 1hr alongside low molecular weight markers. The gel was dried and exposed to x-ray film at -70°C for 4 days. The molecular weight of the bands could then be determined to ensure that the fraction used contained radiolabelled peptide and not free iodine.

2.11. Ligand binding studies.

2.11.1. Calculation of the specific activity of radio-labelled ^{125}I -EGF by self-displacement analysis.

METHOD.

To determine the specific activity of ^{125}I -EGF both a saturation experiment and a displacement experiment were performed in parallel. For the saturation curve, 100 μl of human placental membranes (2 μg protein/eppendorf) were incubated with increasing concentrations of ^{125}I labelled hEGF(ranging from 0.5nM-10nM) in duplicate, in a final volume of 250 μl . The samples were left to equilibrate overnight at 4°C. For the displacement experiment, 100 μl of human placental membrane was incubated in duplicate with a fixed concentration of labelled hEGF (0.5nM) and increasing concentrations of unlabelled ligand (0-200nM) in a total volume of 250 μl , at 4°C overnight. Non-specific binding was determined in the presence of excess hEGF (200nM).

The results obtained from the saturation analysis were converted into bound/total ratios (B/T) and plotted against the log of the radioactivity (cpm) of the total ^{125}I -EGF added to the receptor preparation having subtracted the initial cpm (i.e. the 0.5nM sample) from each subsequent reading. For the displacement experiment, the data were again transformed into B/T ratios but these values were plotted against the log of unlabelled EGF added in nM (i.e. from 0-200nM). The graphs were plotted together and at several B/T ratios in this plot, cpm of ^{125}I -EGF and nM of unlabelled hEGF were read from both curves and plotted against each other to give a straight line. The slope of the line is equal to the specific activity of the radio-labelled peptide.

REAGENTS.

Binding buffer -	25mM sodium phosphate buffer pH 7.6
	150mM NaCl
	0.1% BSA
Na_2HPO_4 (anhydrous)	1.775g in 500ml distilled water
NaH_2PO_4 (anhydrous)	1.5g in 500ml distilled water

The two solutions above were titrated against one another and 8.77g/L of NaCl and 1g/L of BSA added. The pH was adjusted to 7.6 with NaOH and the buffer stored at 4°C. The binding buffer was made up fresh prior to use.

2.11.2. Ligand binding assay.

METHOD.

For each cell line, experiments were performed on whole cells cultured in 24 well collagen coated plates in 10% FCS (in medium) for 72hr, with the medium being changed to that of 2.5% SFCS 24h prior to experimentation. The cells were washed gently, twice, in warm binding buffer and conditions applied to each well as laid out below:

50µl of each concentration of labelled EGF were added to each well and the total volume made up to 250µl with binding buffer. Non-specific binding was determined in an excess of 50µl unlabelled EGF (200nM). Cells were incubated at 4°C for 2hr, after which, they were washed once with ice cold binding buffer to stop the reaction and then twice again with room temperature binding buffer. Care was taken not to dislodge the cells from the plates. 300µl of 0.5M NaOH was added to each well for 2hr at room temperature to solubilize the cells. When the cells were fully solubilised the contents of each well were transferred from the plates to LP4 tubes and read on an LKB Wallac Gamma counter (85% efficiency). 50µl of each concentration of labelled EGF plus 200µl binding buffer was also read to determine the total added prior to incubation. The optimum cell density was determined by incubating cells at varying initial DNA concentrations whilst maintaining a fixed concentration of radio-labelled ligand. The DNA content at the end of 72hr was determined by setting up a separate DNA assay plate in parallel.

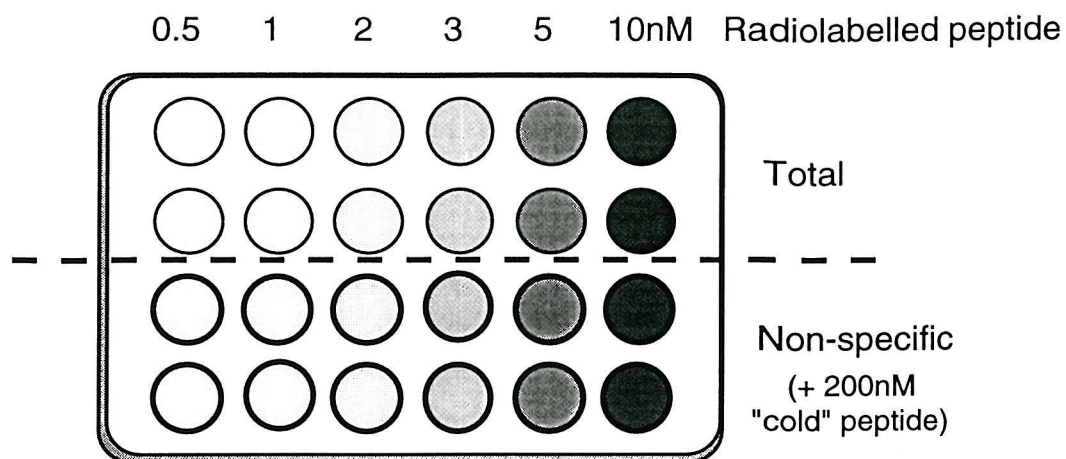


Diagram 2.3. 24 well plate layout for saturation binding study.

Each well contained a fixed concentration of DNA (e.g. 1 μ g/well for BeWo) and 50 μ l of varying concentrations of "hot" peptide in a volume of 250 μ l made up with binding buffer. Non-specific binding was determined in the presence of 200nM unlabelled peptide.

For displacement experiments the incubation conditions were identical to those for saturation. Cells were incubated with a fixed concentration of labelled peptide with increasing concentrations of unlabelled peptide in the range (0-2000nM) at 4°C for 2h. The plate layout is indicated in diagram 2.4.

REAGENTS.

Cell culture reagents, media, plastics etc. as above (section 2.1)

0.5M NaOH

Binding buffer as above.

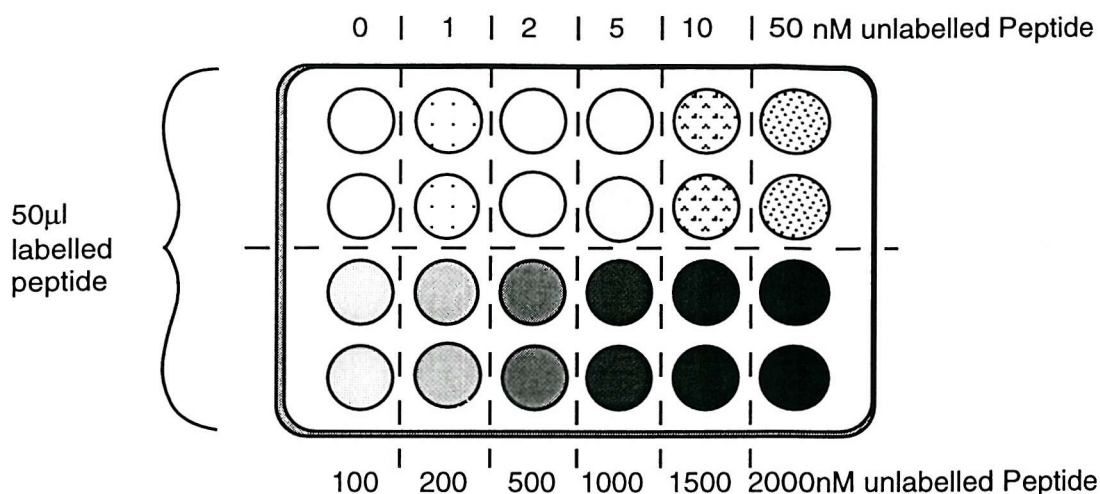
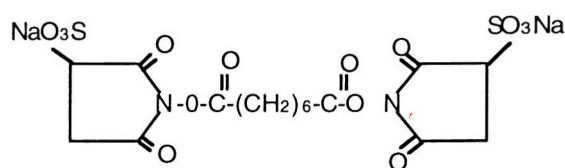


Diagram 2.4. 24 well plate layout for displacement binding study.

Each well contained a fixed concentration of DNA (e.g. 1 µg/well for BeWo), 50 µl of labelled peptide at a fixed concentration and 50 µl of varying concentrations of unlabelled peptide in a volume of 250 µl made up with binding buffer.

2.12. Cross linking of ^{125}I -EGF to EGFR.

Bis (sulfosuccinimidyl) suberate), commonly known as BS³ is a noncleavable, membrane impermeable, water soluble DSS analogue. It is widely used as an amine reactive homobifunctional cross-linker.



Diag 2.5. BS³ Crosslinker.

METHOD

Cells were subcultured into 75ml flasks and grown for 72h in 10% FCS. 24 hour prior to experimentation the media was replaced with serum free medium. In experiments investigating the effect of peptides upon receptor expression, the

medium was supplemented with an appropriate peptide e.g. 10nM PTHrP, for the entire culture period. 2 hours prior to harvesting, the cells were washed with fresh medium and incubated for two hours at 4° C with 10nM radiolabelled EGF either alone or in the presence of an excess of cold EGF (200nM). 2mM BS₃ was added to the flask for 20 minutes with gentle agitation. The cross-linking was terminated after 20 minutes by quenching with 20mM Tris-HCl containing 0.15M NaCl. The cross-linking solution was discarded and replaced with 0.5ml lysis buffer for 30 min at 4°C with frequent agitation. The cells were then scraped from the flask and spun at 1500g for 10 min to remove nuclei and a sample removed for protein assay. The supernatant was mixed with 0.6 v/v of 3x sample buffer, heated for 2 minutes at 60°C and loaded into SDS-PA gels.

REAGENTS

BS³: 6mg/500µl PBS equivalent to stock solution of 20mM

Lysis buffer: 20mM Tris-HCl, 1% Triton-X-100, 2mM PMSF, 5mM EDTA, 5mM iodoacetate.

Terminating buffer: 20mM Tris-HCl, 0.15M NaCl (pH 7.4)

2.13. SDS-polyacrylamide gel electrophoresis.

Sodium Dodecyl sulphate polyacrylamide gel electrophoresis or SDS-PAGE is currently the method of choice for isolation of small amounts of protein. The sample to be investigated is denatured and coated with a detergent by heating in the presence of SDS and a reducing agent (e.g. mercaptoethanol). The SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on a polyacrylamide gel and a high voltage is applied causing the protein components to migrate towards the anode.

The use of a discontinuous gel system favours sharp banding of the protein components and this is achieved by having stacking and separating gels of differing pH or salt concentration (or both!). In the stacking gel of a discontinuous buffer system, the leading ion is chloride and the trailing ion is glycine and between these is an area of lower conductivity and steeper voltage gradient which pulls the peptides from the sample and deposits them on the top of the resolving gel. The higher pH of the resolving gel favours the ionisation of glycine which then moves through the stacking polypeptides and catches up with the chloride ions thus the gap between the two moving 'boundaries' is lost. Therefore in the

resolving gel, the SDS-coated peptides can travel freely in a zone of uniform pH and voltage and be separated by size alone due to the molecular sieving of the gel.

2.13.1. Mini gel SDS-PAGE.

METHOD.

Polyacrylamide gels were cast as outlined in the Hoefer Mighty small dual gel caster catalogue (No. SE245). Briefly, the gel sandwich stack was assembled and loaded into the caster. Stacking and resolving gels of appropriate concentration were prepared with the exception of the polymerising agent TEMED. The TEMED catalyst was added to the resolving monomer solution immediately prior to use, mixed and poured into the gel sandwich leaving a gap of about 3 cm for the stacking gel and taking care not to introduce any air pockets. 0.1% SDS solution was used to overlay the surface of the resolving gel to ensure a clean interface with the stacking gel. The resolving gel was left to polymerise for at least 30 minutes before the addition of TEMED to the stacking gel solution which was then poured carefully on top of the resolving gel. Combs were introduced into the sandwich taking care not to trap air under the teeth and again the gel was left to set for at least 30 minutes.

Following total polymerisation, the combs were removed, the stacking gel wells rinsed with distilled water to remove any gel fragments and the sandwiches removed from the casting unit and inserted into the gel electrophoresis unit.

Samples were prepared by addition of an equal volume of sample buffer, 5 μ l of 0.1% bromophenol blue, heated at 70°C for 10 minutes and then carefully loaded into the stacking gel wells. This method is a modification of the Laemmli procedure (Laemmli, 1970).

Two 10 cm x 10.4 cm polyacrylamide gels were run at a constant current of 40 mA (20 mA per gel) thus ensuring that the electrophoretic migration remains constant throughout the run and minimising heat production. The total run time varied between gels of different percentage but, in general, a 6% gel took about 45 min-1hr, 8% 1hr 20 min and 15-20% 1-2hr. Once the leading buffer front had reached the bottom of the sandwich the current was switched off and the gels carefully removed.

Gels were either blotted onto nitro-cellulose (see below) or fixed and dried. To fix gels they were placed in a solution of 10% acetic acid, 25% methanol for 30 minutes after which they were carefully laid onto 2mm Whatman paper, wrapped in Saran wrap clingfilm and placed in a Biorad gel drier for 1h at 65°C. The dried gels could then be exposed to autoradiography film in cassettes containing intensifying screens.

REAGENTS.

30 % Acrylamide

29.2g Acrylamide and 0.8g bis-acrylamide were made up in 100ml distilled water and stored at 4°C in a dark bottle. This was stable for 2-3 months

Resolving gel buffer.

1.5 M Tris was prepared in distilled water, the pH adjusted to 8.9 with conc. HCl. Stored at 4°C the solution was stable for 2-3 weeks.

Stacking gel buffer

0.5 M Tris was prepared in distilled water, the pH adjusted to 6.7 with conc. HCl and stored at 4°C. Stable for 2-3 weeks.

10 % Ammonium persulphate (APS):

A 10% w/v ammonium persulphate solution was prepared fresh prior to use

TEMED

Stock solution, stored at RT

Sample buffer

In the fume cupboard, 2.5 mls of stacking gel buffer was added to 4 ml of 10% SDS, 2ml of glycerol, 1ml of mercaptoethanol and 0.5ml of distilled water. The buffer was aliquoted into 500µl samples and stored at -20°C until required.

Electrode buffer

0.025 M Tris, 0.19 M glycine and 0.1 % SDS made up in 5 litres of distilled water.

3% Stacking gel (10mls) - sufficient for two mini-gels. For the larger gel system 20ml were made up.

1ml 30% acrylamide, 2.5ml stacking gel buffer, 100 μ l 10% SDS, 130 μ l APS, 7.5mls distilled water, 12 μ l TEMED.

Resolving gels

Several types of resolving gel were used depending on the size of the peptide of interest. Table 2.1. shows the component volumes in ml - 10ml for the mini-gel system and 50ml for the large gel (i.e. multiply each component by 5).

Table 2.3. Ingredient volumes for polyacrylamide gels of different percentages

Gel %	Distilled water (ml)	30% acrylamide (ml)	Resolving gel buffer (ml)	10% SDS (ml)	10% APS (ml)	TEMED (ml)
6	5.3	2.0	2.5	0.1	0.1	0.008
8	4.6	2.7	2.5	0.1	0.1	0.006
10	4.0	3.3	2.5	0.1	0.1	0.004
12	3.3	4.0	2.5	0.1	0.1	0.004
15	2.3	5.0	2.5	0.1	0.1	0.004

Coomassie Staining/Destaining.

Protein captured on SDS-PA gels can be simultaneously fixed with methanol : glacial acetic acid and stained with Coomassie brilliant blue R250 9a triphenylmethane textile dye.

Essentially, the resolving gels are immersed in Coomassie stain (see below) for several hours; then excess dye diffuses from the gel during a prolonged destaining process involving two differing concentrations of Destain (I and II) with several changes of destain left preferably overnight. Destaining for longer periods allows as little as 0.1 μ g of protein to be detected in a single band.

Coomassie Stain

2.5g of Coomassie Brilliant Blue is dissolved in 100ml acetic acid, 450 ml methanol and 450 ml water. The solution is filtered through a Whatman No. 1 filter.

Coomassie Destain I

75ml acetic acid, 200ml methanol, 725 ml water.

2.13.2. Large gel & Gradient SDS-PAGE.

To obtain better separation over a wider range, a large gel system was used. A further advantage of using the large gel system was the capacity to load more protein per lane. When isolation of two distinct protein populations was required, e.g. in identifying the monomeric (170 kDa) and dimeric (340 kDa) forms of the EGF receptor, it was necessary to cast gradient gels to capture both species on the same gel with sufficient separation between them. I employed 5-10% gradient gels poured through the apparatus outlined in diagram 2.6..

Gel solutions were of the same as described in section 2.13.1 but, obviously, of greater volume. After casting and setting the large plates were removed from the casting stand and immersed in an electrophoretic tank filled with electrode buffer. A current of 40 mA was applied perpendicular to the gel and the system kept cool via an integrated cold water cooling system. Depending on the percentage of the gels, the proteins usually took between 4-8 h to migrate to the bottom of the gel. Gels were carefully removed from the running plates and either stained with Coomassie, dried and autoradiographed or used for Western blotting. The protocol for drying, staining and blotting was identical to that used for the mini-gel system.

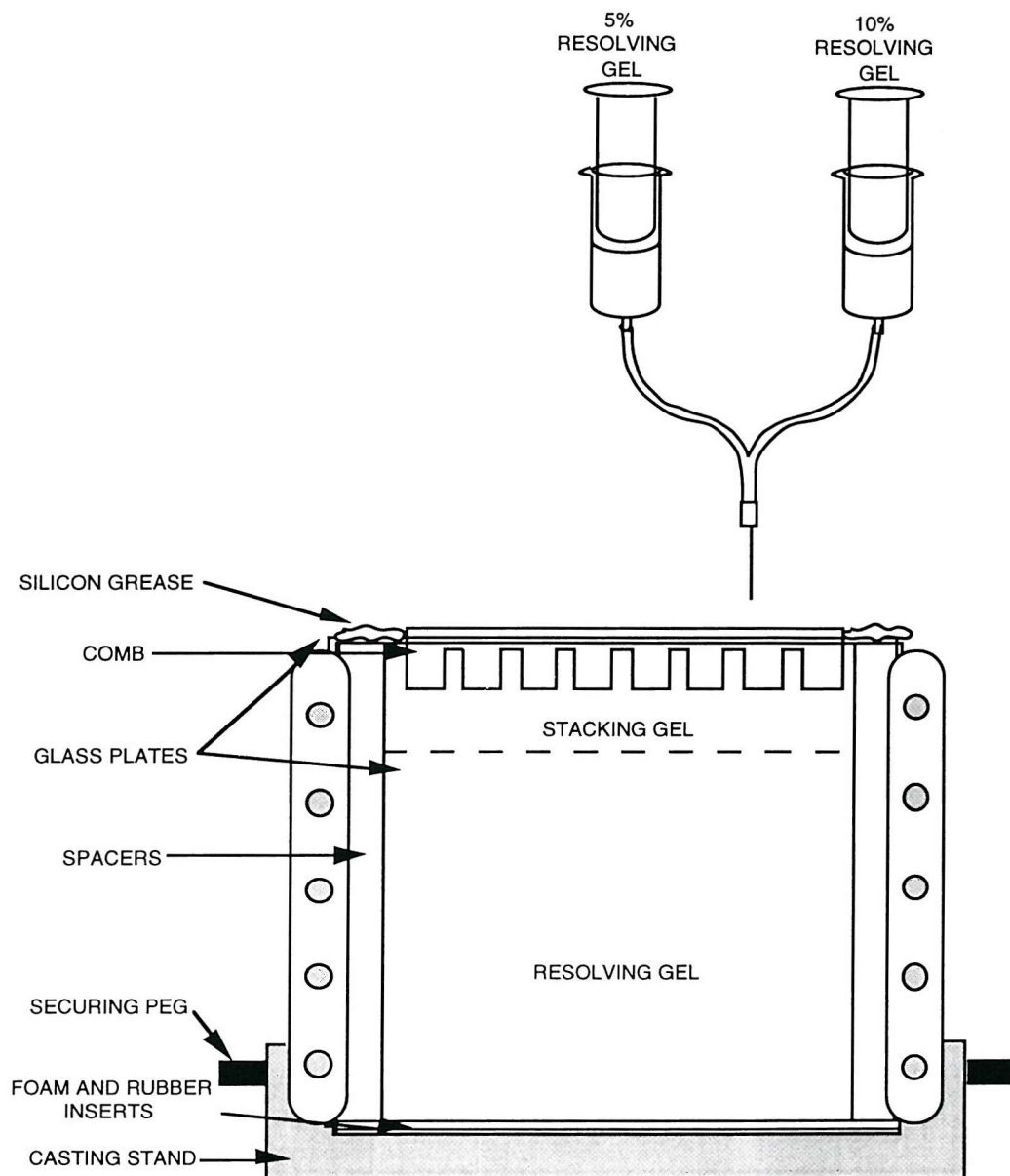


Diagram 2.6. Pouring and casting of a large gradient gel.

Large glass plates were assembled in the casting stand containing two spacers and sealed with silicon grease. Two 50 ml syringes were joined to Y-shaped rubber tubing with a needle attached to the open end. 5% and 10% resolving gel solutions were prepared and the syringe barrels filled with the two solutions. The higher gel percentage was introduced into the Y-shaped tubing first so that the second solution would flow in through the first gel solution. The plungers were depressed ensuring that the rate of depression was identical between the two solutions until the resolving gel was roughly 3 cm from the top of the plates. The gel was overlaid with 0.1% SDS and the stacking gel later cast on top with combs inserted.

2.14. Western blotting.

When a protein is separated by SDS-PAGE it becomes trapped in the matrix of the gel and is therefore inaccessible. Western blotting provides a means for probing protein samples with specific antibodies after electrophoretically transferring them onto a nitro-cellulose or nylon membrane.

METHOD.

Following SDS-PAGE the gels are removed from the glass-alumina plate sandwich and layered onto a nitro-cellulose membrane. A layer of blotting paper was placed on either side of the gel-nitro-cellulose and the entire sandwich placed in foam lined cassettes as supplied by Hoefer. The cassettes were then inserted between two electrodes in an electrophoretic tank containing low ionic strength transfer buffer (this limits the amount of heating in the transfer tank). A voltage gradient was applied perpendicular to the gel at either 1 Amp for 2 hours or 200 mA overnight.

Efficient transfer was determined by a) Coomassie staining of the gel and b) Ponceau staining of the nitro-cellulose membrane. The use of rainbow markers also facilitated easy detection of successful transfer as these could readily be seen on the membrane.

REAGENTS.

Transfer buffer:

0.025 M Tris, 0.19 M glycine, 0.1 % of 10 % (W/V) SDS and 1 litre methanol were made up to 5 litres with distilled water.

Supported nitro-cellulose membranes

0.45 μ M pore size supported nitro-cellulose (Sartorius) was cut to size. The positive charge on the surface of the membrane facilitates protein binding

Amido black

Amido stain was diluted 1:1 with distilled water and stored at RT.

Amido black destain.

15 % w/v isopropanol and 10 % w/v glacial acetic acid were made up in a litre of distilled water and stored at RT.

Ponceau Stain

5 ml of stock solution (Sigma) was applied to the membrane until pink/red protein bands were visible. The dye was readily washed off with distilled water.

Although this staining method lacks the sensitivity of Amido and Coomassie staining, its reversibility makes it a useful tool for checking whether transfer has occurred.

Molecular weight markers

Two kinds of "Rainbow" markers specifically produced for the Laemmli system were used:

A Low Molecular weight marker (Amersham RPN 755) - MW 2350-46000, which was aliquoted out, stored at -20°C and 10µl loaded per lane.

Occasionally the Insulin (a) band was not resolved on the gel.

Wide range Molecular weight markers (6,500-205,000) purchased from Sigma (C 3437) were aliquoted into eppendorfs and stored at -20°C. Approximately 10µl of marker was loaded per lane. Occasionally, the light chain of myosin appeared as an additional blue band with a MW approximately that of Trypsin Inhibitor from Soybean.

Table 2.4. Distribution of proteins in Low molecular weight colour markers

Protein	Native MW of subunits (Daltons)	Colour of conjugate
Ovalbumin	46,000	Yellow
Carbonic Anhydrase	30,000	Orange
Trypsin Inhibitor	21,500	Blue
Lysozyme	14,300	Magenta
Approtinin	6,500	Blue black
Insulin (b) chain	3,400	Blue
Insulin (a) chain	2,350	Blue

Table 2.5. Distribution of proteins in Wide range Molecular weight colour markers

Protein	Native MW of Subunit (Daltons)	Colour of Conjugate
Myosin, Rabbit muscle	205,000	Blue
β -Galactosidase, <i>E. Coli</i>	116,000	Turquoise
Albumin, Bovine serum	66,000	Pink
Ovalbumin, Chicken egg	45,000	Yellow
Carbonic Anhydrase, Bovine erythrocytes	29,000	Orange
Trypsin Inhibitor, Soybean	20,000	Green
α -Lactalbumin, Bovine milk	14,200	Purple
Approtinin, Bovine milk	6,500	Blue

2.15. Immunodetection following Western blotting.

METHOD.

After transfer, the membranes were blocked in a suitable blocking buffer for a minimum of 1hr at RT. or preferably overnight at 4°C. They were then washed 1 x 15 minutes in Tween-Tris buffered Saline (TTBS) followed by 2 washes of 5 minutes with fresh changes of wash buffer. Primary antibody was prepared accordingly and added to the membrane. Incubation was for either 1hr at RT or overnight at 4°C, in hybridisation tubes with shaking. Washing for 15 min in TTBS, to remove excess primary Ab, was followed by two more washes of 5 min before addition of the secondary antibody for 30 min-1h. The membrane was then washed 1 x 15 min and 2 x 5 min in TTBS before detection with either BCIP tablets or the ECL kit.

Detection using the Amersham ECL kit.

The Enhanced Chemiluminescence (ECL) kit comprises a light emitting non-radioactive method for detection of immobilised antigens, conjugated with horse-radish peroxidase-labelled antibodies (Diag 2.7).

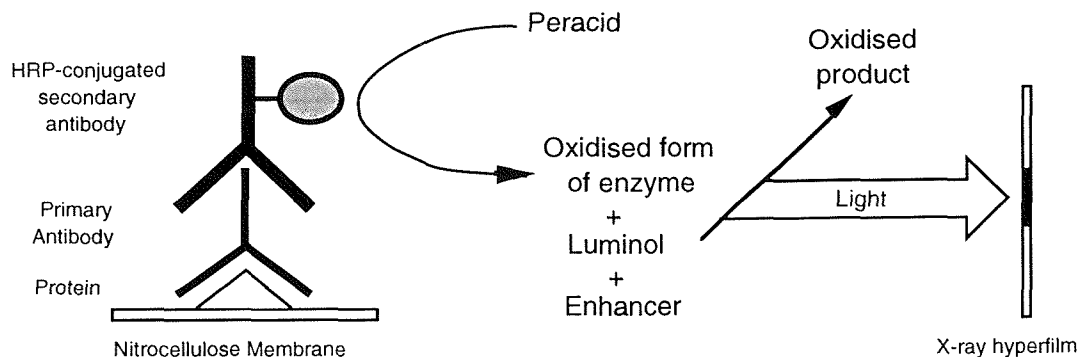


Diagram 2.7. Immunodetection of proteins for Western Blotting using the Amersham ECL system

Cartoon representing the chemiluminescent detection procedure. HRP - horse radish peroxidase.

Membranes were prepared as outlined above, excess buffer drained and the blots placed on a piece of Saran Wrap clingfilm. Equal volumes of detection reagents 1 and 2 were prepared and pipetted onto the membrane ensuring the whole area was covered. After an incubation period of 1 minute without agitation, the detection reagent was drained off and the membrane wrapped in Saran wrap. Any air bubbles present were smoothed out and the membrane placed, protein side up, in a film cassette. A sheet of autoradiography film was placed on top of the membrane, the cassette closed, and the film exposed for 15 seconds. The film was then removed and inserted into an X-graph GRI automated film developer and replaced immediately with a fresh piece of unexposed film. The first exposure gives an indication of how long to continue the exposure of the second piece of film which sometimes varied between 1 minute and 1 hour depending upon the amount of target protein on the membrane.

Sequential re-probing of membranes with a variety of antibodies was possible with the ECL system. To strip and re-probe, membranes were first incubated in stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris HCl, pH 6.7) for 30 min at 50°C with gentle agitation. The membranes were then washed 2 x 10 min in TTBS, blocked in 5% dried milk for 1 hour at RT and the immunodetection procedure performed as outlined above.

REAGENTS.

Tris Buffered Saline (TBS)/ Tris Buffered Saline + Tween (TTBS):

100 mM Tris in distilled water, adjusted to pH 7.4 with conc. HCl plus 150 mM NaCl. Tween-20 0.1 % w/w was added, if required, and the solution stored at 4°C.

Blocking Reagents:

Goat serum:

10 % of goat serum in TTBS is used to block all the non-specific binding sites.

10% BSA:

10g of Bovine Serum Albumin is dissolved in 100ml of TTBS

10% Powdered milk solution:

10g of 'Safeways' Fat-free powdered milk was dissolved in 100ml TTBS.

IW Ab:

This 19mer anti-EGFR polyclonal antibody was raised in rabbits to a synthetic peptide sequence which was made “in-house” by Ian Woolveridge. The sequence was contained within the intracellular portion of the EGFR and is detailed below:



Table 2.6. Antibody concentrations for western blotting

1° ANTIBODY	2° ANTIBODY	BLOCKING AGENT
Anti- EGF: IW Ab 1:200	Anti-rabbit -HRP conjugated IgG 1:30,000	10% BSA/Powdered milk (0.05% Tween-20)
Anti EGF: 12E Ab 1:300	Anti-rabbit -HRP conjugated IgG 1:30,000	10% Goat serum (0.05% Tween-20)
Anti Ptyr: PY69 Ab 1:1000	Anti Mouse HRP-conjugated 1:1,000	10% BSA
Anti c-erbB-2: 21N Ab 1:300	Anti rabbit IgG 1:30,000	10% Goat serum (0.05% Tween-20)

2.16. Cell lines.

BeWo

Medium - Ham's F12, 90%; FCS 10%.

This was the first human endocrine cell type to be maintained in continuous cultivation and was set up by Patillo and Gey in 1968 from a malignant choriocarcinoma of placental origin (Patillo, 1968). Hertz transplanted it to the hamster cheek pouch where it was maintained for 8 years by 304 serial passages before being established in vitro (Hertz, 1959).

BeWo have been shown to secrete many placental hormones, including human chorionic gonadotrophin (hCG), polypeptide hormones, human placental lactogen

(hPL), estrone, estradiol, estriol and progesterone. Cellular differentiation is possible if hormonal precursors are added to the incubation medium. The cells have an epithelial like morphology.

JAR

Medium - RPMI 1640, 90%; FCS, 10%.

The JAR choriocarcinoma was set up by Patillo directly from a trophoblastic tumour of the placenta. It releases progesterone hCG and hPL. It is epithelial like, but does not form a uniform monolayer.

JEG-3

Medium - Eagle's MEM, 90%; FCS 10%.

This is a clonally derived choriocarcinoma isolated by Kohler set up from fragments of the Wood's strain of the Erwin-Turner tumour (387th passage). It was transplanted into hamster cheek pouch, isolated and recloned after propagation. Jeg-3 secretes hCG, hPL and progesterone and can transform steroid precursors. It is epithelial like.

A431

Medium - Dulbecco's modified Eagle's medium with 4.5g/L glucose, 90%; FCS, 10%.

This is a human epidermoid carcinoma cell line established from solid tumours by Giard from a vulval carcinoma.

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CHAPTER THREE

THE PROLIFERATIVE EFFECT OF EPIDERMAL GROWTH FACTOR ON THREE CHORIOCARCINOMA CELL LINES - BEWO, JAR AND JEG-3.

3.1. Introduction.

The development of an invasive trophoblast early in pregnancy is vital for placental function. Indeed, implantation may be a deciding factor between fertile and non-fertile cycles, and incorrect placental function can lead to complications of pregnancy such as gestational diabetes and pre-eclampsia (Redman *et al*, 1991). Recent work has suggested that poor placentation may result in fetal growth retardation which may, in turn, affect the long-term morbidity of the adult. Furthermore, restricted or accelerated growth in utero (with mismatch of placental and infant size) has been linked to the adult development of coronary heart disease, stroke, hypertension and diabetes. (Barker and Martyn, 1992; Barker *et al*, 1992).

Invasion of the placenta is balanced by the proliferation/differentiation and controlled growth arrest of trophoblast cells. Several trophoblast phenotypes exist including the proliferative cytotrophoblast cells which differentiate into the hormone producing syncytiotrophoblasts. These can then further differentiate into the highly invasive intermediate or extravillous trophoblast cells and the apparently 'inert' giant binucleate cells of the placenta.

Growth factors and protooncogenes are implicated in placental and endometrial function (Ohlsson *et al*, 1989) and I have chosen to look at Epidermal Growth Factor (EGF) and its receptor, in particular, as they have been identified in reproductive tissues in many species including humans (Hoffmann *et al*, 1992 and 1993). EGF, acting through its receptor, can cause the release of human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) from differentiated trophoblast cells, suggesting an autocrine/paracrine role for this growth factor and its receptor during implantation (Barnea *et al*, 1990).

Divergent expression of EGFR (erbB-1) and its homologue erbB-2 proteins during trophoblast invasion/growth arrest has been associated with the proliferation of cytotrophoblast where the EGFR is predominant - and conversely the expression of erbB-2 in the intermediate and syncytial cells is associated with differentiation (Muhlhauser *et al*, 1993). This suggests that EGF can have multiple effects in the same tissue depending upon the proportion of erbB-1/erbB-2 present.

EGF causes dramatic changes in morphology and a several-fold increase in invasive capacity of cultured first trimester cytotrophoblast cells and is associated with alterations in cell adhesion (Bass *et al*, 1994).

Clearly, opinion is divided on EGF's precise role in the placenta and our knowledge of human implantation remains incomplete, mainly due to a lack of adequate experimental models. The study of mechanisms underlying this process in humans is restricted by nature and for ethical reasons to in vitro models.

Three trophoblastic cell lines of placental origin, JAr, Jeg-3 and BeWo, have been established making it possible to look at the effects of EGF upon these cells and to interpret the results to partially explain the growth factor's role in vivo.

In the work in this chapter I have investigated the proliferative response of choriocarcinoma cells to EGF. It was necessary to optimise the growing conditions of the cell lines, to determine the most sensitive assay system and ultimately to ensure that the most appropriate cell line was selected for future experiments.

3.2. Aims.

The principle aim of this work was to investigate the effects of EGF upon proliferation of the BeWo, JAr and Jeg-3 choriocarcinoma cells in culture. In order to do this the growth of the cell lines had to be maintained and the conditions optimised so that a consistent effect of EGF could be observed. It was also necessary to look at different assay systems to determine which method was best suited to these particular cells. This preliminary work was essential so that future investigations into regulation of receptor expression could be undertaken. This work has also served as the basis of other work in the laboratory in which interactions between EGF and potential modulators of its effects have been studied.

3.3. Materials and methods.

Materials.

As described in materials and methods (chapter 2, section 2.1-2.5)

Methods.

Initially, four assays were chosen - DNA assay, MTT, CV and 3H-thymidine incorporation and the principles behind these are outlined in section 2.2-2.5. Cells were maintained in culture as outlined in section 2.1. For experimentation, cells were deprived of serum for 24 h prior to use and harvested by trypsinisation. Total cell concentration was determined by DNA assay of 3 x 100µl aliquots (see section 2.2) and re-suspended in appropriate supplemented medium to give the required total stock concentration.

To determine the effect of "growth arresting" (GA) the cells, the incubation medium was discarded 24h prior to harvesting and replaced with serum free medium and compared with control cells run in parallel.

For standardisation experiments, cells were plated out at varying initial DNA concentrations into 24 well tissue culture plates and grown in 2.5% SFCS for 72h at 37°C in 5% CO₂ in air. At the end of the culture period the cell density was determined by DNA, MTT or CV assay.

For the dose response curves, cells were plated out at their predetermined optimal plating densities in the appropriate medium containing 2.5% SFCS. 50µl of required dilution of hEGF (Bachem) was added to each well and medium added to give a total well volume of 500µl. The plates were then gassed with 5% CO₂ in air and incubated at 37°C for 72h, after which time they were assayed by one of the methods mentioned previously.

3.4. Results.

3.4.1. The effect of growth arresting BeWo cells in the presence or absence of EGF.

In the case of all the data shown, fluorescence readings were converted to µg DNA/well from the standard curve (an example of which is given in section 2.2).

Figure 3.1 shows the comparison between growth arrested (GA) and control BeWo cells at varying initial plating concentrations of DNA measured as μg DNA/well after 72h, in 2.5 % SFCS, 5% CO_2 in air at 37°C . One can see clearly that both control BeWo cells and GA BeWo cells, not surprisingly, follow the same trend, i.e. the greater the initial DNA, the greater the final DNA concentration as measured by fluorescence of the intercalated Hoescht 33258 compound. It also appears that the GA BeWo cells proliferate more readily when compared with the control BeWo cells; in the case of $1\mu\text{g}$ initial DNA, the final DNA concentration increasing from $2.11\mu\text{g}$ DNA/well to $2.84\mu\text{g}$ DNA/well an increase of 35% and this is observed at all three DNA concentrations, although the results are not statistically significant when compared to controls using Student's t-test.

In Fig 3.2, the response of BeWo cells to EGF (5ng/ml) (in triplicate) is shown at differing concentrations without being growth arrested. Cells were not deprived of serum prior to sub-culturing into 24 well plates for 72h in 2.5 % SFCS, 5% CO_2 in air at 37°C . At all initial DNA plating densities - 0.5, 1, and $1.5\mu\text{g}$ DNA/well there is an increase in BeWo cell proliferation in response to EGF as measured by the amount of DNA ($\mu\text{g/well}$). At both initial DNA densities of 1 and $1.5\mu\text{g}$ DNA/well there is a significant increase ($p < 0.05$) in cell proliferation on addition of EGF, with, for example an increase from $2.12\mu\text{g}$ DNA/well (at $1\mu\text{g}$ initial DNA) to $2.76\mu\text{g}$ DNA/well following treatment - an increase of 30% ($n=3$).

The effect of EGF (5ng/ml) upon the GA BeWo cells is shown in figure 3.3. Again, cells were subcultured into 24 well plates for 72h, in 5% CO_2 in air at 37°C with 2.5% SFCS but 24h prior to harvesting were growth arrested by serum deprivation. The data show the arithmetic mean \pm s.e.m. of triplicates for three experiments and is expressed as μg DNA /well. At each concentration of initial DNA the presence of EGF causes an increase in total DNA after 72h. For example, at $0.5\mu\text{g}$ initial DNA the control BeWo cells had proliferated from $1.81\mu\text{g}$ DNA/well to $2.17\mu\text{g}$ DNA/well, a 20% increase which can be attributed to increased cell number. However, the increase was not statistically significant.

If one compares the response to EGF by both control and GA BeWo cells in comparison to untreated BeWo cells (Figure 3.4), then one can see that at an initial plating density of $0.5\mu\text{g}$ DNA the control cells proliferate 31% more than control and the GA cells by 34%; at an initial concentration of $1\mu\text{g}$ DNA the control cells proliferate by 31% and the GA cells by 46%. The cells plated out at $1.5\mu\text{g}$

DNA/well initially, show the opposite effect with the non-GA cells proliferating up to 52% and the GA BeWo cells proliferating slightly less at an increase of 38%. The increase in proliferation of both control and GA cells at 1 and 1.5µg initial DNA/ well were statistically significant ($p<0.05$) when compared with their respective controls on application of Student's t-test.

In conclusion, it appears Growth Arrested (GA) BeWo cells respond better to EGF than those which have been supplemented with serum prior to harvesting, with 1µg DNA seeming to be the most suitable initial plating density for the BeWo cell line. The decrease in response to EGF by the GA BeWo cells at 1.5µg DNA/well could be due to the fact that at such high levels of DNA/well and hence greatly increased cell number, the cells had neared confluence and therefore their growth rate would be inhibited.

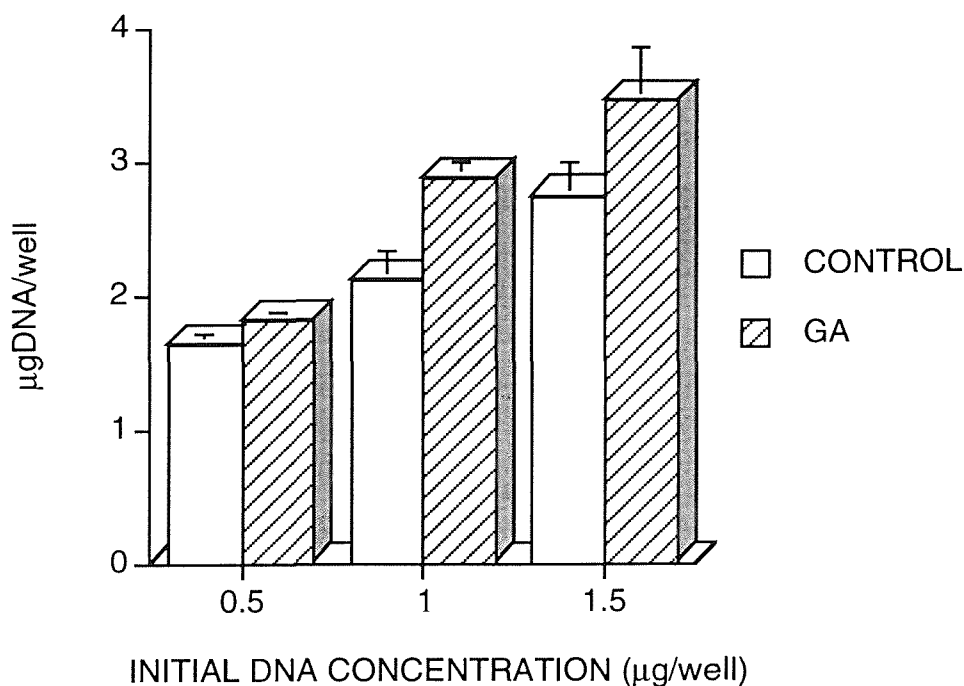


Figure 3.1. The effect of growth arresting (GA) BeWo cells.

A comparison of control and GA BeWo cells at three different initial DNA plating densities - 0.5, 1 and 1.5µg DNA/well. Cells were cultured for 72h in 2.5%SFCS in 24 well plates in 5% CO₂ in air, either with or without serum deprivation 24h prior to sub-culturing. Proliferation was assessed by DNA assay and the results are expressed as mean µg DNA/well for triplicates ± s.e.m. Statistical analysis was performed using Students' t-test where $*=p<0.05$ compared with the equivalent control, (n=3), but results were not statistically significant in this experiment.

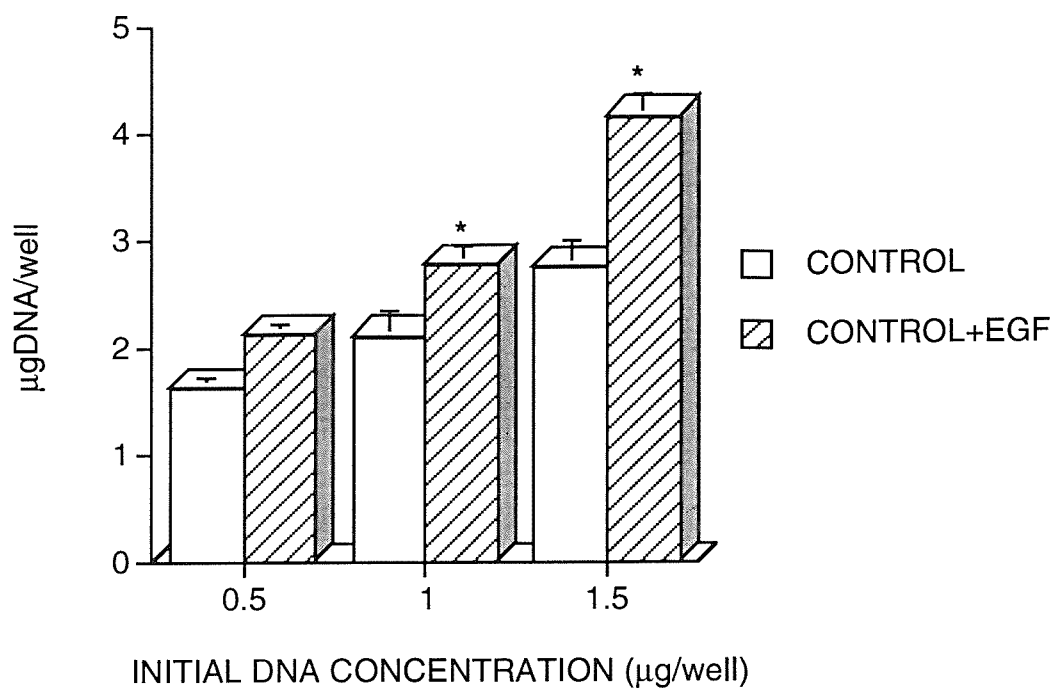


Figure 3.2. The effect of EGF upon control BeWo cells at differing initial DNA concentrations

BeWo cells were grown in 24 well plates for 72h in 2.5% SFCS and 5% CO₂ in air, either with or without 5ng/ml hEGF. The differences between EGF treated and untreated BeWo cells are shown at three different initial DNA plating densities - 0.5, 1 and 1.5µg DNA/well. Proliferation was assessed by DNA assay and the results are expressed as mean µg DNA/well for triplicates \pm s.e.m. Statistical analysis was performed using Students' t-test where $\ast=p<0.05$ compared with the equivalent control, (n=3).

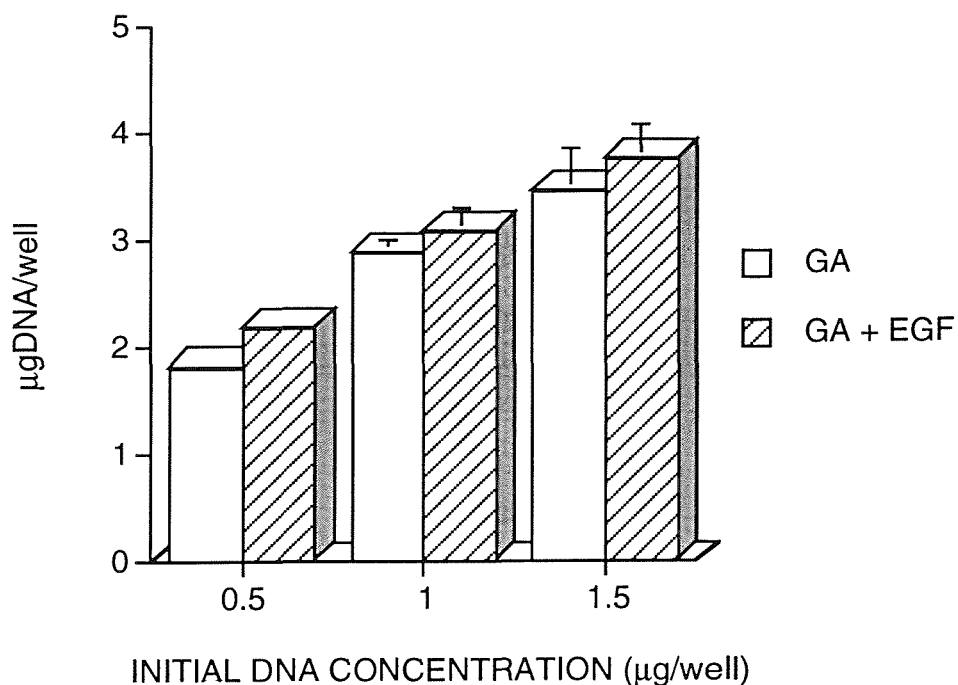


Figure 3.3. The effect of EGF upon growth arrested BeWo cells.

BeWo cells were growth arrested (GA) (by serum deprivation) 24 prior to harvesting and recultured in 24 well plates for 72h in 2.5% SFCS and 5% CO₂ in air, either with or without 5ng/ml hEGF. The differences between EGF treated and untreated BeWo cells are shown at three different initial DNA plating densities - 0.5, 1 and 1.5µg DNA/well. Proliferation was assessed by DNA assay and the results are expressed as mean µg DNA/well for triplicates \pm s.e.m. Statistical analysis was performed using Students' t-test where $\ast=p<0.05$ compared with the equivalent control. (n=3).

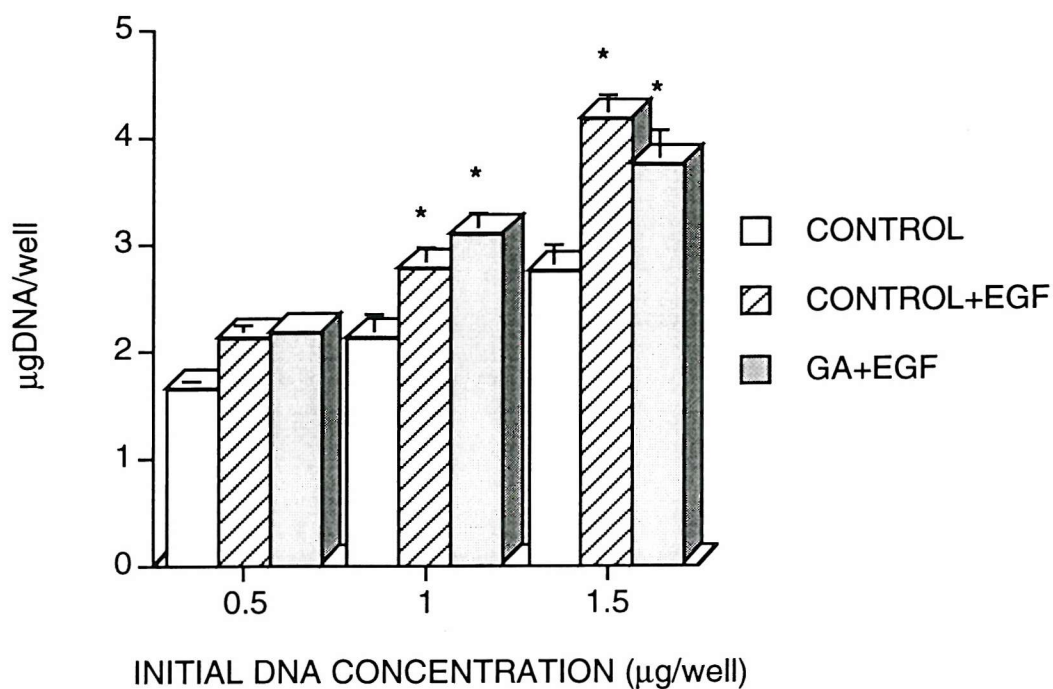


Figure 3.4. A comparison between the response of control and growth arrested (GA) BeWo cells to EGF.

BeWo cells, either control or GA, were grown in 24 well plates for 72h in 2.5% SFCS and 5% CO₂ in air either with or without 5ng/ml hEGF. The differences between EGF treated and untreated, GA or control BeWo cells are shown at three different initial DNA plating densities - 0.5, 1 and 1.5µg DNA/well. Proliferation was assessed by DNA assay and the results are expressed as mean µg DNA/well for triplicates \pm s.e.m. Statistical analysis was performed using Students' t-test where $\ast = p < 0.05$ compared with the equivalent control. (n=3).

3.4.2. Comparison of control and EGF treated BeWo cells at different densities by Crystal Violet Dye Elution (CVDE).

BeWo cells were GA 24h prior to being sub-cultured into 24 well-plates. Varying initial DNA concentrations ($\mu\text{g}/\text{well}$) were set up and grown for 72h in 2.5% SFCS, 5% CO_2 in air at 37°C - either with or without exposure to EGF (5ng/ml).

After 72h the cell number was assessed by incorporation of Crystal Violet Dye and the amount of dye taken up by the cells measured on an Argus plate reader (see section 2.4 for detailed method). The absorbances were converted to CV absorbed/ μg DNA and subsequently transformed into a percentage of the control untreated cells. This was to allow comparison between two different experiments where the absorbance was markedly different yet the observed trend was the same.

Figure 3.5 shows increasing initial DNA plating densities for the BeWo cells against the amount of CV Dye absorbed/ μg DNA and this is displayed as a percentage of the control value. The data are the mean of two experiments and shows that with increasing initial DNA concentration, the amount of dye incorporated into the BeWo cells also increases e.g. from 0.5-2 μg the absorbance is increased by 76%. The relationship is not linear and appears to saturate at 1.5-2 μg initial DNA. It is clearly shown that the addition of 5ng/ml EGF results in an increase in crystal violet dye incorporation across all initial DNA plating densities by an average 66%. The errors are somewhat larger than those observed for the DNA assay and it should be noted that the CV assay results were, on the whole, inconsistent. However, the general trends were similar to those observed in the DNA assay. The results were not statistically significant.

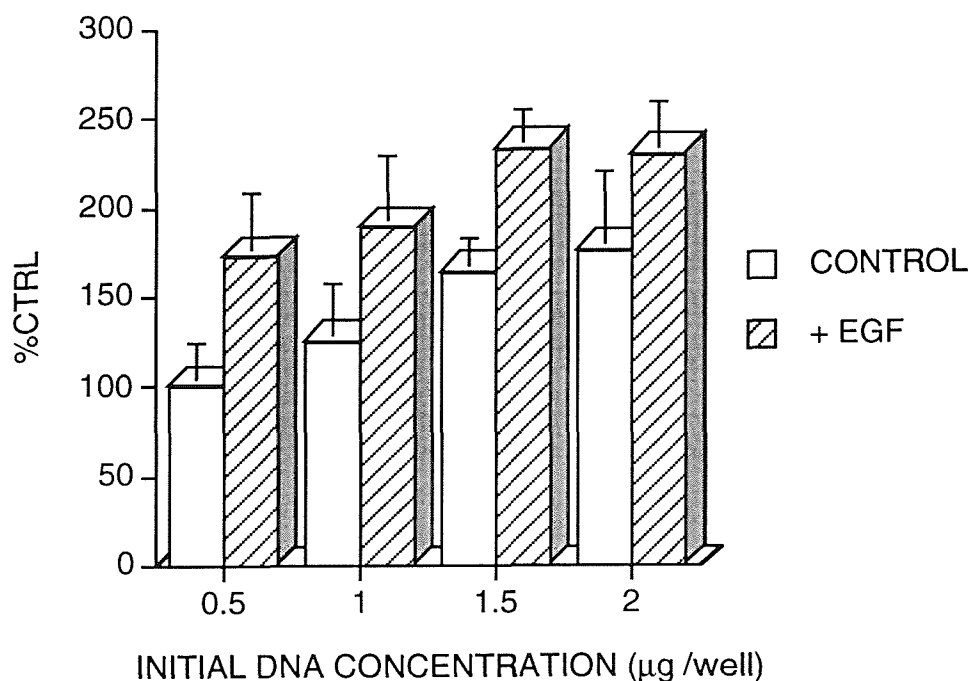


Figure 3.5. Incorporation of Crystal Violet Dye into BeWo cells at differing DNA concentrations in the presence/absence of EGF.

BeWo cells were growth arrested 24h before harvesting and sub-cultured at different initial DNA concentrations (0.5-2µg/well) in 24 well plates in 2.5%SFCS, 5%CO₂ in air, at 37°C for 72h in the presence or absence of 5ng/ml EGF. At the end of the culture period, increase in cell number was assayed (in triplicate) by Crystal Violet Dye Elution (see section 2.4) and the data expressed as the mean percentage of the control untreated cells. Statistical analysis was performed on the data but the number of replicates were insufficient for any significant conclusions to be made (n=2).

3.4.3. *MTT and 3H-Thymidine incorporation.*

The results from the thymidine incorporation assay were inconclusive and the replicates inconsistent (data not shown) therefore this assay was deemed unsuitable for the BeWo cell-line. The MTT assay indicated similar trends to those seen with both the CV and DNA assays but again, the results were equivocal and are not shown here.

3.4.4. *Growth rates of BeWo, JAr and Jeg-3 as determined by DNA assay.*

Figure 3.6. indicates the rates of growth of JAr, BeWo and Jeg-3 plated out in the appropriate media (see section 2.1) at differing initial DNA concentrations after 72h culture, in 2.5 % SFCS, 5% CO₂ in air at 37°C. After 72h the cells were assayed, in triplicate, for their DNA content (n=2). Increasing initial plating densities of DNA (µg/well) are shown against mean final DNA concentrations (µg/well) ± s.e.m.

From the data it is clear that, under our culture conditions, all three cell-lines proliferate readily. The rate of growth varies between cells with the JAr cells proliferating most rapidly, BeWo proliferating the slowest with Jeg-3 lying in between the two. In Fig. 3.6.a) the JAr cell-line plated at 2µg initial DNA/well had increased to 6.8µg DNA/well, over a 3 fold increase, with the cells approaching confluence. Clearly an initial plating density of 1µg DNA/well or above is unsuitable for this rapidly proliferating cell-line because as cells in culture near confluence their responsiveness to growth factors is diminished. Therefore an optimal plating density of 0.5µg/well initial DNA was selected for the JAr cell line for further experiments.

For the Jeg-3 cells, indicated in Fig 3.6.c), 1.5µg/well initial DNA had grown to 5.4µg/well after 72h, again indicating that this would be too high an initial plating density. Therefore a half-maximal plating density was chosen of 0.5µg DNA/well yielding 1.7µgDNA/well after 72h. Similarly for the BeWo cell line, shown in Fig 3.6.c), 1µg initial DNA was chosen as the initial plating density as at 1.5µg DNA /well (initial) the cells had increased to 5.3µg DNA/well after 72h which is too high and therefore a lower initial plating density was chosen.

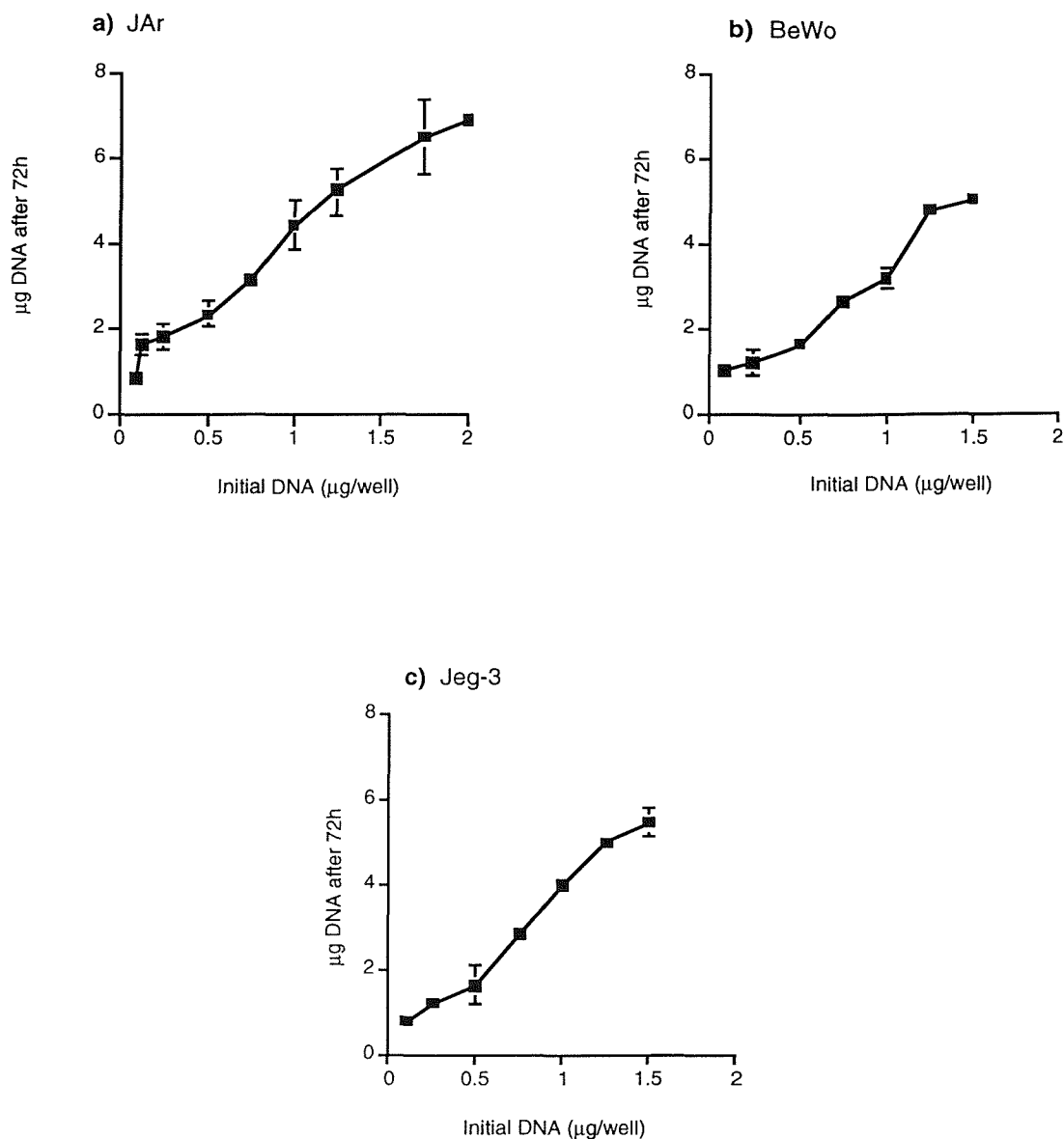


Figure 3.6. The comparative growth rates of JAr, BeWo and Jeg-3 cells.

a) JAr, b) BeWo and c) Jeg-3 choriocarcinoma cell-lines were growth arrested 24h prior to use and sub-cultured at varying initial DNA concentrations (0.125-2µg DNA/well) in the appropriate media (see section 2.1). Cells were grown for 72h in 2.5% SFCS, 5%CO₂ in air at 37°C after which time they were assayed for DNA content in triplicate. The results are the mean of two experiments (assayed in triplicate).

3.4.5. Comparison of dose response curves of BeWo to EGF as determined by three assay systems.

BeWo choriocarcinoma cells were set up as outlined previously and exposed to increasing concentrations of EGF (0-20ng/ml) for 72h and subsequently assayed for their DNA content, the incorporation of CV dye and the ability to oxidise MTT (an indicator of mitochondrial activity).

Figure 3.7 shows a comparison between three assay techniques in the BeWo cell line. For each assay system used, the data were transformed to % control/ μ gDNA (mean \pm s.e.m.) and plotted against increasing concentrations of EGF, where $n=3$. The data were transformed in such a way as to enable comparisons to be made among the three assays. 100% is proportional to 1.54 μ gDNA/well for the DNA assay and 0.6 CV Abs/ μ g DNA and 0.11 MTT / μ g DNA for the CV and MTT assays, respectively.

The cells were exposed to increasing concentrations of hEGF (0-20ng/ml) for 72h after which time they were analysed by DNA, MTT or CV assay (see section 2.2, 2.3, and 2.4 for methodologies).

The DNA assay produced a bell-shaped dose-response curve with a maximum significant response ($p<0.05$, Student's t -test) to EGF at 5ng/ml (20%). Although stimulation of proliferation was observed at all EGF concentrations this was not significant. The CV and MTT assays showed a similar stimulation at 5ng/ml but this was not significant in either assay. The latter dose response curves were dissimilar from the DNA dose-response curve, with EGF at higher doses appearing to cause an inhibition of cell number.

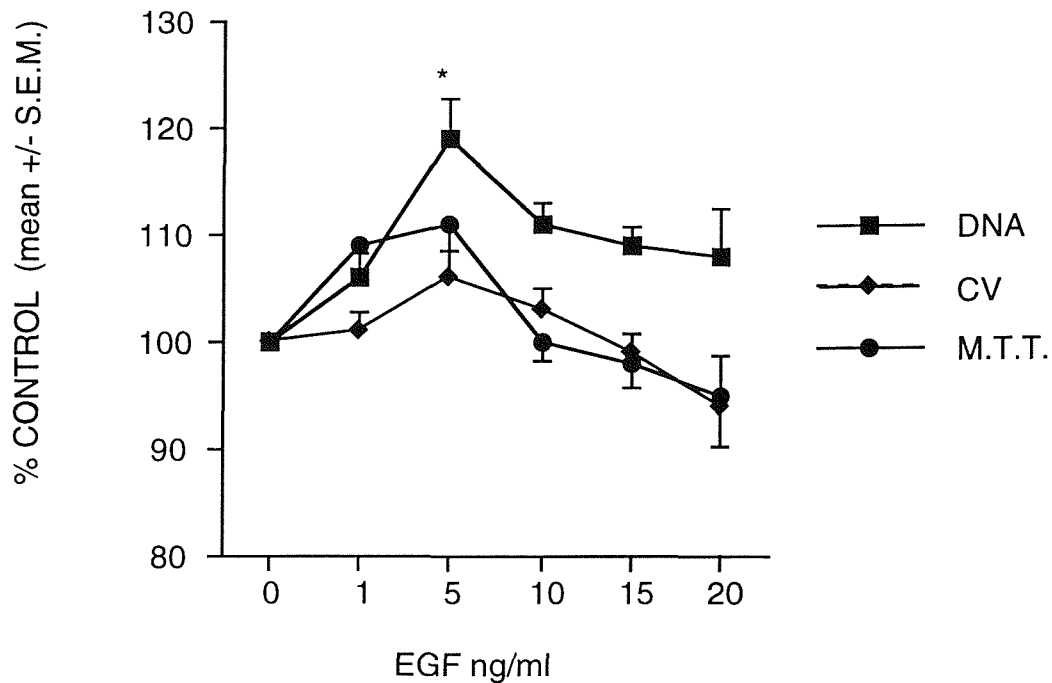


Figure 3.7. Dose response curve of BeWo to EGF analysed by DNA, CV and MTT assay.

Proliferation of the trophoblast cell line BeWo in response to EGF. Cells were plated at $1\mu\text{g}$ DNA/well into 24 well plates and cultured for 72 hours with hEGF (0-20ng/ml). Cell proliferation was measured as an increase in DNA/well (a) or increase in crystal violet absorption (b) or (c) MTT metabolism. In all cases, EGF caused a 10-20% increase in the apparent number of cells, over control values (in the absence of EGF). Statistical analysis was carried out on the data using a Student's t-test ($*=p<0.05$).

3.4.6. The effect of EGF upon proliferation of JAr, BeWo and Jeg-3 cell-lines.

JAr, Jeg-3 and BeWo cell-lines were cultured at initial DNA concentrations of 0.5µg DNA/well, 0.5µg DNA/ well and 1µg DNA/ well, respectively for 72h in the appropriate culture medium supplemented with 2.5%SFCS and grown in 5% CO₂ in air at 37°C. The dose response curves for increasing concentrations of EGF (0-20ng/ml) for all three cell lines, following DNA assay, are shown as mean µg DNA ± s.e.m. expressed as a percentage of control. All assays were carried out in triplicate and analysed statistically by Student's t-test (n=3).

For JAr, BeWo and Jeg-3 the value of 100% can be interpreted as the amount of DNA present after 72h growth in the absence of any EGF and was 2.32, 3.24 and 1.89µg DNA/well, respectively, bearing in mind that the initial plating density of the BeWo cell line was double that of the other two.

The BeWo cell-line displayed the same trend as observed earlier, i.e. a bell-shaped dose-response curve, with a significant maximal stimulation at 5ng/ml EGF ($p<0.05$). JAr cells proliferated significantly (*= $p<0.05$, **= $p<0.005$, ***= $p<0.001$) in response to EGF at all concentrations and yielded a bell-shaped dose response curve, not dissimilar from that observed in BeWo. However, the stimulatory effect of EGF upon growth was remarkably more so than that seen in BeWo with a maximal stimulation at 5ng/ml of almost 90% ($p<0.001$), with concentrations in excess of this having a lesser effect. Clearly this cell-line was proliferating rapidly and readily in response to EGF at all concentrations. Conversely, the Jeg-3 cell-line appeared to show no significant proliferative response to EGF, in any assay system, at any concentration.

In summary, from the DNA assay data it is clear that:

- a) EGF causes increased proliferation of BeWo cells at all concentrations when expressed as a percentage of control with a significant 26% increase in growth at 5ng/ml ($p<0.05$);
- b) EGF causes a marked, significant increase in proliferation of JAr cells at all doses. Growth is potentiated up to 90% in the presence of EGF (5ng/ml) ($p<0.001$) and the resulting dose response curve is bell shaped.
- c) EGF does not appear to have any effect upon the proliferation of Jeg-3 cells in culture.

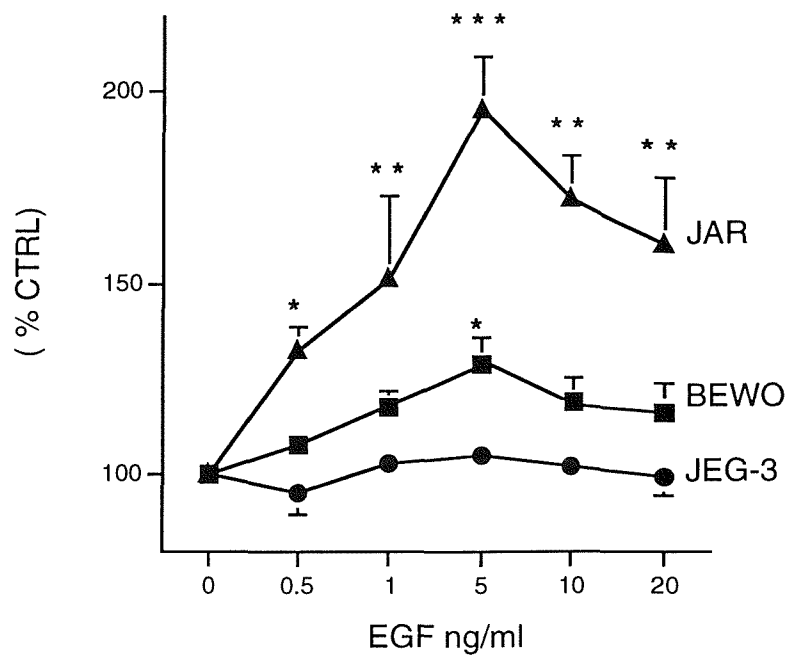


Figure 3.8. Dose response curves for JAr, BeWo and Jeg-3 in response to EGF.

JAr, BeWo and Jeg-3 cells were grown for 72h in 24 well plates, 5% CO₂ in air, 2.5% SFCS at 37°C in the appropriate media (section 2.1) with the addition of various concentrations of hEGF (0-20ng/ml). Initial plating densities were 0.5µg DNA /well for JAr and Jeg-3 and 1µg DNA/well for the BeWo cell line. After 72h the cells were assayed for DNA content in triplicate and the data are expressed as a mean percentage of control \pm s.e.m. For JAr, BeWo and Jeg-3 100% is equivalent to 2.32, 3.24 and 1.89µg DNA/well respectively. Data were analysed using Student's t-test (*P<0.05 **P<0.01 ***P<0.001), n=3 for Jeg-3 and BeWo and n=6 for JAr.

3.5. Discussion.

The establishment of pure, long-term cultures of differentiating placental trophoblast cells is extremely difficult to achieve. Various methods have been developed to isolate, purify and culture human trophoblast (Loke *et al*, 1983; Kliman *et al*, 1986). Fisher *et al* managed to isolate cytotrophoblasts from first trimester placenta which maintained a certain amount of mitogenic and invasive properties (Fisher *et al*, 1989). The presence of serum, divalent cations, and association with adhesion molecules have been shown to induce aggregation of isolated cytotrophoblasts to form a syncytium in vitro (Kao *et al*, 1988; Babalola *et al*, 1990; Coutifaris *et al*, 1991).

A major pitfall in the isolation of trophoblast is the risk of contamination by other cells e.g. placental fibroblasts, which will not only proliferate much more rapidly than the trophoblast but as a result may give false positive results. i.e. the effect observed originates from the fibroblast phenotype rather than trophoblast.

The advantage in using transformed choriocarcinoma cell lines is that they represent a homogenous population of pure trophoblast, replicate readily in culture, can be cloned easily if required, and retain similarities with non-transformed trophoblast cell lines. Several choriocarcinoma cell-lines have been used in previous work because a) morphologically they resemble cytotrophoblasts, b) they are capable of adhering to and degrading ECM and c) they express a variety of different receptors (Aplin, 1991).

The three cell lines employed in this thesis have been widely used to look at various interactions in implantation as outlined in Table 3.1.

Table 3.1. The use of choriocarcinoma cell-lines as experimental models.

Cell Line	Experimental use
BeWo	Cell migration (Aplin and Charlton 1990); Invasion model (Kliman <i>et al</i> 1990); Invasion of uterine blood vessels (Aplin, 1991); Expression of ECM components (Fisher <i>et al</i> 1989).
JAr	Blastocyst outgrowth (White <i>et al</i> 1988);
Jeg-3	Effects of Interferon (Toth <i>et al</i> 1991); hCG production (Bahn and Speeg, 1981) Effects of TNF α (Feinberg <i>et al</i> 1994)

The role of serum in the control of cell multiplication remains something of a mystery with work by Robert Holley on 3T3 cells (1975) providing some answers. He noted that if 3T3 cells were placed in culture with serum, they multiplied; then the population plateaued and entered quiescence. (Quiescence is the phenomenon whereby cells cease to proliferate yet retain the capacity to divide further - see diagram 3.1).

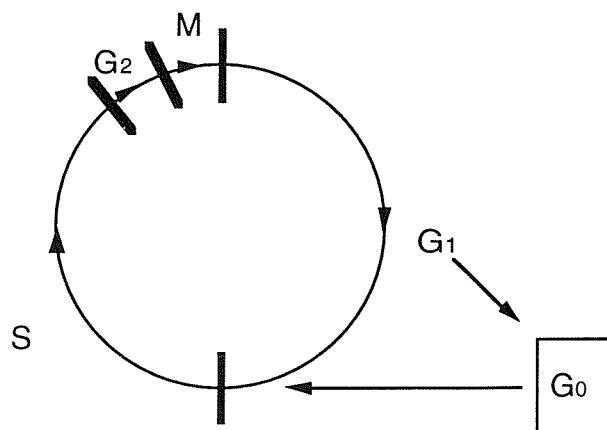
Furthermore, the amount of division prior to quiescence was directly related to the amount of serum present and, more importantly, the addition of fresh serum could 'kick-start' the cells into the cell-cycle, causing them to multiply further. This implies that serum contains substances which induce the cell to multiply and in doing so are used up in the process.

As an experimental tool, the removal of serum from cultured cells will arrest their growth at G1, causing them to enter G₀ and hence the cell-population will become synchronised. Thus, any effects observed by addition of external growth factors can be said to be acting at the same point in the cell cycle i.e. the presence or absence of serum can regulate entry and exit from the cell cycle in a controlled fashion. Cells only appear to require serum whilst in the G1 phase and will still proceed into the cell cycle if serum is withdrawn (Pardee, 1974) after a period corresponding to a few hours before the onset of S-phase.

From the growth arrested data (section 3.4.1.), one can see that serum deprivation of the BeWo cells for 24h prior to harvesting enhanced both their subsequent proliferation and their proliferative response to EGF. This may be due to the fact that having been halted at the same restriction point, the GA cells were 'primed' and on exposure to 2.5% SFCS rapidly underwent cell division. The control cells, on the other hand, would have been at various stages of the cell cycle and although they still proliferated, the overall effect was not as marked as that seen in the GA cells.

Crystal Violet Dye Elution proved to be difficult to assess for these cell lines. The results were inconsistent with the previous two assays and were often erratic. One of the problems encountered was that the assay seemed to 'saturate' readily, even with initial DNA concentrations as low as 0.75µg. This draw-back has been encountered by colleagues in the laboratory (F. Safadi, personal communication) and could be overcome by reducing the initial cell density. However, the JAr cells for example, proliferate so rapidly as to exceed the saturation point for the assay

after 24 h, at which time the effect of EGF may not be observed. Cells plated at too low a density initially will in fact not grow and therefore the C.V. assay is deemed unsuitable for these cells. The exception of course being the BeWo cells which, due to their slow growth rate, could be assessed using this method. The results obtained by C.V. for the BeWo were in accordance with the DNA and MTT assay although the consistency between replicates was poor.



G0=Non-proliferating cells which can re-enter the cell-cycle
G1=Interphase period prior to DNA synthesis (cells increase in size)
S =DNA synthesis (6-8h)
G2=Period prior to mitosis (2h)
M =Mitosis

Diagram 3.1. The Cell Cycle.

At G₀ the cell is quiescent. A signal allows it to enter the G₁ phase and to proceed to DNA replication and mitosis resulting in two daughter cells.

Subsequent literature searches have lead me to believe that crystal violet is in fact not specifically incorporated into the cell membrane, but is capable of interacting with other cellular components e.g. intercalation in DNA (Fox *et al*, 1992).

Therefore, it is difficult to assess what one is actually measuring with this assay. In addition, changes in cell size and hence lipid bilayer surface area could mislead one's interpretation of an increase/decrease in dye incorporation.

The MTT assay produced general trends that were the same as that for the DNA results but the margin of error was greatly increased and no conclusive results could be drawn from the data. A recent publication has emphasised the error of concluding 'false' positive results from the MTT assay (Rollino *et al*, 1995) due to interference from serum proteins such as heparin. This is overcome in our assay by using stripped FCS and by incorporating a cell-free control to be subtracted from optical density.

Tritiated thymidine incorporation was also tried out as a suitable method for analysing the cell growth, however, this failed to produce any consistent results (although more recent work has shown that the ^3H -thymidine assay and DNA assay give virtually identical results with the JAr cells (Umar, personal communication) .

The reason that so many of the assay systems may have failed to produce convincing results can be attributed to the fragile nature of the trophoblast cells. The MTT, CV and ^3H assays all involve several washing steps and it was apparent when conducting the experiments that cells were being lost in the assay procedure. One could see quite clearly, with the naked eye, cells being dislodged from the base of the plate and floating away! Obviously, this produces extremely erratic and misleading results and therefore the three alternative assays were relinquished in favour of the DNA assay, which provided consistent replicates.

In an attempt to counter the fragility of the cells the percentage serum can be increased but this introduces new problems in that endogenous serum components may not only mask the effects of the growth factor being investigated, but will cause the cells to proliferate too readily and reach confluence prior to analysis.

DNA content was preferred over protein assay for several reasons. Firstly, the in-house assay system for measuring DNA is quick, sensitive and produces reliable replicates. Secondly, the presence and turnover of proteins in the supplemented culture medium could give misleading results. Thirdly, the Bradford/Lowry method depends upon the presence of tyrosine and phenylalanine residues and will give underestimates if there are few of these amino acid residues present. As an estimate, $1\mu\text{g}$ DNA is proportional to 1×10^6 cells.

The fastest growing cell line, under our culture conditions was the JAr cell-line, followed by the Jeg-3 with BeWo growing the slowest. Light microscope studies showed quite different patterns of growth between the three types: BeWo cells seemed to send out individual cells across the plate which were capable of surviving independently from the larger cell colonies; JAr cells tended to proliferate rapidly and form large colonies with the cells piling up on top of one another before reaching confluence; and Jeg-3 tended to favour the monolayer configuration and did not form the cell clumps seen in JAr. Clearly, despite their

common origins the cell-lines displayed quite different phenotypes and these differences were echoed when the cells were treated with EGF.

Four members of the EGF family are found in large amounts in uterine tissue in the peri-implantation period - EGF, TGF α , HB-EGF and amphiregulin (Cross, *et al* 1994) and are reported to be secreted in response to the actions of oestrogen upon the uterine epithelium (Das, *et al* 1994). Several lines of evidence suggest that EGF is involved in human pregnancies (Chegini and Rao, 1985; Rao, *et al* 1985; Chen, *et al* 1988) with much of the data coming from binding studies (see Chapter 4). Local expression of EGF within the placenta has been suggested (Bissonnette, *et al* 1992) and immunohistochemistry has shown that EGF immunoreactivity is predominantly localised to the syncytiotrophoblast (Hoffman, *et al* 1992). More recent work by Amemiya *et al* has shown that EGF mRNA is expressed at all stages of trophoblast differentiation (1994).

It is uncertain whether TGF α plays an important role in implantation, as mice lacking a functional TGF α gene are still able to maintain successful implantation (Luetkeke, *et al* 1993), yet it is present in large amounts in uterine tissue (Han, *et al* 1987) and is also (along with HB-EGF) expressed in macrophages (Derynck, *et al* 1988; Higashiyama, *et al* 1991).

HB-EGF is expressed only in macrophages (Higashiyama, *et al* 1991) and in the luminal epithelium at the site of apposition of the blastocyst about 7 hours prior to attachment. However, during delayed implantation it is not expressed but on injection of oestrogen its expression is rapidly induced (Das, *et al* 1994).

Previous work has emphasised a differentiative role for EGF in implanting and invading trophoblast (Genbachev 1988), but it is clearly acting as a mitogen in the JAr and to a lesser extent in BeWo. This is in agreement with Truman and Ford (1986) who showed that EGF caused proliferation of cultured human trophoblast and encouraged the formation of a continuous monolayer.

EGF is a potent mitogen in many cell types (Van de Geer, *et al* 1994) and exerts its effects by activation of the many intracellular pathways triggered by substrate phosphorylation. As discussed in Chapter 1, activation of the EGFR by EGF leads to a number of biological events, including relatively rapid events such as tyrosine phosphorylation of a number of cellular substrates including the EGFR

itself, activation of ion transport systems such as induction of calcium influx, and after a few hours, the induction of DNA synthesis followed by cell division.

Clearly the Jeg-3 cells, in the presence of EGF, showed no increase in proliferation yet they do express EGF receptors and one would therefore expect some sort of response. It appears that EGF is not acting as a mitogen in this cell-line. In fact, it has previously been shown that the effect of EGF upon Jeg-3 leads to increased production of hCG and has little or no effect upon their growth (Bahn *et al* 1981). It would be interesting to measure any changes in hCG levels between the three cell-lines following stimulation with EGF.

It is evident that EGF is capable of eliciting pleiotropic responses in the choriocarcinoma cell -lines yet how this is achieved is unknown. Several explanations can be put forward :

i) Activation of different intracellular signalling pathways. Cellular substrates which display high affinity receptor tyrosine kinase association include PLC-gamma, PI-kinase, c-raf and ras-GAP (Wahl, *et al* 1989; Coughlin, *et al* 1989; Ellis, *et al* 1990). There is very good experimental evidence that activation of PKC by PLC-gamma mediates mitogenic responses (Nishizuka, 1988; Coussens, *et al* 1986) and PKC activation has been identified as a significant event for the induction of DNA synthesis in quiescent cells (Heath, 1993). Stimulation of the MAP kinase pathway, through ras, activates transcription factors which could not only play a role in the mitogenic responses of JAr and Jeg-3 to EGF, but could also be responsible for the specific transcription of hCG in the Jeg-3 cells. A more recent nuclear signalling pathway is beginning to emerge involving the Jak non-receptor PTK family and the Stat (signal transducer and activator of transcription) transcription family. EGF activates Jak 1 (Shuai, *et al* 1993) and Stat 91 binds to the EGFR via its SH2 domain (Fu and Zhang, 1993) and these in turn bind to a response element called GAS (IFN-gamma activated site) (Lew, *et al* 1991). If, for example, the hCG gene is GAS-regulated then the occurrence of Jak/Stat pathways in Jeg-3 and not JAr and BeWo would confer the different behaviour in response to EGF because an important feature of the Jak/Stat pathway is that it is ras independent (Silvennoinen *et al*, 1993). Diagram 3.2. shows several pathways that can be stimulated upon receptor binding and it is clear that activation of the EGFR provides enormous capacity for activating pleiotropic signalling paths which in turn could lead to a multitude of biological responses.

The presence and recruitment of SH2 proteins (Grb2, Sos, etc.) and different adapter proteins (CRK, NCK etc.) can also increase signal diversity.

It is difficult to predict which intracellular pathways will be activated because SH2 domains have some overlapping binding specificity and can bind more than one consensus sequence and it seems likely that co-operation between pathways is important in EGFR signalling.

ii) In addition the strength and duration of the signal can affect the outcome of receptor activation. For example, transient activation of the Ras/MAP kinase pathway in PC12 cells results in cell growth. However, if there is persistent activation the cells differentiate (Traverse *et al*, 1992).

iii) Receptor Homodimerisation/Heterodimerisation - EGFR can exist as a monomer, homodimer or as a heterodimer with other members of the type 1 TKR e.g. erbB-2, erbB-3, erbB-4. Signal diversity can be increased by heterodimerisation as outlined in Chapter 1. The differences observed between the cell-lines could therefore be due to the differential expression of receptors e.g. in the JAr, and to a lesser extent in the BeWo cell-line, EGFR-EGFR could be the predominant combination whereas in Jeg-3 EGFR-erbB-2 is favourable. In the trophoblast, expression of EGFR has been localised, by immunohistochemistry with an anti-EGFR antibody and Ki67 (a marker of proliferation) to the proliferative cytotrophoblast cells with erbB-2 being absent (Muhlhauser *et al*, 1993).

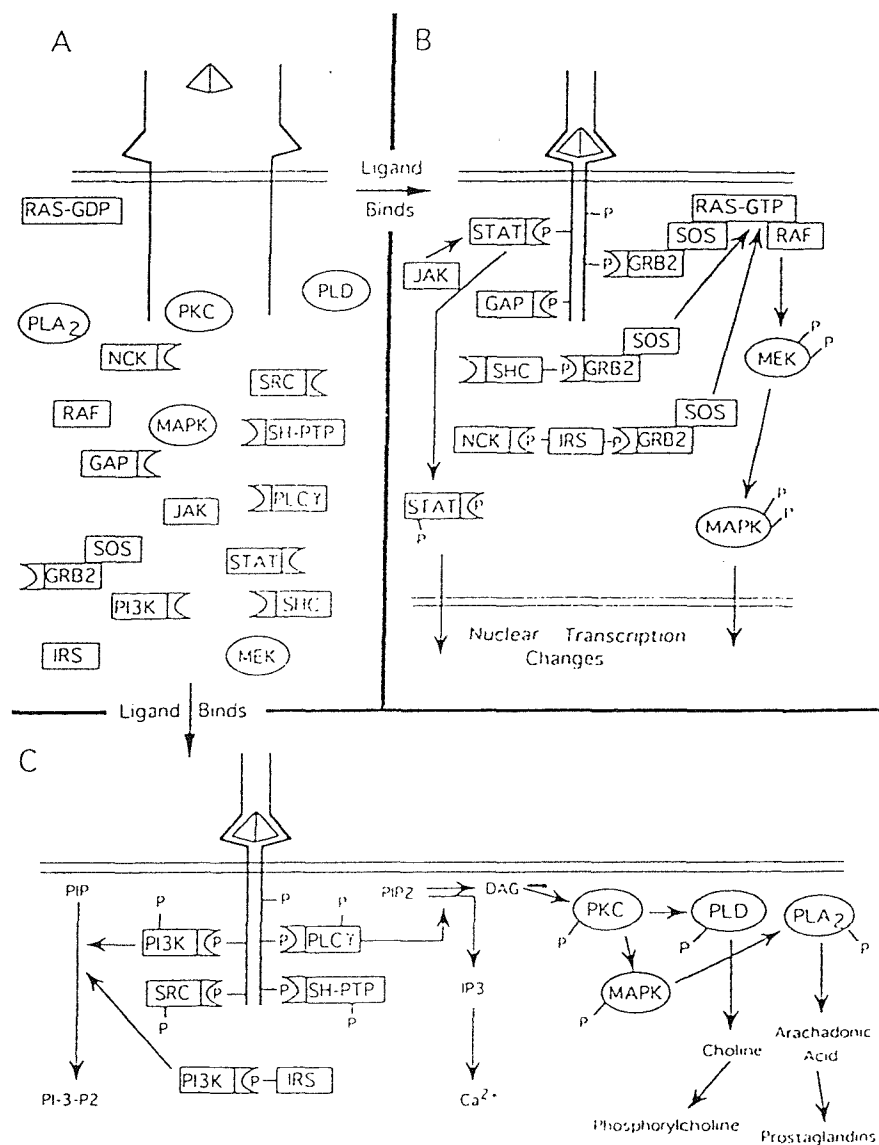


Diagram 3.2. Cartoon of Receptor Protein Tyrosine Kinase (RPTK) signalling pathways.

Several pathways that become stimulated upon RPTK activation are shown. (A) In an unstimulated cell, the EGFR exists as a kinase -inactive monomer, is not tyrosine phosphorylated and has few associated proteins. (B) Following ligand binding, EGFRs dimerise and autophosphorylate leading to target protein association and/or phosphorylation. The ras and stat pathways lead to transcriptional changes. (C) EGFR activation stimulates many additional pathways that involve phospholipid messengers. (Taken from Van de Geer *et al*, 1994)

Conversely, erbB-2 is only expressed as the cytotrophoblast differentiates and loses the ability to proliferate (Muhlhauser *et al* 1993). Similar studies have shown that as villous trophoblasts migrate off their basement membrane to form cell columns they lose their EGF-R and express erbB-2 instead and co-expression of both receptors is seen in terminally differentiated trophoblast populations (Jokhi *et al* 1994). Therefore, EGFR and erbB-2 seem to follow a reciprocal expression pattern with the EGFR appearing to play an important role in trophoblast proliferation whereas erbB-2 may be important for trophoblast invasion and differentiation.

The preliminary work outlined in this chapter contributes somewhat to an understanding of EGF and its receptors role in the choriocarcinoma cell lines but clearly underlines how little is known about the role of growth factors in the placenta. Nevertheless, I have shown that EGF can positively influence trophoblast cell growth in the JAr and BeWo choriocarcinoma cell lines , which may reflect the situation in vivo - an important concept when dealing with both normal pregnancy and with pregnancy failure.

CHAPTER FOUR

CHARACTERISATION OF EGF BINDING TO ITS RECEPTOR USING RADIORECEPTOR-LIGAND BINDING ASSAYS.

4.1. Introduction.

Epidermal growth factor (EGF) mediates its mitogenic response through association with a specific membrane bound receptor - the Epidermal Growth Factor Receptor (EGFR). The binding of EGF to the extracellular domain of the EGFR activates its cytoplasmic tyrosine kinase that not only autophosphorylates the receptor itself but also phosphorylates various cellular proteins (Downward *et al*, 1984).

Following binding of the receptor on target cells an array of biological responses are triggered including both short term and long term effects. Short term effects initiated within minutes include ruffling of the plasma membrane (Chinkers *et al*, 1979). The long term effects e.g. DNA, RNA and protein synthesis, require continuous occupancy of the receptor by the ligand by up to as much as 12-16h (Hollenberg and Cuatrecasas, 1973).

Since the response to EGF is determined, to some extent, by the number of receptors occupied, the magnitude of the effect is likely to depend not only upon the concentration of the ligand but on the number of available functional receptors.

Previous workers have looked at EGF binding to its receptor in midterm and term placental cell cultures and human placental membranes (Lai and Guyda, 1984; Hock and Hollenberg, 1980). Because human cytotrophoblast cell cultures differentiate into a syncytium after 48h in culture, the binding capacity also changes (Alsat *et al*, 1993) and therefore it is difficult to assess the effects of other growth factors/cytokines on EGF binding. Human placental membranes have been widely used in ligand-binding studies as they provide a plentiful source of EGFR but it is equivocal whether the receptors in membrane preparations behave as they would in whole cell cultures (Yarden and Schlessinger, 1985).

I thought it would be valuable to characterise the binding of EGF in a choriocarcinoma cell line so that factors regulating either a) the affinity of EGF for its receptor or b) the actual number of receptors could be studied, in stable whole cell cultures, with ease.

In the previous chapter I observed a difference in the effect of EGF upon cells of the same origin. It was my intention to investigate the binding affinities and receptor number in each cell line to see if any differences in response to EGF could be attributed to differences at the level of receptor binding.

In this chapter, I have investigated EGF binding to its receptor in human placental membranes, JAr, BeWo and Jeg-3 cells. The binding constants and the absolute receptor numbers per cell have been determined. This provides a standard for the basis of future work where regulation of the EGFR by other growth factors can be investigated.

Prior to carrying out ligand-binding studies it was necessary, firstly, to determine that the iodinated ligand was behaving as if it were un-labelled; secondly, to determine the specific activity of the labelled peptide; and thirdly, to optimise the binding conditions. Once these criteria were met, then characterisation of the receptor-ligand binding could be undertaken.

4.2. Aim.

The main objectives of this chapter were, firstly, to radiolabel EGF to a high specific activity whilst still retaining the behaviour of the natural ligand. Once this had been achieved I wanted to determine the optimal binding conditions (temperature, time, pH, receptor density) for the JAr cell-line so that maximal binding would be obtained; thus characterisation of EGF binding to its receptor in all three cell lines could be achieved.

4.3. Materials and Methods.

Unless otherwise stated, as described in section 2.10.

4.4. Results.

4.4.1. *Determination of the specific activity and ligand equivalence of iodinated EGF in human placental membranes (HPM).*

Human placenta provides a convenient source of membranes (HPM) which bind EGF in a specific manner (O'Keefe *et al*, 1974; (Hock and Hollenberg, 1980). The membranes are stable for at least 3 months at -20°C.

Two types of binding experiment were performed. The first set provided the saturation curve whereby 2µg HPM/eppendorf were incubated with 50µl increasing concentrations of ¹²⁵I-EGF (0.5,1,2,3,5,10nM) and 200µl binding buffer - 25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin - overnight at 4°C (Non-specific binding was determined in the presence of 50µl 200nM excess cold EGF and 150µl binding buffer.). In the second experiment, which provided the competitive binding curve, 2µg HPM/eppendorf were incubated with a fixed concentration of labelled EGF (1nM) and increasing concentrations of cold EGF (0,0.1,0.2,0.5,1,2,5,10,20,50,100,200nM) again at 4°C overnight.

From the saturation curve (Fig 4.1), the binding data were transformed into B/T ratios and plotted on a semilogarithmic scale against the radioactivity added to the eppendorfs (following subtraction of the initial cpm of the first tube).

For the competitive binding curve (Fig 4.2), the binding data were transformed into Bound over Total added ratios (B/T ratios) and plotted against the log of nanomoles of unlabelled EGF. At several BT ratios in this plot, cpm of ¹²⁵I-EGF and nanomoles of unlabelled EGF were read from the saturation and competition curves respectively. The values obtained were then plotted against one another and the specific activity read from the slope of the resulting straight line.

For the ligand equivalence plot, the apparent specific radioactivity calculated at various B/T ratios ($\log \text{cpm} - \log(\text{nmol}) = \log \text{SA in cpm/nmol}$) was converted into specific radioactivity in µCi/µg and plotted versus the B/T ratio at which each specific radioactivity was obtained. According to Hollemans and Touber, (1974), if a radioligand has the same affinity as the unlabelled ligand, the line obtained in

the above plot is horizontal. If the slope of this line differs significantly from zero then there is nonequivalence.

Figure 4.1 shows the saturation curve for EGF binding to HPM following overnight incubation at 4°C, pH 7.4. One can clearly see that on increasing the concentration of ^{125}I -EGF the amount of EGF specifically bound to HPM increases to a maximum saturable level of binding at just over 20000 counts minute⁻¹. Non-specific binding remains low throughout, never rising above 40%.

Figure 4.2 shows the displacement of 1nM radio-labelled EGF from its receptor in placental membranes by increasing concentrations of unlabelled EGF (0.1-200nM) following overnight incubation at pH 7.4, 4°C. One can see that at cold EGF concentrations less than 1nM, the labelled EGF has fully displaced the cold from the receptors. Interestingly, the labelled EGF begins to displace at concentrations as high as 10nM unlabelled, but only by 15-20%.

The saturation and binding curves were transformed into bound/free ratios and plotted on a semi-logarithmic plot (Figure 4.3). Following the procedure outlined above, ligand equivalence and the specific activity were determined (shown in figs 4.4 and 4.5, respectively). The LE plot produced a straight line and the specific activity, which was calculated from the gradient of the line in Fig 4.5, was estimated at 21 $\mu\text{Ci}/\mu\text{g}$ protein for this particular iodination.

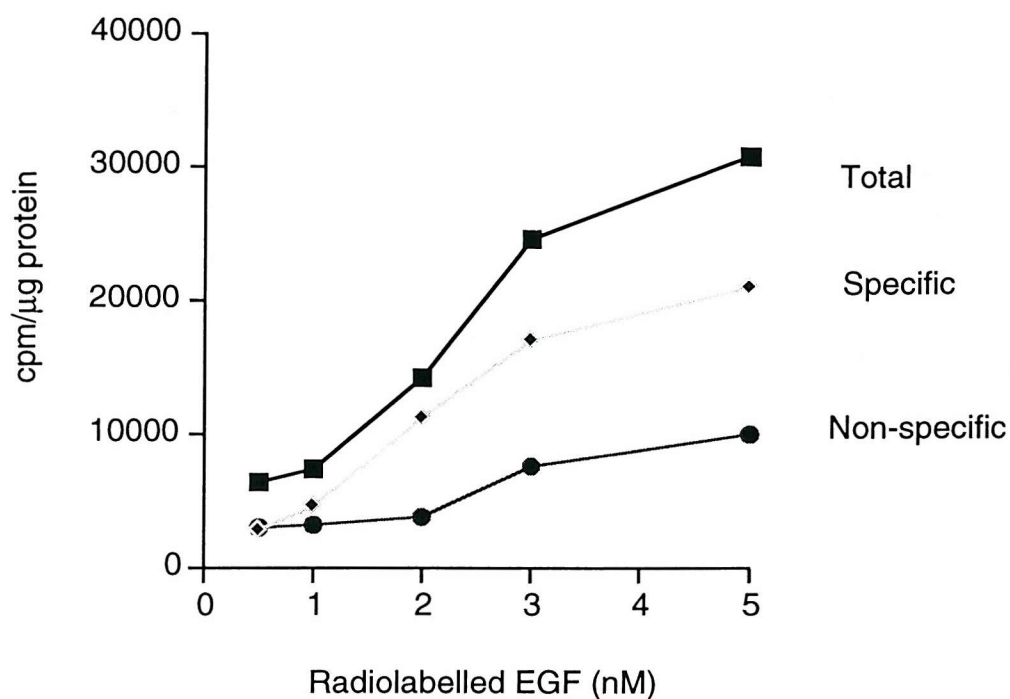


Figure 4.1. Binding of ^{125}I -EGF to human placental membranes.

Placental membrane aliquots (2μg protein/eppendorf) were incubated overnight at 4°C with increasing concentrations of ^{125}I -EGF (0-5nM) in 0.2ml of 25mM sodium phosphate buffer, pH 7.6 containing 150mM NaCl and 0.1% (w/v) bovine serum albumin. Non specific binding was determined in the presence of an excess of unlabelled EGF (200nM). Specific binding was calculated by subtracting the non-specific binding from the total bound (cpm/μg protein)

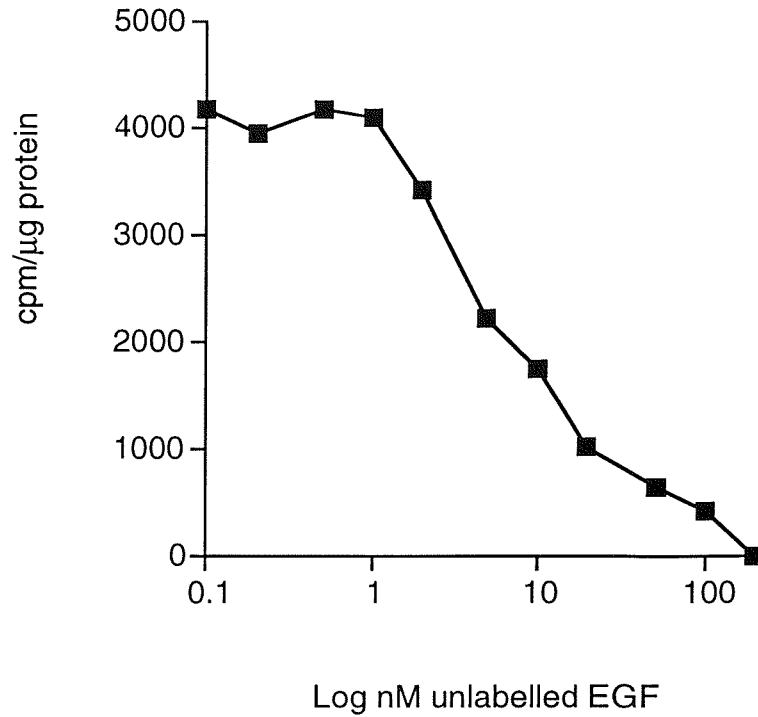


Figure 4.2. Binding competition curve for ^{125}I -EGF in human placental membranes.

Placental membrane aliquots (2μg protein/eppendorf) in 0.2ml of 25mM sodium phosphate buffer, pH 7.6 containing 150mM NaCl and 0.1% (w/v) bovine serum albumin, were incubated with 1nM ^{125}I -labelled EGF and increasing concentrations of unlabelled hEGF (1-200nM). After being left overnight at 4°C, the amount of radioligand bound was determined (as outlined in section 2.11.). Values are corrected for non-specific binding which was measured in the presence of an excess of unlabelled EGF (200nM).

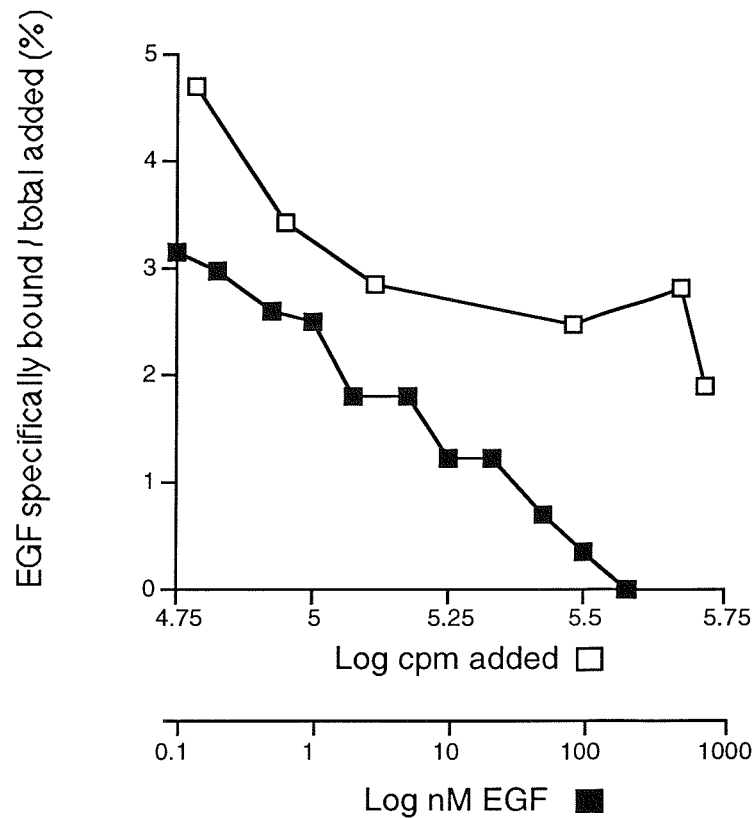


Figure 4.3. Calculation of the Specific Activity of ^{125}I -hEGF by self-displacement analysis.

The specific binding curve from Fig 4.1. was transformed into Bound/Total (B/T) ratios and plotted on a semi-logarithmic scale against the radioactivity added to the receptor preparation (in cpm) following subtraction of the initial cpm added in the first tube (white boxes). For the competition binding curve, the data were transformed into B/T ratios and plotted Vs the log of unlabelled EGF (1-200nM) in the same semi-logarithmic plot (black boxes).

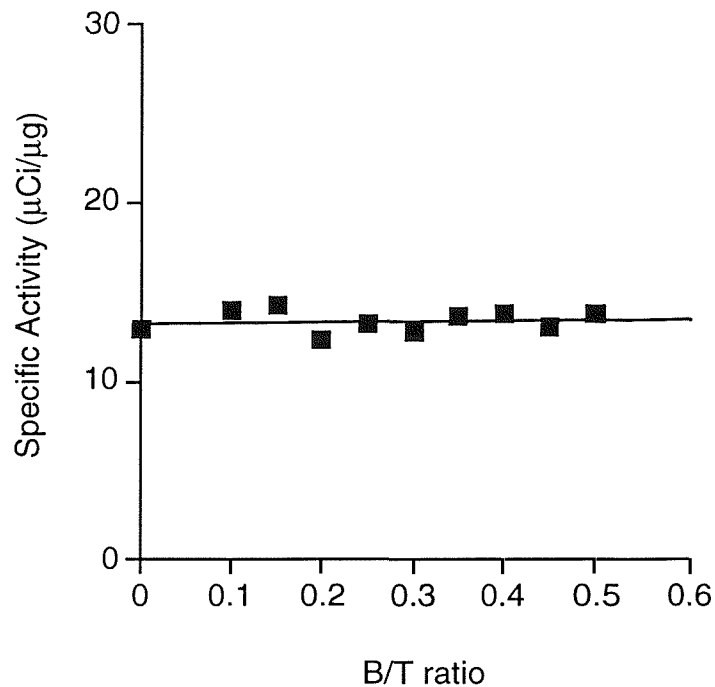


Figure 4.4. Ligand equivalence plot obtained for ^{125}I -EGF labelled by the lactoperoxidase method.

To ensure that the radiolabelled ligand is behaving the same as if it were unlabelled an LE plot is obtained from the saturation and competitive binding curves. The apparent SA calculated at various B/T ratios was converted into specific activity in $\mu\text{Ci}/\mu\text{g}$ and plotted Vs the B/T ratio at which this SA was obtained. The horizontal line suggests that, for this particular iodination, the radio-ligand has the same affinity for the EGFR as the unlabelled ligand (Holleman and Touber, 1974).

($\log \text{cpm}/\log \text{nM} = \log \text{SA in cpm/nM}$)

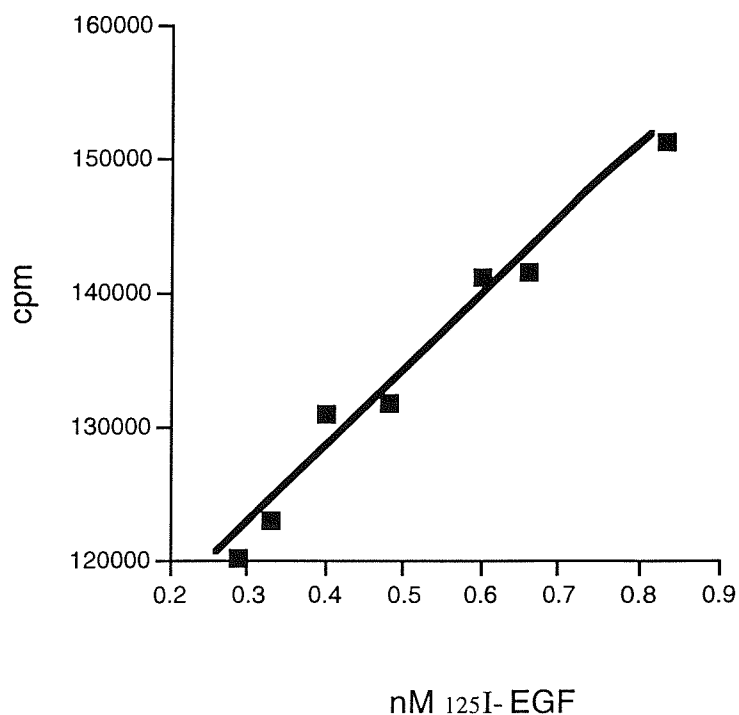


Figure 4.5. Calculation of Specific Activity of ^{125}I -EGF by self-displacement analysis.

At several B/T ratios in Fig 4.3., cpm of ^{125}I -EGF and nM unlabelled EGF were read from the saturation curve and the competition curve respectively. The cpm of ^{125}I -EGF were plotted against corresponding log nM unlabelled EGF. From the straight line obtained, the specific radioactivity of the ligand preparation can be obtained as the slope of this line. For this particular iodination, SA = 57,000 cpm/nM (21 $\mu\text{Ci}/\mu\text{g}$ protein).

4.4.2. Determination of optimum pH of EGF binding to JAr cells.

JAr cells were plated out at an initial concentration of 0.5 µg/DNA per well and grown for 72h in 5%CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5%SFCS for the latter 24h) before the binding assay was performed. The cells were incubated with increasing concentrations of labelled ¹²⁵I-EGF overnight at 4°C in a total volume of 250µl binding buffer (25mM sodium phosphate buffer containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) at pH 6, 7 or 8. The specific binding was determined by subtracting the non-specific binding (determined in the presence of 200nM excess cold EGF) from the total bound and expressed as a percentage of the total bound.

From Figure 4.6. one can clearly see that 71% maximum binding was obtained with 10nM EGF at pH 7. The percentage of specifically bound EGF at both pH 7 and pH 8 were not significantly different from each other. The cells incubated at pH 6 showed erratic binding over the concentration range used, only achieving a maximum binding of 42%, and at all EGF concentrations the non-specific binding was higher than the specific.

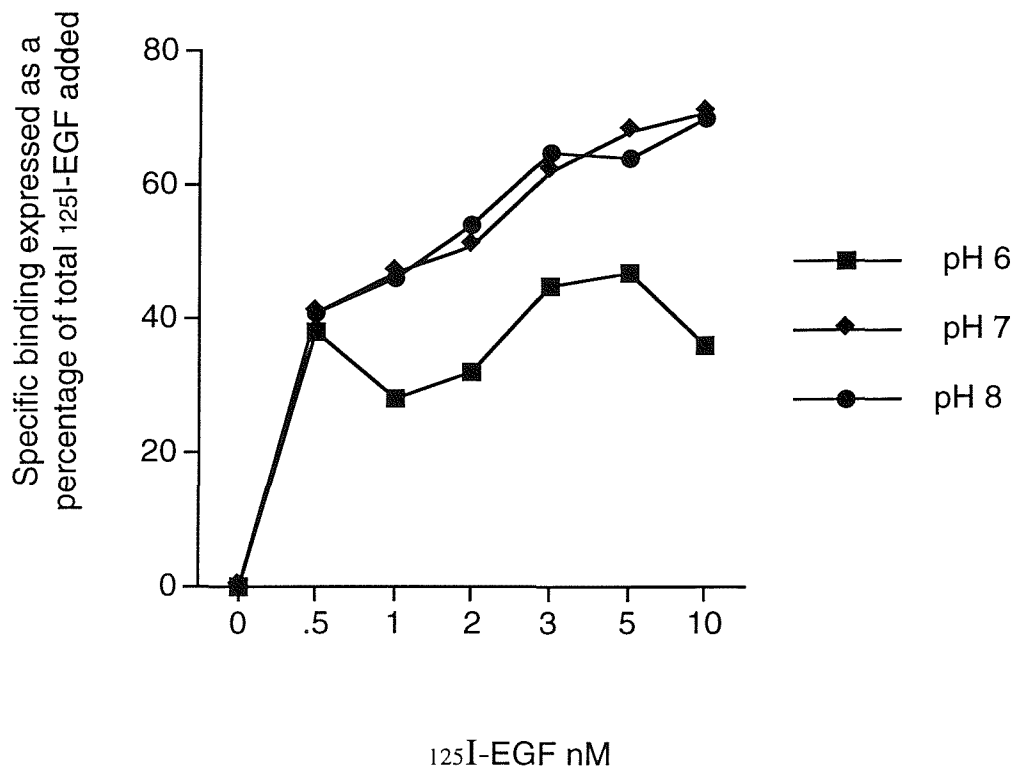


Figure 4.6. Dependence of ^{125}I -EGF binding in JAR cells on pH.

JAr choriocarcinoma cells (initial DNA 0.5 μg /well) in 250 μl of 25mM sodium phosphate buffer adjusted to the indicated pH (6,7, or 8) , containing 150mM NaCl and 0.1% (w/v) bovine serum albumin, were equilibrated overnight at 4°C with increasing concentrations of ^{125}I -EGF (0-10nM). Cell associated radioactivity was assessed following washing and solubilisation as outlined in section 2.11. Non-specific binding was obtained in the presence of 200nM excess unlabelled EGF and specific binding calculated by subtracting NS from the total radioactivity bound. Specific binding (SB) is expressed as a percentage of the Total radiolabel bound at each ^{125}I -EGF concentration (nM)

4.4.3. Optimisation of temperature for binding of EGF to its receptor in JAr cells.

JAr cells were plated out at a concentration of 0.5 µg/DNA per well and grown for 72h in 5% CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5%SFCS for the latter 24h) before the binding assay was performed. The cells were incubated in a total volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled¹²⁵I-EGF for 2h. Incubation temperature varied between the three plates ranging from 4, 22, and 37°C. The specific binding was determined by subtracting the non-specific binding (determined in the presence of 200nM excess cold EGF) from the total bound and expressed as a percentage of the total bound. Following incubation, cells were washed, lysed and the incorporated radioactivity counted according to the protocol laid out in 2.10.2

Fig 4.7 shows the percentage of EGF specifically bound after two hours at different temperatures. One can observe that at 37°C binding is rapid and saturation is achieved at 1nM EGF. At this point, 26% of the total bound EGF is specifically bound. Increasing the concentration of EGF at this temperature did not cause any further incorporation of radioactivity and the percentage specifically bound remained constant around 26-29%.

At 22°C saturable binding was achieved at 3nM (33%) after which concentration there was a slight increase in binding to 48% at 10nM.

At 4°C initial binding was less rapid than at the previous temperatures. Saturation of binding was greatly increased (76%) but did not occur until concentrations as high as 5nM after which time there was no evidence for an increase in binding and the curve plateaued to 10nM.

As a result of these experiments, 4°C was chosen as the optimum binding temperature for future experiments as it yielded the highest amount of specific binding.

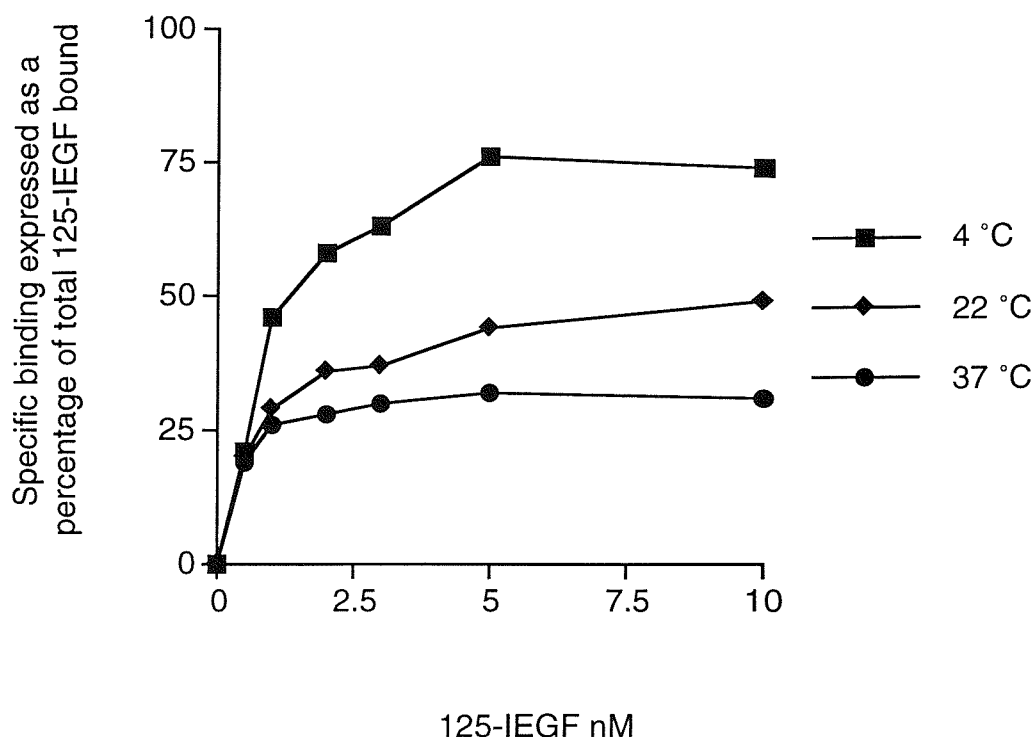


Figure 4.7. Dependence on temperature of ^{125}I -EGF binding in JAr cells.

JAr cells (initial DNA 0.5 $\mu\text{g}/\text{well}$) were incubated in 250 μl of 25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% BSA (w/v) and equilibrated with increasing concentrations of radiolabelled ^{125}I -EGF(0-10nM) for 2h at varying temperatures - 4°C, 22°C and 37°C. Receptor associated radioactivity was measured on an LKB Wallac gamma counter following washing and solubilising (see section 2.11.). Specific binding (SB) was expressed as a percentage of the total bound following subtraction of Non Specific binding (determined in the presence of 200nM excess unlabelled EGF).

4.4.4. Time course of EGF binding to its receptor in JAr cells.

To investigate the time course of EGF binding to its receptor in JAr cells, 0.5 μg initial DNA was plated out in 24 well plates, cultured for 72h in 2.5%SFCS, 5% CO_2 in air at 37°C. The cells were incubated in a total volume of 250 μl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled ^{125}I -EGF. The binding was terminated by addition of ice-cold binding buffer at various time periods - 60, 90, 120, 180, and 720 minutes, and the resulting cell lysates measured for associated radioactivity.

Figure 4.8 shows the effect of 5nM radiolabelled EGF binding at 4°C at varying time intervals (0-720 minutes). Specific binding at this temperature was rapid up to 2h, reaching a value of 33% of total bound, after which time it plateaued at around 35 %. As mentioned above, this experiment was carried out for all EGF concentrations (0-10nM) which showed the same trend (data not shown). Subsequently, an optimum incubation time of 2h was chosen for further experiments.



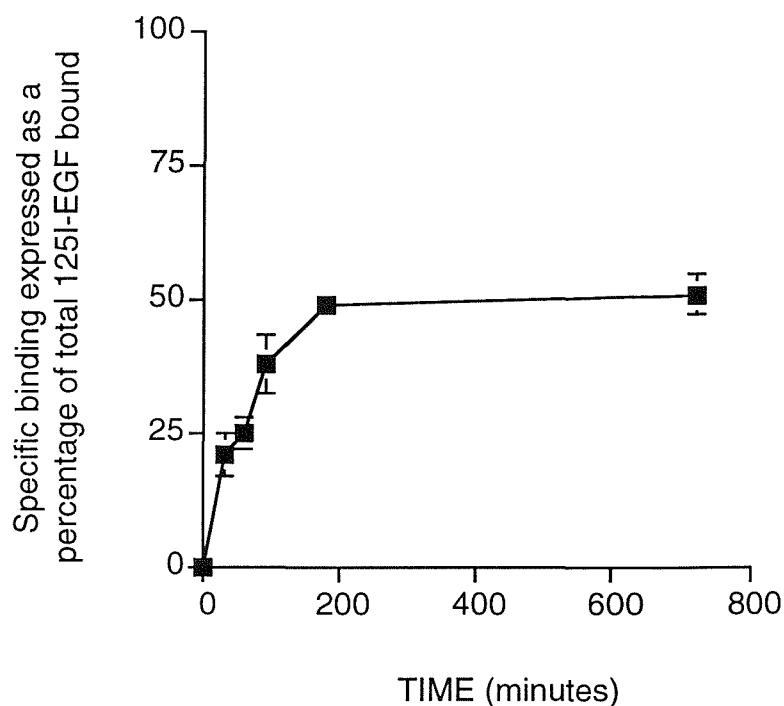


Figure 4.8. Time course of ^{125}I -EGF binding in Jar choriocarcinoma cell line.

JAr cells were grown for 72h as outlined in section 2.10. at an initial plating density of $0.5\mu\text{g}$ DNA/well. Cells were incubated at 4°C in 24 well plates with $50\mu\text{l}$ of 5nM ^{125}I -EGF in a total volume of $250\mu\text{l}$ of Binding Buffer (25mM sodium phosphate with 150mM NaCl and 0.1% (w/v) BSA. Incubations were stopped at the indicated times. Non specific binding was measured in the presence of 200nM excess unlabelled EGF and subtracted from the Total bound to yield SB. Specifically Bound (SB) radioactivity is expressed as the percentage of the total radioactivity added for 3 experiments in triplicate where points represent the mean \pm s.e.m.

4.4.5. Saturable binding of EGF to JAr cells.

Having determined the optimum binding conditions for the JAr cell-line it was possible to conduct further experiments to investigate the binding affinity of EGF for its receptor and the number of binding sites present.

Cells were grown for 72h, in 24 well plates at 37°C in 10%SFCS (replaced with 2.5%SFCS for the final 24h) and subjected to a ligand binding assay as outlined previously i.e. the cells were incubated in a total volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled ¹²⁵I-EGF for 2h. Figure 4.9 shows the Total, Specific, and Non-specific binding of EGF bound to its receptor in the JAr cell line at 4°C, pH 7.6 following 2h incubation. The data shown are actual cpm and indicate that total binding was high (up to 10,000 cpm at 10nM), Specific binding was saturable (at 5-10nM) and at all concentrations non-specific binding remained lower than specific. This is shown in Figure 4.10. where the specific and non-specific binding have been calculated as a percentage of the total radioactivity bound.

In order to obtain K_d and B_{Max} values it was necessary to transform the data into Bound/Free ratios and carry out Scatchard analysis. Data are from a representative experiment (of 8). From the Scatchard plot in Fig 4.11. a curvilinear plot, characteristic of multiple binding sites, yielded two K_ds - a high affinity site ($3 \pm 72 \times 10^{10} \text{M}^{-1}$) and a low affinity site ($55 \pm 1.4 \times 10^9 \text{M}^{-1}$) with $19,874 \pm 799$ and $37,318 \pm 1,435$ receptors per cell respectively.

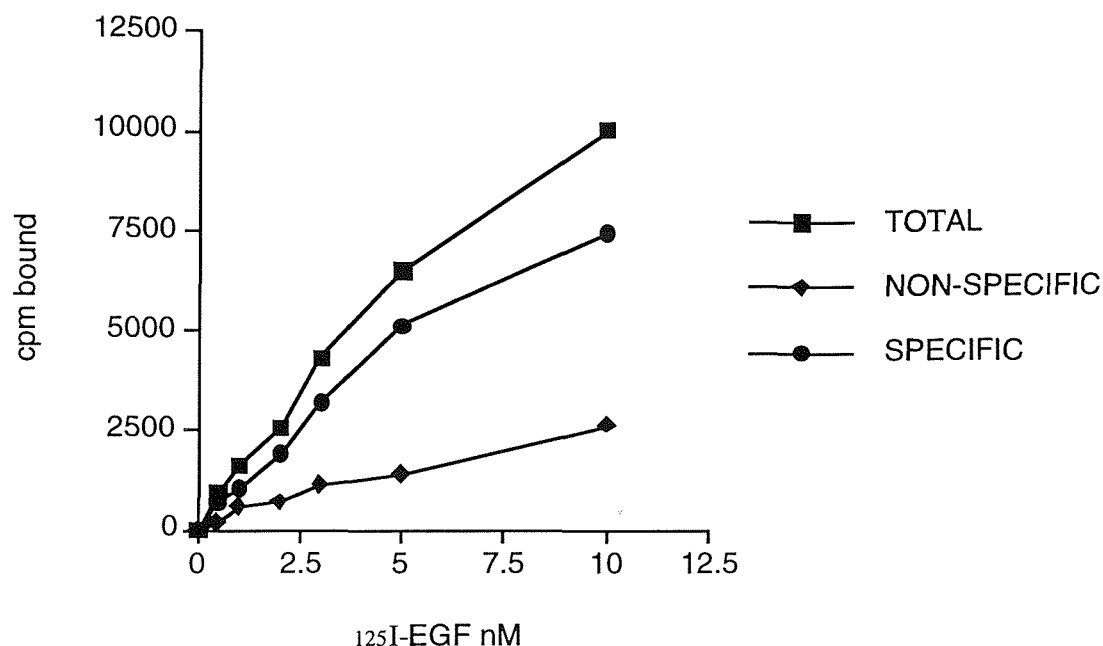


Figure 4.9. Binding of ^{125}I -EGF to JAr choriocarcinoma cells.

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11. The cells were incubated with various concentrations of ^{125}I -EGF over the range (0-10nM) to yield total binding. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB). Each point represents the mean of eight experiments in duplicate

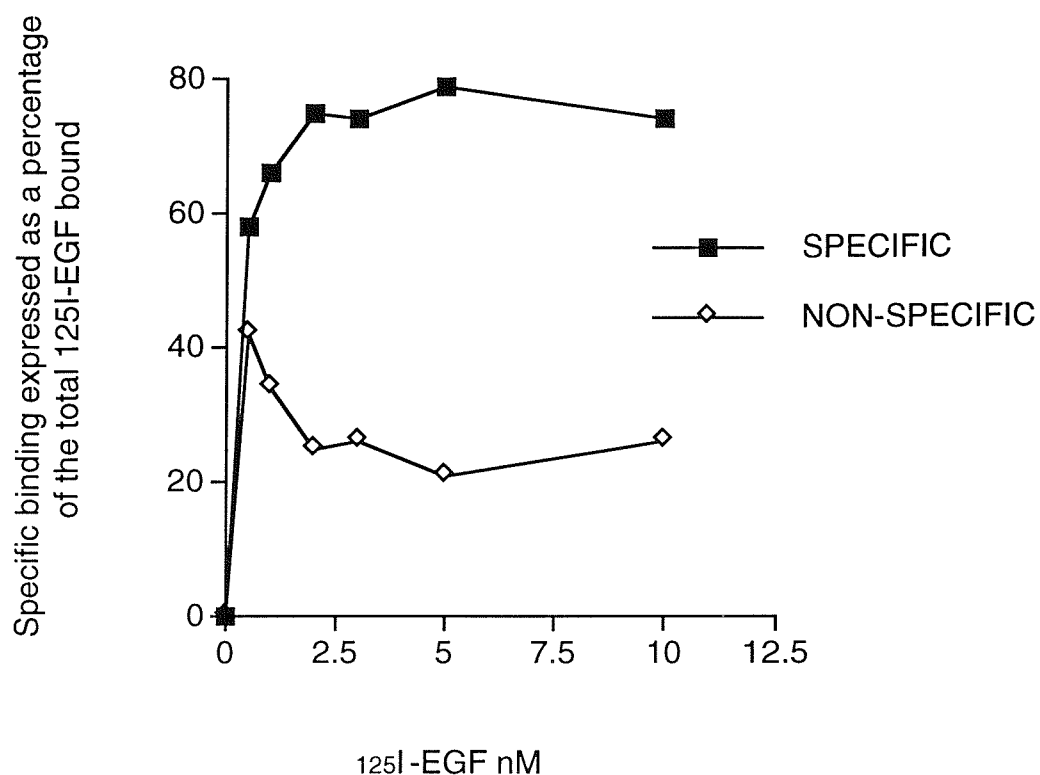


Figure 4.10. Saturation curve of specific binding of $^{125}\text{I-EGF}$ to JAr cells.

The data obtained from figure 4.9. are transformed into the percentage of specific and non-specific binding of the total bound at each EGF concentration, where total bound = 100%. Non-specific binding remains low at around 25% of the total radioactivity bound.

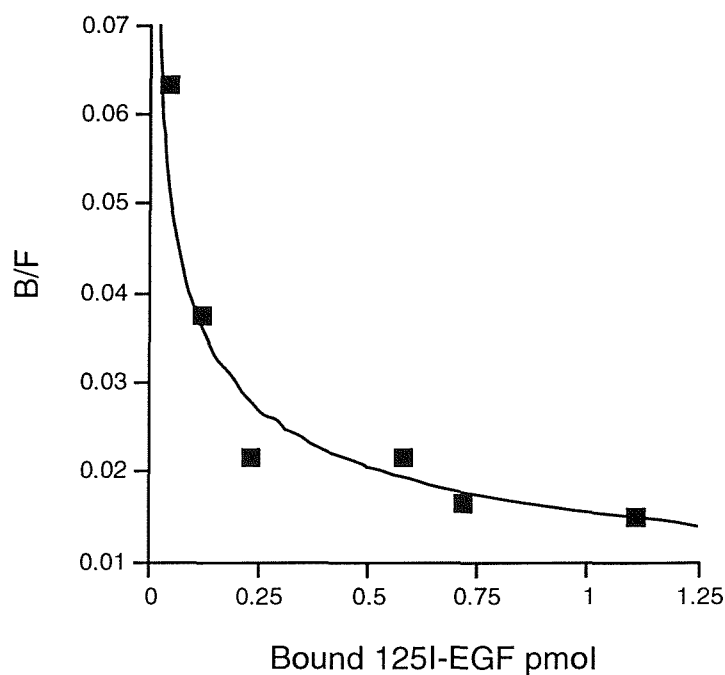


Figure 4.11. Scatchard analysis of ^{125}I -EGF binding data in JAr cells

JAr cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C, 5% CO₂ in air before the start of the binding assay. The cells were incubated for 2h at 4°C, pH 7.6 with increasing concentrations of ^{125}I -EGF (0-10nM) and cell associated radioactivity measured as outlined in section 2.11. Data are from a representative experiment (of 8) calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

4.4.6. Saturable binding of EGF to BeWo cells.

BeWo cells were grown for 72h (initial plating density 1µgDNA/well) in 24 well-culture plates gassed in 5% CO₂ in air at 37°C. For the first 48h of growth the medium was supplemented with 10% FCS and for the final 24h this was substituted for 2.5% SFCS. Figure 4.12 shows the Total, Specific, and Non-Specific binding of increasing concentrations of ¹²⁵I-EGF bound to its receptor in the BeWo cell line at 4°C, pH 7.6 following 2h incubation. The data shown are actual cpm and indicate that total binding was ten fold lower than that observed in JAr. In figure 4.13. the specific and non-specific binding was calculated as a percentage of the total ¹²⁵I-EGF bound and shows that despite the comparatively low counts/minute the specific binding was saturable (at 5-10nM) and that at all concentrations non-specific binding remained lower than specific.

Scatchard analysis was performed on the data and again a curvilinear plot was obtained for each experiment with figure 4.14. representing one of three experiments. This was again interpreted as multiple binding sites, yielding two Kds which relate to a high affinity binding site ($2.2 \pm 1.65 \times 10^9 \text{M}^{-1}$) and a low affinity site ($17.0 \pm 2.15 \times 10^9 \text{M}^{-1}$) with $3,601 \pm 572$ and $8,091 \pm 484$ binding sites per cell, respectively.

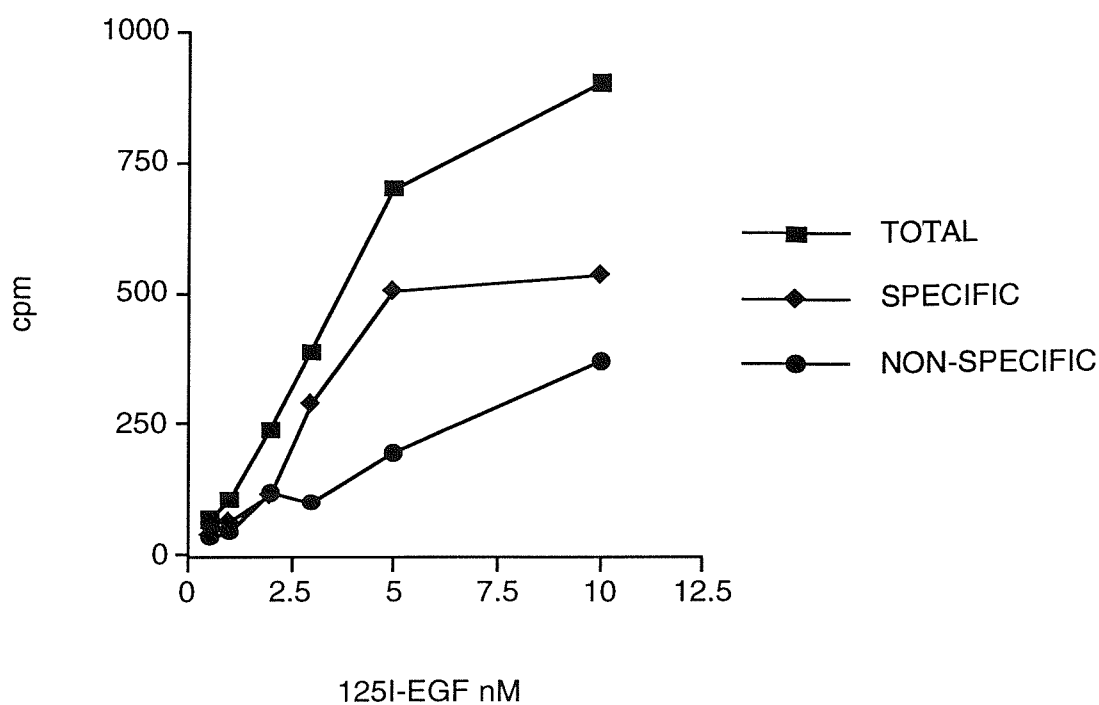


Figure 4.12. Binding of ^{125}I -EGF to BeWo choriocarcinoma cells.

BeWo cells were plated in 24 well culture plates (1 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11. The cells were incubated with various concentrations of ^{125}I -EGF over the range (0-10nM) to yield total binding. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB). Each point represents the mean of three experiments in duplicate.

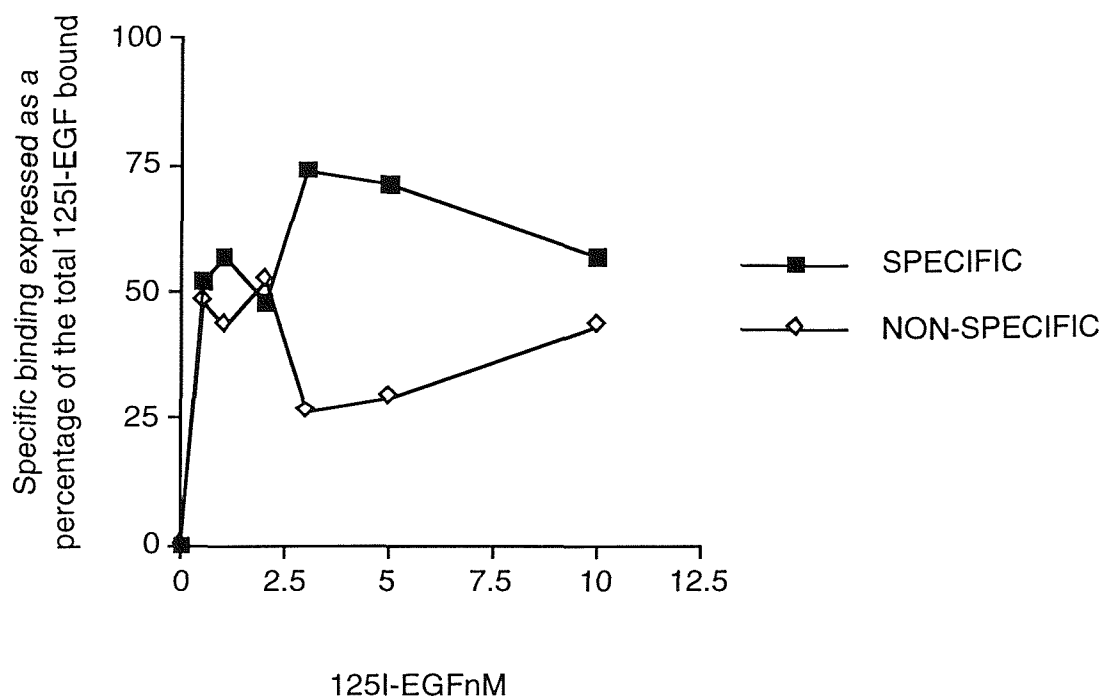


Figure 4.13. Saturation curve of specific binding of ^{125}I -EGF to BeWo cells.

The data obtained from figure 4.12. are transformed into the percentage of specific and non-specific binding of the total bound at each EGF concentration, where total bound =100%.

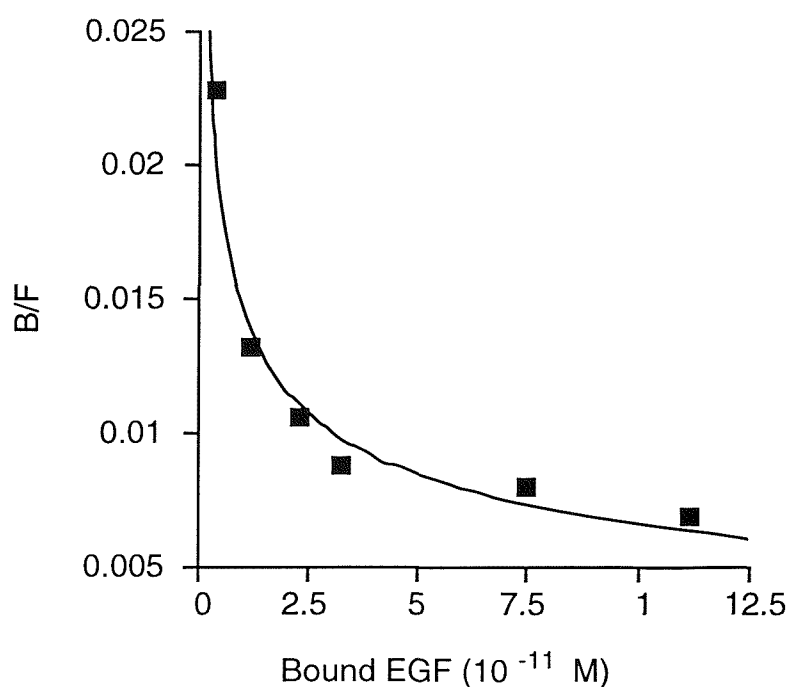


Figure 4.14. Scatchard analysis of ^{125}I -EGF binding data in BeWo cells

BeWo cells were plated in 24 well culture plates ($1\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C , 5% CO_2 in air before the start of the binding assay. The cells were incubated for 2h at 4°C , pH 7.6 with increasing concentrations of ^{125}I -EGF (0-10nM) and cell associated radioactivity measured as outlined in section 2.11. Data are from a representative experiment (of 3) calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

4.4.7. Saturable binding of EGF to Jeg-3 cells.

Jeg-3 cells were grown for 72h (initial plating density 0.5 μ gDNA/well) in 24 well-culture plates gassed in 5% CO₂ in air at 37°C. For the first 48h of growth the media was supplemented with 10% FCS and for the final 24h this was substituted for 2.5% SFCS. The binding assay was carried out as outlined in section 2.11. Figure 4.15. shows the Total, Specific, and Non-specific binding of increasing concentrations of I¹²⁵-EGF bound to its receptor in the Jeg-3 cell line at 4°C, pH 7.6 following 2h incubation. The data shown are actual cpm and indicate that total binding was again low with similar values as observed in BeWo. Specific binding was saturable (at 5-10nM) and that at all concentrations non-specific binding remained lower than specific. This is shown in figure 4.16. where the specific and non-specific binding is expressed as a percentage of the total cell associated radioactivity.

To determine the number of binding sites, Scatchard analysis was performed on the data (Fig 4.17.) and again a curvilinear plot was obtained (Fig.4.17. is from a representative experiment (of 3)). This was taken to represent multiple binding sites and analysis with a non-linear curve fitting Ligand computer programme (McPherson, 1985) using a two site model yielding two K_ds - a high affinity site ($2.7 \pm 1.43 \times 10^9 \text{M}^{-1}$) and a low affinity site ($18.2 \pm 1.19 \times 10^9 \text{M}^{-1}$) with the equivalent of $5,994 \pm 1,321$ and $11,752 \pm 1,904$ ligand binding sites per cell, respectively.

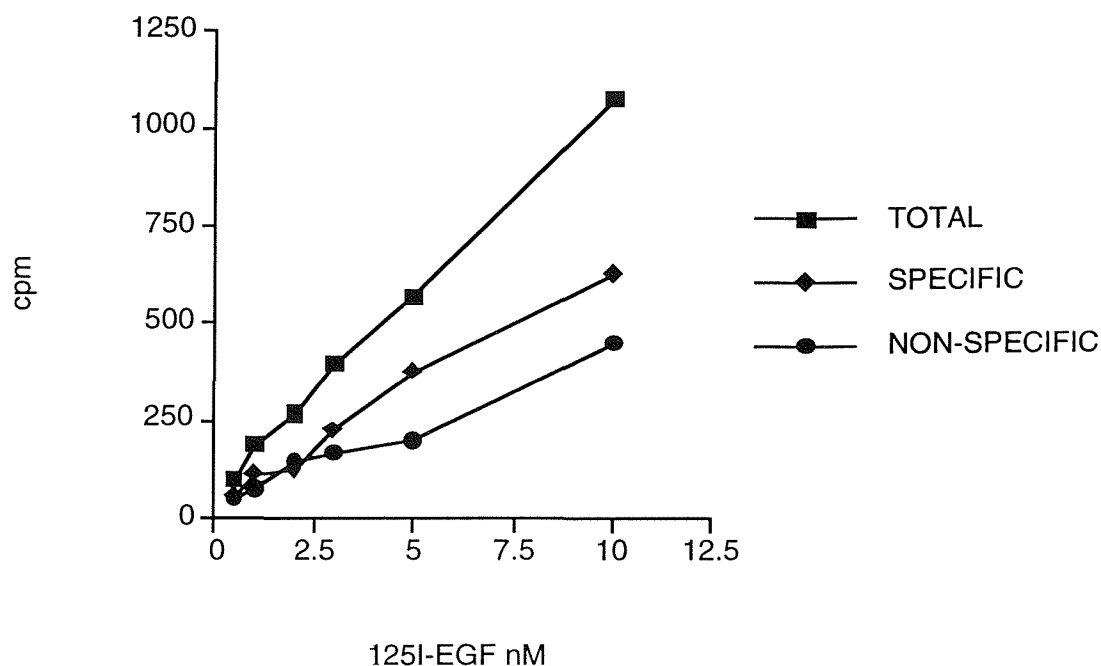


Figure 4.15. Binding of ^{125}I -EGF to Jeg-3 choriocarcinoma cells.

Jeg-3 cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11. The cells were incubated with various concentrations of ^{125}I -EGF over the range (0-10nM) to yield total binding. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB). Each point represents the mean of three experiments in duplicate.

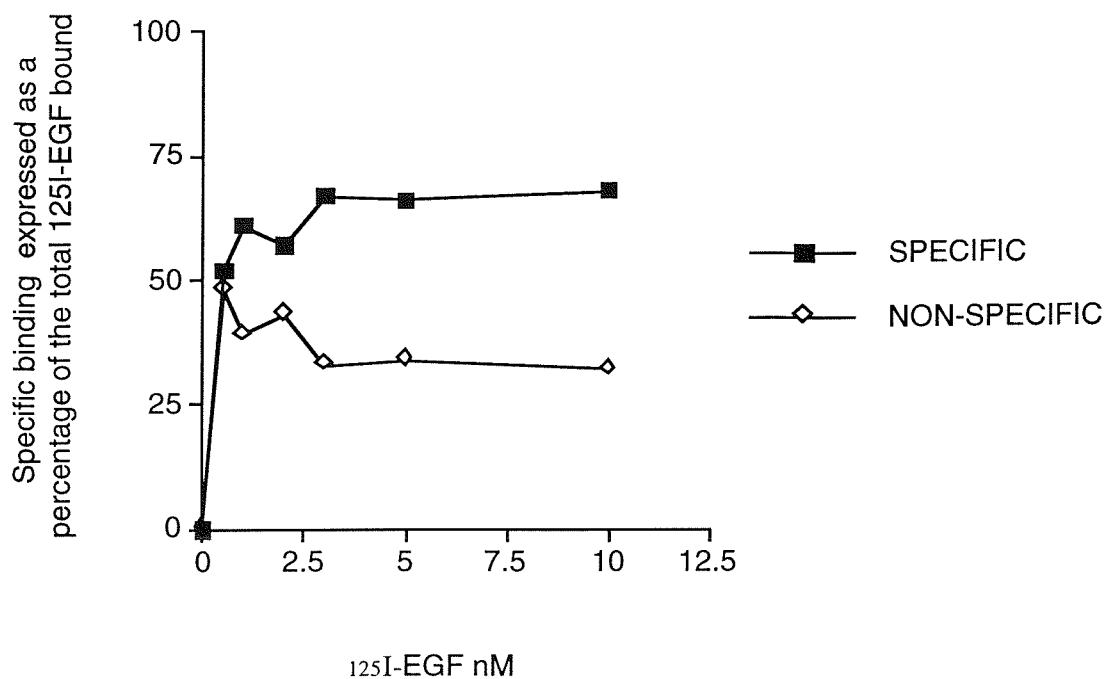


Figure 4.16. Saturation curve of specific binding of $^{125}\text{I-EGF}$ to Jeg-3 cells.

The data obtained from figure 4.15. are transformed into the percentage of specific and non-specific binding of the total bound at each EGF concentration, where total bound = 100%.

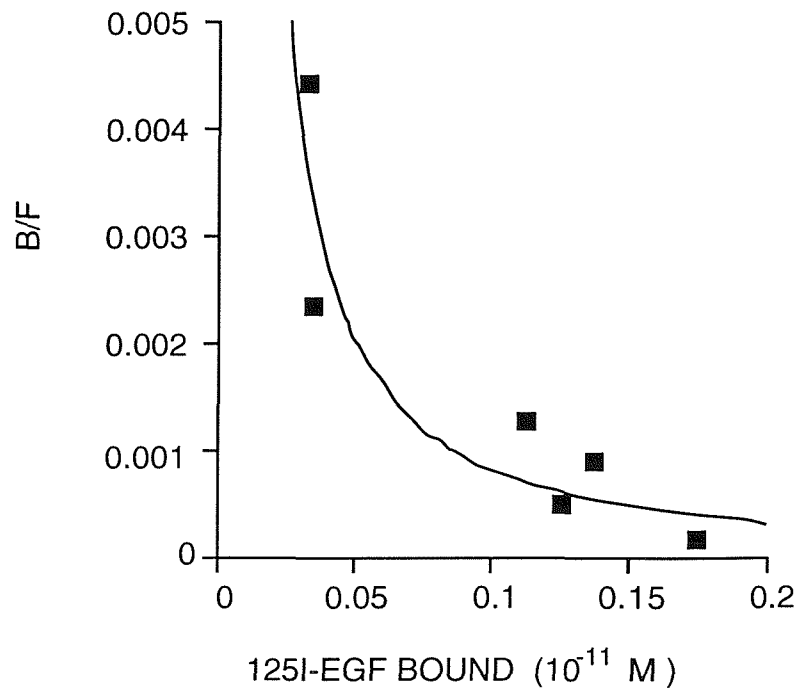


Figure 4.17. Scatchard analysis of ^{125}I -EGF binding data in Jeg-3 cells

Jeg-3 cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C, 5% CO₂ in air before the start of the binding assay. The cells were incubated for 2h at 4°C, pH 7.6 with increasing concentrations of ^{125}I -EGF (0-10nM) and cell associated radioactivity measured as outlined in section 2.11. Data are from a representative experiment (of 3) calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

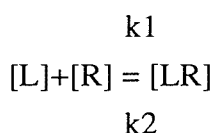
4.5. Discussion.

EGF can be iodinated on up to five tyrosine residues (Matrisian, 1985) some of which may be involved in receptor binding. Therefore in iodinating EGF one must ensure that the labelled peptide behaves the same as the natural ligand in receptor binding. Failure to establish this could result in erroneous receptor affinity and capacity estimates. To ensure that the radiolabelled ligand was behaving the same as un-labelled EGF a Ligand Equivalence Analysis (LEA) was carried out. This allows an accurate determination of the Specific Activity if the affinity of the unlabelled and labelled ligand towards the receptor are identical. If these affinities are not identical then the slope of the LE plot differs from zero (Kienhuis *et al*, 1991). In this instance, the LE plot generated a straight line indicating that the radiolabelled EGF was representative of un-labelled EGF.

The procedure must also label homogeneously and strongly enough to allow good assay sensitivity but avoiding strong oxidation or reduction. The lactoperoxidase method was initially favoured in this work because it has been shown to oxidise mildly, however, a relatively high amount of hydrogen peroxide is added to initiate the reaction which could be harmful to the peptide. This did not apparently affect the binding characteristics of EGF suggesting that there was no substantial damage to the protein. One could then presume that the tyrosine residues targeted by this method are not involved in receptor binding.

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) is another mild iodination reagent which has four chloramine-T like groups yet is insoluble in water. When dissolved in water, chloramine T generates hypochlorous acid (HOCl) a soluble and strong oxidative molecule. Due to its insoluble nature, the iodogen method has many of the advantages of the classical chloramine-T method without the release of harmful oxidising agents such as HOCl thus yielding a labelled peptide with considerably higher specific activity.

The interaction of EGF with its receptor can best be described by the following equation:



Where L is the free EGF, R is the unoccupied EGFR and LR is the EGF-EGFR complex. k_1 and k_2 are the association and dissociation constants respectively. At equilibrium, the law of mass action states that:

$$K_d = \frac{[L].[R]}{[B]} \quad \text{where } K_d = k_2/k_1$$

If the total receptor concentration = B_{max} then

$$K_d = \frac{[L].(B_{max}-[LR])}{[B]}$$

which is the same as:

$$[B] = \frac{B_{max}.[L]}{K_d + [L]}$$

This equation is identical to the Michaelis Menten equation used in enzyme-substrate kinetics and describes a hyperbolic relationship between the amount of EGF bound to its receptor at a given free concentration. If the amount bound is plotted against the amount free, then accurate estimates of the required constants (K_d and B_{max}) can not be calculated since it is virtually impossible to estimate the asymptotic value of B_{max} .

However, by performing a double reciprocal plot as described by Scatchard (1949) (similar to that performed by Lineweaver and Burke) one can linearise the curve:

$$[B] = B_{max} - K_d.([B]/[L])$$

Thus a Scatchard plot yields a straight line with a slope = $-1/K_d$ and an x intercept of B_{max} . A linear plot suggests that the ligand is binding to a homogeneous population of receptors yet for many systems, including EGF, this is not the case. The following equation relates the total amount of ligand bound when binding to two sites simultaneously and incorporates non-specific binding:

$$[B] = \sum_{i=1}^n \frac{B_{\max i} [L]}{K_{di} + [L]} + N [L]$$

Where K_{di} and $B_{\max i}$ are the constants for the ligand binding to site i , N is the ratio of bound/free at infinite free concentration.

The complexity of the above equations is overcome, fortunately (!), by the availability of computer programmes such as LIGAND which have been developed using the above model (Munson and Rodbard, 1980). LIGAND is a non-linear curve fitting programme specifically designed to cope with radioligand binding site interactions.

In order to investigate and characterise the binding of EGF to its receptor it was necessary to optimise the binding conditions. This included determination of the optimum time of incubation, temperature, protein/DNA concentration and pH.

The optimum conditions for looking at EGF binding to its receptor in human placental membranes had already been determined in our laboratory and therefore the experiments were not repeated (Onagbesan *et al*, 1995).

However for the choriocarcinoma cells (JAr, Jeg-3 and BeWo) it was important that all conditions were optimised.

From the pH data, 71% maximum binding was obtained with 10nM EGF at pH 7. The cells incubated at pH 6 showed erratic binding over the concentration range used and only achieved a maximum binding of 42%. In all cases the non-specific binding was higher than the specific and hence it was concluded that pH 6 was not suitable for binding of EGF to its receptor in this cell line.

Lai and Guyda (1984) showed that in human placental cell cultures, binding of EGF to its receptor declined rapidly at pH values below 7. Although this decline is less dramatic in my experiment, the trend is nevertheless the same.

The percentage of specifically bound EGF at both pH 7 and pH 8 were not significantly different from one another, therefore a compromise was made and a pH of 7.6 chosen for further studies. This value is in accordance with the

previously mentioned work of Lai and Guyda (1984) and also with several other publications (Brabyn and Kleine, 1994). Korc and Finman (1989) looked at the effect of pH on EGF binding in T3M4, human pancreatic carcinoma cells, and found that decreasing the pH of medium from pH 7.4 to 5.0 resulted in a gradual dissociation of EGF from its receptor with complete dissociation at pH 4.5. They later showed, in the same cells, that, an increase to pH 8.5 results in a decrease in the number of high affinity binding sites for EGFR (Korc *et al*, 1991). Therefore it is evident that pH plays an important role in EGF binding but precisely why is not known.

In order to ascertain the optimum pH more accurately it would have perhaps been advisable to maintain a fixed concentration of labelled EGF and to increase the range of pH values being investigated. However, because this was the preliminary experiment it was difficult to know which variables could be kept constant/moving without altering the binding. For example, the optimum incubation time chosen for the experiments was quite different from that used here (2h instead of overnight) but having not yet performed the time course experiments it was necessary to set an incubation period albeit the wrong one.

Since the binding of EGF to its receptor is a thermodynamic process, the correct temperature plays an important role in receptor-ligand studies. In fact it is not necessarily the binding which is temperature dependent but the aggregation of receptors in the lipid bilayer. Immunohistochemical studies have shown that at 4°C, cultured syncytiotrophoblasts incubated with ¹²⁵I-EGF revealed a cell surface location for the majority of the ligand whereas at 37°C a shift in the distribution of labelled EGF was observed; in continuous labelling experiments, a progressive intracellular accumulation of EGF was observed at this temperature (Lai *et al*, 1986).

Following binding, the ligand-receptor complex is supposedly internalised via clathrin coated pits by endocytosis and both the ligand and receptor then degraded (Stoscheck and Carpenter, 1984). However, other workers have shown involvement of non-lysosomal intracellular components suggesting that endosomes may capture the free EGF and make it available for recycling (Lai *et al*, 1986). In the above experiments only surface bound EGF is being measured therefore any internal EGF will not be taken into account. Binding of EGF to its receptor is rapid at 37°C, taking place within the first 5 minutes, therefore what one assumes to be a lack of binding is in fact due to highly efficient binding and subsequent

internalisation. In terms of ligand binding studies this is not suitable since equilibrium must be reached in order to analyse the data. Clearly, in such a short period of time equilibrium is not achieved and hence the Lineweaver-Burke calculations cannot be applied. Similarly at 22°C a large proportion of the radiolabelled EGF can be assumed to be internalised and although a slightly higher amount of EGF remains surface bound, the specific binding is still relatively low in comparison with that at 4°C. At 4 °C it is clear that very little EGF is being internalised with almost 75% remaining specifically bound after 2h.

Thus, after 2h at pH 7.6 and 4°C, binding of EGF to its receptor in JAr cells had reached maximum specific binding with receptor and ligand complexes being in equilibrium with free ligand and free receptor. Consequently these parameters were chosen as the optimum binding conditions for further experiments.

It is generally accepted that the EGF receptor has two binding affinities for EGF but there is debate over the physiological relevance of this. The existence of two binding sites is well published and the values obtained here were not dissimilar from those previously reported (Lai *et al*, 1986; Hurwitz *et al*, 1991; Schechter *et al*, 1991; Alsat *et al*, 1991; Earp *et al*, 1995). Based on the literature it was assumed that there were two binding sites present in all three cell lines and the data were analysed bearing this in mind.

As described in Chapter one, a model of EGF binding has been established with binding of EGF to monomeric receptors leading to enhanced receptor aggregation. The dimerised or aggregated receptors have a higher affinity for EGF than the monomeric, non-aggregated, receptor and also possess increased PTK activity. This is supported by the work of Hurwitz *et al* whereby they produced and purified a recombinant partial EGFR (Extracellular domain) using the baculovirus/insect expression system and demonstrated that their EGF-Rx specifically bound EGF with a dissociation constant of 100nM (the Scatchard plot was linear). They also cross-linked EGF-Rx monomer to give DSS-stabilised dimers. Both monomers and dimers cross-linked in the absence of EGF had a low affinity for ligand (154, and 264nM respectively) whereas dimers cross-linked in the presence of EGF had a ten fold higher affinity for ligand (14.6nM). This suggests that binding of EGF to its receptor increases the affinity of the receptor i.e. it results in conversion to a high affinity conformation and favours the conversion of the receptor from a monomeric to a dimeric form (Hurwitz *et al*, 1991).

The low affinity binding site has been taken to be the monomeric receptor and the high affinity site the dimeric receptor. However, a recent paper by Wofsy *et al.* throws doubt on this by re-examining EGF receptor Scatchard plots. She suggests that interpretation as two binding sites is incorrect since it predicts that the Scatchard plot will display positive cooperativity. This is the opposite to what is seen experimentally (Brabyn and Kleine, 1995). They offer the alternative suggestion that EGF will bind with a higher affinity to a receptor in a dimer with both sites free rather than to a receptor in a dimer with one site already occupied. This would allow for the negatively curved Scatchard plot since there is negative cooperativity between the receptors in each dimer. This means that both high and low affinity sites could affect the signal transduction of the EGF R. This is unusual since biological activities are in most cases transduced via the high affinity complex but in the case of EGF, binding and biological activity correlate strongly and suggest that low and high affinity binding are affected similarly (Walker *et al.*, 1990).

The dissociation constants, for both low and high affinity sites, between the three cell lines were, surprisingly, not the same. One would readily expect the number of receptors to vary but not necessarily the affinity of EGF for its receptor. However, if one looks at a few examples of the previous work conducted in placental cells, as outlined in Table 4.1., one can see that a wide range of values has been reported by different authors. The constants obtained in these experiments are consistent with previously published data (Lai and Guyda, 1984; Alsat *et al.*, 1993). More importantly, what is consistent across all the whole cell culture experiments is the existence of more than one binding site for EGF and the presence of more low affinity sites than high affinity sites.

If one summates the number of binding sites (i.e. both high and low affinity) the JAr, BeWo and Jeg-3 cells possess 57,282, 11,692 and 17,746 receptors per cell respectively. In comparison, rat granulosa cells have approximately 5,000 receptors/cell, fibroblasts 100,000/cell and A431 cells approximately 2 million receptors/cell (Carpenter *et al.*, 1975; Haigler *et al.*, 1978). The magnitude of the cell response to EGF could be due directly to the differences in receptor number.

The difference in affinities could be explained by the existence of different combinations of dimers. In the introduction to this work I explained the existence of homodimers and heterodimers (section 1). Perhaps EGF has different affinities for erbB-1/erbB-1 dimers in comparison with erbB-1/erbB-2 or erbB-1/erbB-3

heterodimers. If this were true then the affinity and subsequent activation by action of EGF would depend upon the ratio of EGFR to homologues on the cell surface and therefore the differences observed between my cell lines could depend upon the differential expression of erbB-1 to erbB-4. Recent work has shown that EGFR does in fact have an increased affinity for dimerising with erbB-2 in preference to homodimer formation (Qian *et al* 1994) so this is a feasible explanation.

Alternatively, the differences in affinity could be accounted for by the degree of glycosylation on the extracellular portion of the EGFR of which there are 12 potential sites. In the Hurwitz paper mentioned above, EGF was shown to have different affinities for the synthesised EGFRx and the EGFR in A431 cells. On closer investigation it was revealed that the EGF-Rx that was produced was N-linked glycosylated and had a high mannose pattern whereas the EGFR isolated from A431 cells has a complex pattern of glycosylation (Hurwitz *et al*, 1991). Some growth factors, (e.g. HB-EGF and FGF) can bind with low affinity to cell surface proteoglycans which cannot transmit signals alone but somehow modulate the ability of the growth factor /receptor to generate a response (Schlessinger *et al*, 1995).

It is evident that there are many unanswered questions regarding the precise mechanism of EGF binding to its receptor. In the work outlined in this chapter I have found differences in EGFR number and EGF affinity for its receptor in cell-lines of similar origin. Whether this is due to discrepancies in expression of EGFR family receptors, alterations in glycosylation or simply difference in membrane composition between the trophoblast cells remains unclear.

Table 4.1. Summary of binding experiments for EGF and its receptor.

Name	Reference	System	No of sites	K _d i	K _d ii	B _{max} i	B _{Max} ii
Hurwitz	J. Biol. Chem (1991) 266: 32	Baculovirus generated EFR _x	1	100nM			
King and Cuetracasas	J. Biol. Chem (1982) 257: 3053-60	Living cells	2	100pM	2-5nM		
Yarden and Schlessinger	Biochemistry (1987) 26: 1434-1442	Detergent solubilised receptor	1	100nM			
Yarden and Schlessinger	Biochemistry (1987) 26: 1443-1451	Detergent solubilised receptor	1	40nM			
Alsat	J. Clin. Endocr. Metab (1991) 73:288-295	Cultured human trophoblast cells from					
		a) Early placenta and b) Term placenta	2 2	$1.53 \pm .21 \times 10^{-10}$ $1.41 \pm .27 \times 10^{-10}$	$4.07 \pm .035 \times 10^{-9}$ $3.72 \pm .28 \times 10^{-9}$	$3.4 \pm .04$ pmol / μ g protein $.61 \pm .07$ pmol/ μ g protein	2.32 ± 0.3 pmol/ μ g protein $3.48 \pm .27$ pmol/ μ g protein
Cao	Endocrinology (1995) 136: 3163-3172	Jeg-3 nuclei	1	No quantitative	data	supplied	
Alsat	J.Cell Physiol (1993) 154: 122-128	a) Early placenta	2	1.34×10^{-10}	3.8×10^{-9}	$.33 \pm 0.04$	$3.5 \pm .45$
		and b) Term placenta	2	1.42×10^{-10}	3.8×10^{-9}	$.63 \pm 0.07$	$3.5 \pm .45$
O'Keefe	Arch Biocem & Biophys (1974) 164: 518-526	Rat liver membranes	1	1.5×10^{-9}			
		Human placental membranes	1	6×10^{-10}			
Wofsy	Biophys. J. (1992) 63: 98-110	c'1022R on vesicles	2	2.5×10^{-11}	3.2×10^{-9}		
Lai and Guyda	J. Clin. Endoc. Metab. (1984) 58: 344	Human placental cell cultures	2	$1.5-2.3 \times 10^{-9}$ (Mid) $1.2-9.5 \times 10^{-9}$ (Term)	$13.1-16.8 \times 10^{-7}$ $14.8-16.0 \times 10^{-7}$	Total receptor number 300-460(Mid) & 9000-12,000(term)fmol/mg	
Hock & Hollenberg	J. Biol. Chem. (1980) 255: 10737-43	HPM	1	4×10^{-10}		20pmol/mg protein	

CHAPTER FIVE

THE EFFECT OF PARATHYROID HORMONE (1-34) AND PARATHYROID HORMONE RELATED PEPTIDE (1-34) ON EPIDERMAL GROWTH FACTOR RECEPTOR EXPRESSION.

5.1. Introduction.

In the previous two chapters I established the presence of and characterised epidermal growth factor receptors in the choriocarcinoma cells. Having determined the nature of these receptors I decided it would be of paramount interest to investigate possible interactions with other hormones found in the placenta. Therefore this chapter concentrates on the effect of factors which may affect EGFR expression.

It is well known that the rate of transcription of the EGFR gene can be regulated by EGF itself (Clark *et al*, 1985; Earp *et al*, 1988) and by several other factors such as phorbol esters (Clark *et al*, 1985), retinoic acid (Thompson and Rosner, 1989), angiotensin II, vasopressin (Earp *et al*, 1988), TGF β (Thompson *et al*, 1988) and testosterone (Noguchi, 1991) - although the mechanisms behind regulation of receptor synthesis remain unknown.

Recent studies have suggested that the human placenta is a target organ for Parathyroid hormone (PTH) (Lafond *et al*, 1988) (Rodda *et al*, 1988) and Parathyroid Hormone Related Peptide (PTHrP) (Asa *et al*, 1990) (Kramer *et al*, 1991). In cultured human trophoblastic cells from early and term placentae, PTH (1-34) and PTHrP (1-34) increase EGF binding to its receptor and an increase in EGFR mRNA (Alsat *et al*, 1991, Alsat *et al*, 1993b).

I wanted to see if similar effects of PTH(1-34) and PTHrP(1-34) upon EGFR expression were observed in the JAr choriocarcinoma cell line. Also, since the work of Alsat *et al*, new research into the actions of PTH and PTHrP have

suggested that the two peptides may act via different receptors (Inomata *et al*, 1995) (Behar *et al*, 1996) which may in turn result in different effects upon the cell - a phenomenon which would have been overlooked in earlier studies.

Qualitative experiments were conducted by covalently cross-linking ^{125}I -EGF to its receptor and isolating the peptide-receptor complexes by SDS-PAGE and autoradiography. The effects of PTH (1-34) and PTHrP (1-34) were looked at more closely by receptor-ligand binding studies which provided quantitative data regarding ligand affinity and receptor number.

Modulation of receptor number in vitro and perhaps subsequently in vivo could provide a handle on the control of trophoblast invasion. Also, alterations in the affinity of EGF for its receptor by paracrine influences could go some way to explaining the balance being maintained during trophoblast invasion/growth arrest.

5.2. Aims.

The principle objective was to determine the precise nature of EGF binding to its receptor in the JAr cell line in the presence of PTH (1-34) and PTHrP (1-34) and to quantify any changes observed in receptor binding.

5.3. Materials and Methods.

As outlined in chapter 2.

5.4. Results.

5.4.1. SDS-Polyacrylamide Mini-gel isolation of EGF-EGFR complex, from JAr cells, following covalent crosslinking of ^{125}I -EGF. The effect of pre-incubation with PTH (1-34) and PTHrP (1-34).

The purpose of this preliminary experiment was to qualitatively investigate the binding of ^{125}I -EGF to its receptor in JAr cells in the presence of the two peptides (PTH and PTHrP).

JAr cells were sub-cultured into 75ml flasks and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) in the absence (c) or presence of 10nM PTH 1-34 (d) and 10nM PTHrP 1-34 (e). Prior to harvesting the medium was replaced with serum free RPMI for 2h, the cells washed twice with PBS (pH 7.4) and incubated with 125 I EGF for 2h at 4°C. 2mM cross-linking reagent, BS3, was added for 20 min after which the reaction was quenched with 20mM Tris-HCl. Cells were harvested, lysed and assayed for protein content. As a control 125 I EGF was cross-linked to A431 cells (a). To determine whether the binding was specific, A431 cells were incubated with 125 I-EGF and an excess of unlabelled EGF (200nM) (b). 10µg of protein was loaded onto a 6% mini-gel and separated by SDS-PAGE, dried and autoradiographed.

In figure 5.1., under all conditions a single band is observed at 170 kDa which corresponds with the molecular weight for the EGFR. However, a doublet is observed in the A431 lysate in lane a, a phenomenon which has been previously described by several workers. In A431 cells, the presence of an excess of cold unlabelled EGF (200nM) totally displaces the 125 I-EGF from its receptor and the radioactive band is lost (lane d), thus confirming that the cell-associated radioactivity is specific for the EGFR.

The autoradiograph was scanned using a GS300 transmittance reflectance scanning densitometer (Hoeffer), and analysed with Hoeffer gs365w software to give a Gaussian integration index representing the band density. This was converted to a percentage of control (100%) for cells which had no treatment.

Figure 5.2. shows the percentage change in 125 I-EGF receptor association in the JAr cells as determined by scanning densitometry. Untreated JAr cells were standardised to give a control value of 100% and the subsequent increase in band density following treatment with PTH or PTHrP calculated as a percentage of this control from the Gaussian integration value obtained. In the presence of 10nM PTH and PTHrP, band density decreased by 9% and increased by 41% respectively. This suggested that PTH caused a decrease, whereas PTHrP caused an increase, in 125 I-EGF -EGFR association. However, this gave no indication whether the differences were due to alterations in receptor number or changes in affinity of the ligand for its receptor.

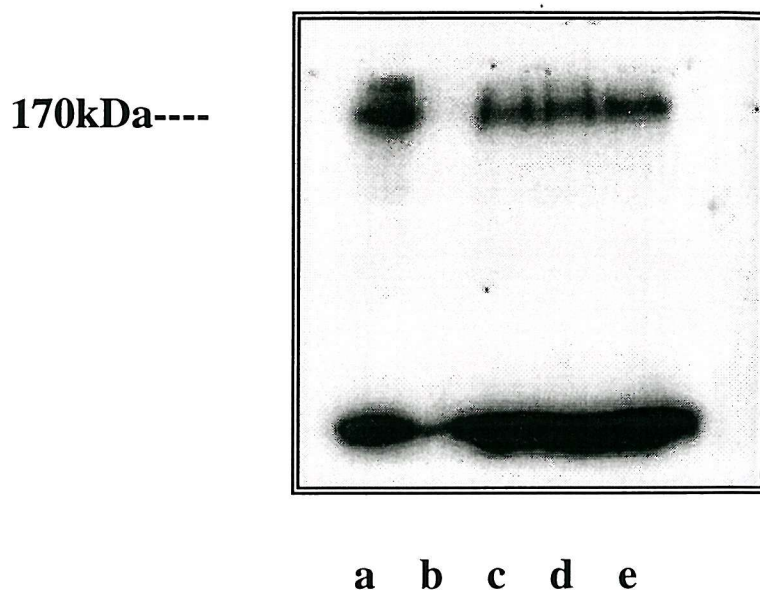


Figure 5.1. Affinity cross-linking of ^{125}I -EGF to its receptor in JAr cells in the presence of PTH (1-34) and PTHrP (1-34).

Identification of the EGFR in JAr cells by affinity cross-linking of ^{125}I -EGF. JAr cells were cultured in RPMI supplemented with 10%FCS for 48h and replaced with 2.5%SFCS for the final 24h, in the absence (Lane **c**) or presence of 10nM PTH (1-34) (Lane **d**) and 10nM PTHrP (1-34) (Lane **e**). Washed cells were incubated for 2h at 4°C with 20ng/ml ^{125}I -EGF and 2mM cross-linker reagent BS_3 as described. The samples (10 μg protein) were solubilised, subjected to SDS-PAGE (6%) and autoradiographed. A431 cells were incubated with 20ng/ml ^{125}I -EGF (**a**) and in the presence of an excess of unlabelled cold EGF (200nM) (**b**).

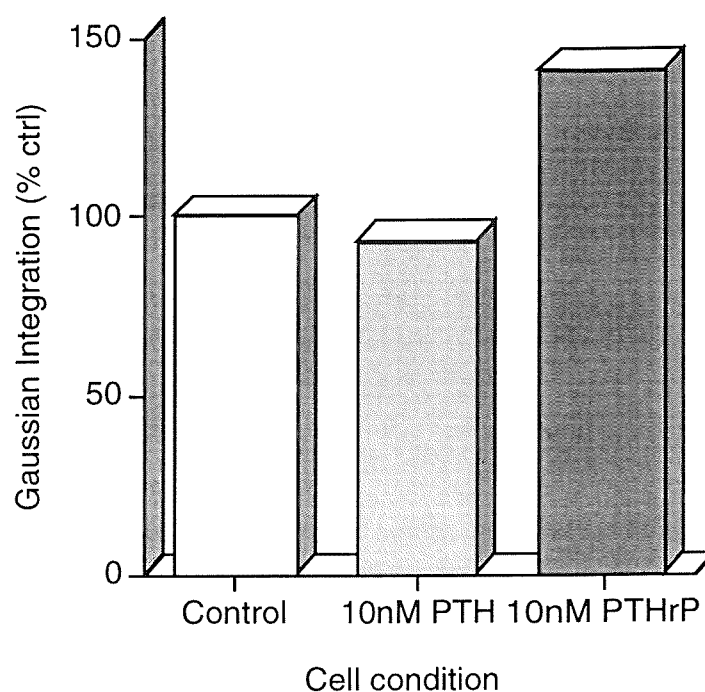


Figure 5.2. Scanning densitometry analysis of the effect of PTH and PTHrP on ^{125}I -EGF association with its receptor in JAr cells.

The data were generated from Figure 5.1. using a GS300 Hoeffer transmittance reflectance scanning densitometer. The graph shows the Gaussian integration index, as a percentage of control (100%), for JAr cells in the absence (white) or presence of 10nM PTH (shaded) and 10 nM PTHrP (dark shaded).

5.4.2. Isolation of ^{125}I -EGF-EGFR complex, from JAr and cells, by SDS-PAGE on a gradient gel (5-10%) The effect of pre-incubation with PTH (1-34) and PTHrP (1-34) on the formation of monomeric and dimeric forms of the receptor.

Having determined that PTH and PTHrP(1-34) caused a decrease and increase in the EGF receptor monomer, respectively, I subjected the cell lysates to separation on a large gradient gel (5-10%) to determine if any differences were observed in the formation of receptor dimers.

JAr cells were sub-cultured into 75ml flasks and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) in the absence (**c**) or presence of 10nM PTH 1-34 (**d**) and 10nM PTHrP 1-34 (**e**). As a further control, ^{125}I -EGF was cross-linked to A431 cells (**a**). 10 μg of protein was loaded onto a 5-10% gradient and subjected to SDS-PAGE, dried and autoradiographed.

In each case, a parallel control set of cells were incubated in the presence of an excess of unlabelled EGF (200nM) to ensure that the bands observed represented specific EGF receptor-associated radioactivity - (A431 (**b**); control (**d**), PTH (**f**) and PTHrP (**h**)).

In figure 5.3., under all treatments two bands were observed at 170 kDa and 340kDa which corresponds with the molecular weight for the EGFR in its monomeric and dimeric forms, respectively. Again, a doublet is observed for the EGFR monomer in the A431 lysate in lane **a**.

Under all conditions, in the presence of an excess of unlabelled EGF the cell-associated radioactivity is diminished, confirming that the bands observed contain specifically bound ^{125}I -EGF.

As before, the gel was subjected to scanning densitometry to determine the relative changes in band density under each condition with untreated cells being taken as control (100%). In Figure 5.4.a) one can see that in the presence of PTH, the monomeric form of the receptor (that seen at 170kDa) decreased by 9% whereas in the presence of PTHrP the monomeric band density had increased by 53%. The

results for the dimeric form of the receptor are indicated in figure 5.4.b) and show a 1% decrease and 29% increases from control when cells were treated with PTH and PTHrP respectively.

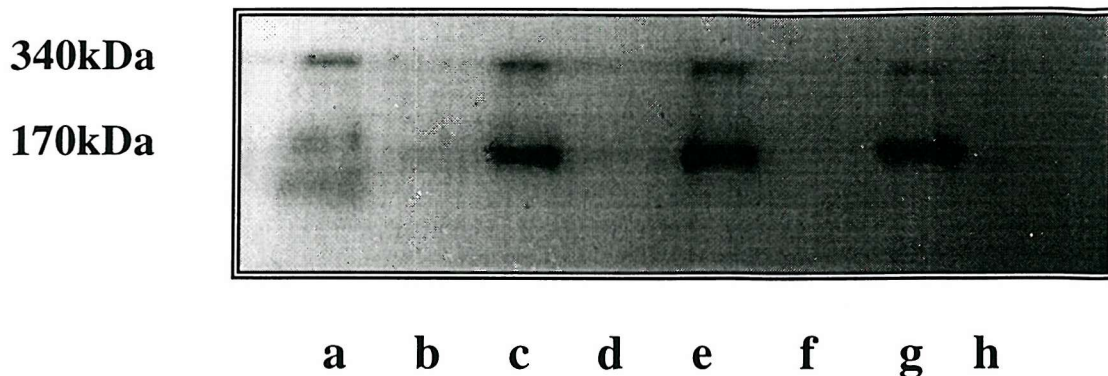


Figure 5.3. Affinity cross-linking of 125 -EGF to its monomer and dimer receptor complexes in JAr cells in the presence of PTH (1-34) and PTHrP (1-34). Separation by 5-10% gradient SDS-PAGE.

Identification of the monomeric and dimeric forms of the EGFR in JAr cells by affinity cross-linking of 125 I-EGF. JAr cells were cultured in RPMI supplemented with 10%FCS for 48h and replaced with 2.5%SFCS for the final 24h, in the absence (Lane **c**) or presence of 10nM PTH (1-34) (Lane **e**) and 10nM PTHrP (1-34) (Lane **g**). Washed cells were incubated for 2h at 4°C with 20ng/ml 125 I-EGF and 2mM cross-linker reagent BS3 as described. The samples (10µg protein) were solubilised, subjected to 5-10% gradient SDS-PAGE and autoradiographed. Specific binding was confirmed by crosslinking in the presence of 200nM excess unlabelled ligand (Ctrl (**d**); PTH (**f**) and PTHrP (**h**)). A431 cells were incubated with 20ng/ml 125 I-EGF (**a**) and in the presence of an excess of unlabelled cold EGF (200nM) (**b**). Cell associated radioactivity was observed at 170kDa and 340kDa corresponding to the monomeric and dimeric forms of EGFR respectively.

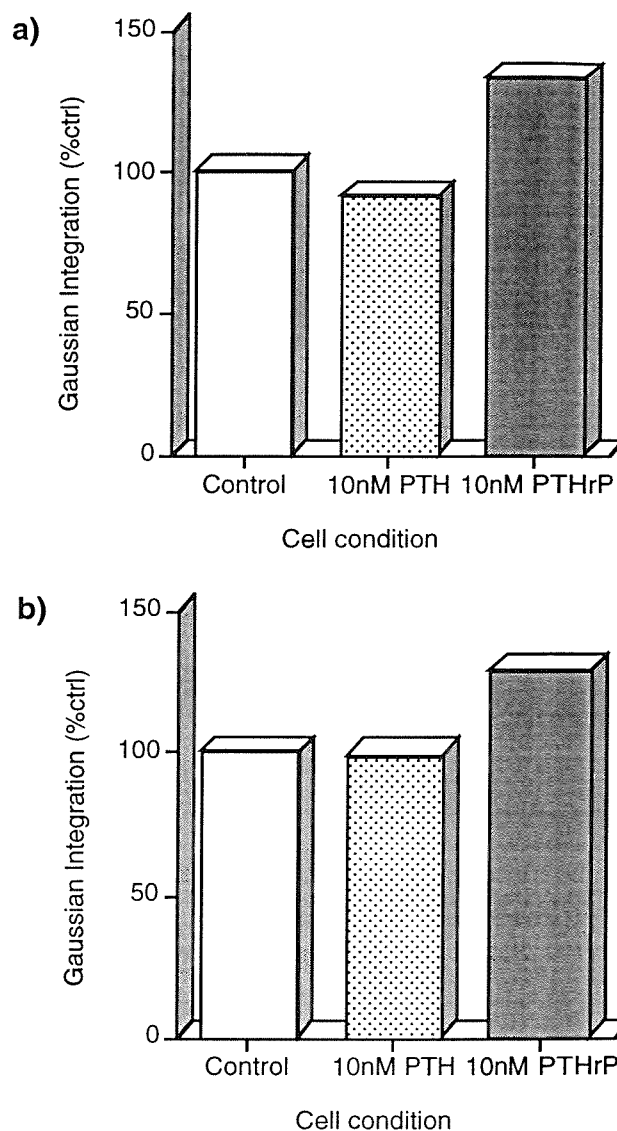


Figure 5.4. Scanning densitometry analysis of the effect of PTH and PTHrP on ^{125}I -EGF association with its monomeric and dimeric receptor species in JAr cells.

The data were generated from Figure 5.3., using a GS300 Hoeffler transmittance reflectance scanning densitometer, and show the Gaussian integration index for JAr cells in the absence (white) or presence of 10nM PTH (shaded) and 10nM PTHrP (dark shaded). Figure 5.4. a) shows the band density as a percentage of control for the EGFR monomer whereas 5.4.b) shows the changes in band density of the dimeric receptor complex.

5.4.3. Saturable binding of ^{125}I -EGF binding to EGFR in JAr cells.

Although SDS-PAGE gave an indication of changes in receptor ligand association, it only provided qualitative information. To determine precisely what was going on it was necessary to perform ligand binding studies to provide quantitative analysis of receptor-ligand kinetics.

The binding affinity of ^{125}I -EGF for its receptor and the number of binding sites present in control untreated JAr cells was determined.

Cells were grown for 72h, in 24 well plates at 37°C in 10%SFCS (replaced with 2.5%SFCS for the final 24h) and subjected to a ligand binding assay as outlined previously i.e. the cells were incubated in a total volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled ^{125}I -EGF for 2h. Figure 5.5 shows the Specific, and Non-specific binding of ^{125}I -EGF bound to its receptor in the JAr cell line at 4°C, pH 7.6 following 2h incubation. The data shown are expressed as a percentage of total binding and indicates that total binding was high (up to 76%), Specific binding was saturable (at 1-1.5 nM) and that at all concentrations non-specific binding remained lower than specific binding.

In order to obtain Kd and BMax values it was necessary to transform the data into Bound/Free ratios and carry out Scatchard analysis. Data are from a representative experiments (of 3). From the Scatchard plot in Fig 5.6. a curvilinear plot, characteristic of multiple binding sites, yielded two Kds - a high affinity site ($3.0 \pm 0.12 \times 10^{10} \text{M}^{-1}$) and a low affinity site ($59.0 \pm 1.4 \times 10^9 \text{M}^{-1}$) with 21,231 and 40,280 receptors per cell respectively.

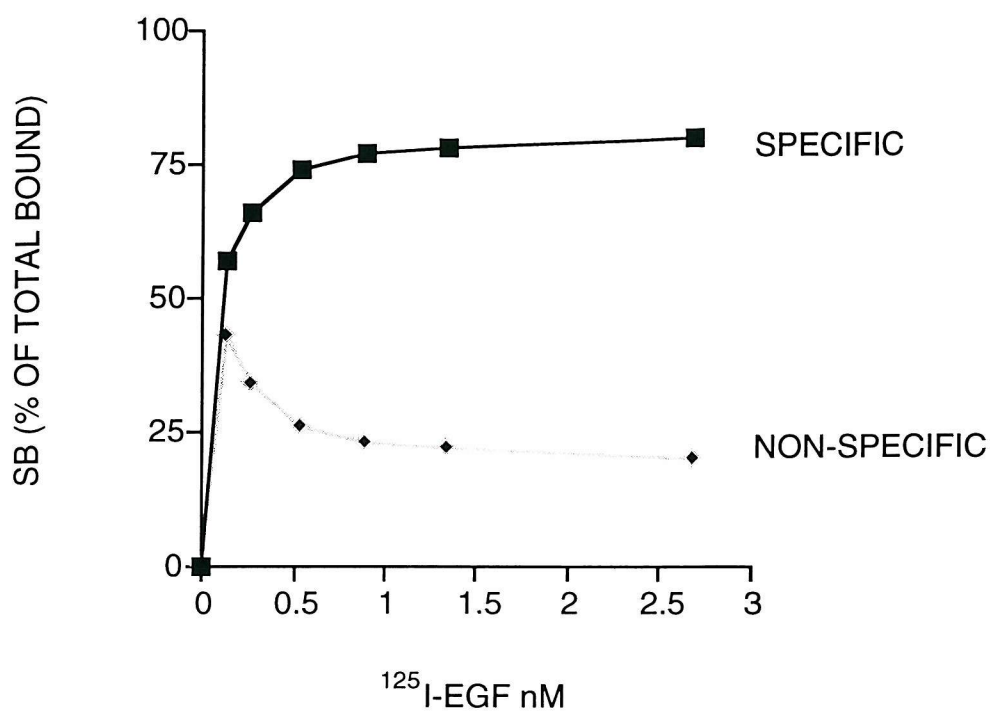


Figure 5.5. Control binding of $^{125}\text{I-EGF}$ to Jar choriocarcinoma cells.

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.10. The cells were incubated with various concentrations of $^{125}\text{I-EGF}$ over the range (0-3nM) to yield total binding. Data shown is representative of one experiment, which was repeated in triplicate. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB).

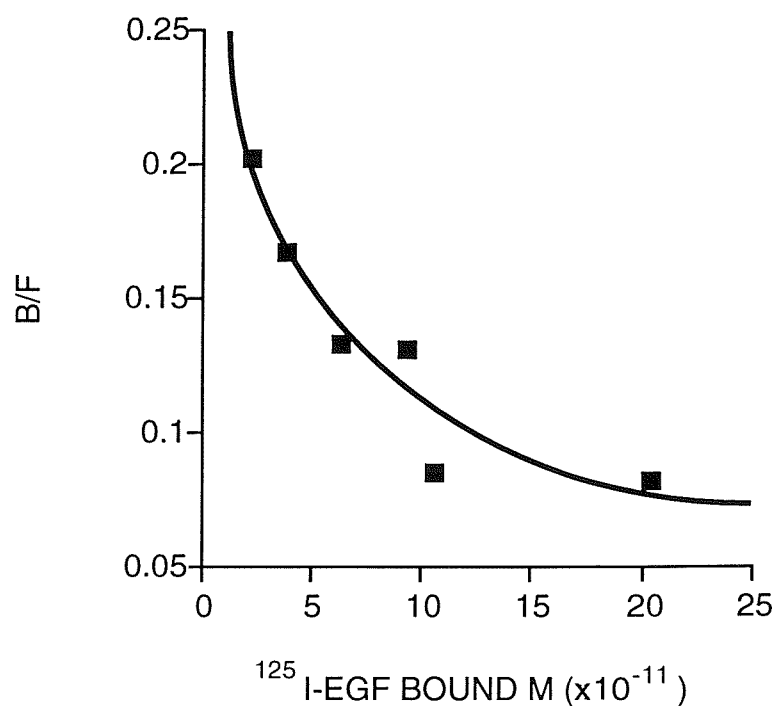


Figure 5.6. Scatchard analysis of $^{125}\text{I-EGF}$ binding data in JAr cells

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C , 5% CO_2 in air before the start of the binding assay. The cells were incubated for 2h at 4°C , pH 7.6 with increasing concentrations of $^{125}\text{I-EGF}$ (0-3nM) and cell associated radioactivity measured as outlined in section 2.10. Data are from a representative experiment calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

5.4.4. The effect of PTH (1-34) upon ^{125}I -EGF binding to its receptor in JAr cells.

To examine the effects of PTH (1-34), cells were grown for 72h, in 24 well plates at 37°C in 10%SFCS (replaced with 2.5%SFCS for the final 24h) in the presence of 10 nM PTH (1-34). The medium was changed after 24h and fresh PTH was added for the final incubation period. The cell-line was subjected to a ligand binding assay as outlined previously i.e. the cells were incubated in a total volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled ^{125}I -EGF for 2h. Figure 5.7 shows the Specific, and Non-specific binding of ^{125}I -EGF bound to its receptor in the JAr cell line in the presence of 10nM PTH (1-34). The data shown are expressed as a percentage of total binding and indicates that total binding was high (up to 80%), specific binding was saturable (at 1.5-2 nM) and at all concentrations non-specific binding remained lower than specific binding.

The data were transformed into Bound/Free ratios to perform Scatchard analysis in order to obtain Kd and BMax values (Fig 5.8). Data are from a representative experiment (of 3). Surprisingly, a linear plot was obtained, unlike that observed in control experiments, suggesting that only a single binding site was present. The Kd of $1.5 \pm 0.09 \times 10^{10} \text{M}^{-1}$ suggested a high affinity receptor class with approximately 41,800 binding sites per cell.

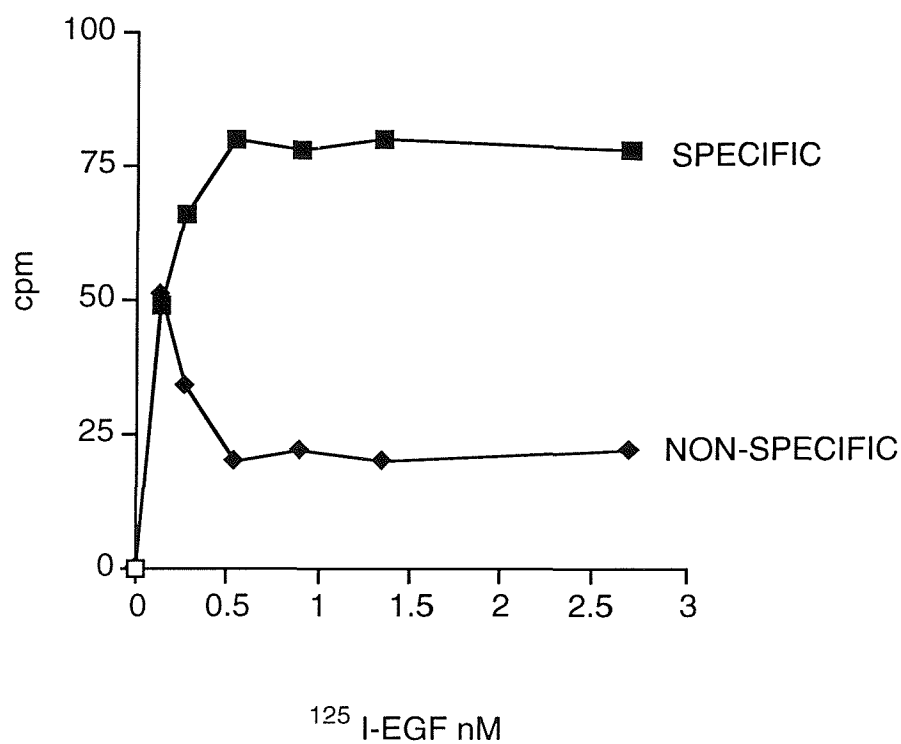


Figure 5.7. Effect of PTH (1-34) on binding of $^{125}\text{I-EGF}$ to JAr choriocarcinoma cells.

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay in the presence of 10nM PTH (1-34). The assay was carried out as described in section 2.10. The cells were incubated with various concentrations of $^{125}\text{I-EGF}$ over the range (0-3nM) to yield total binding. . Data shown are representative of one experiment, which was repeated in triplicate. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB).

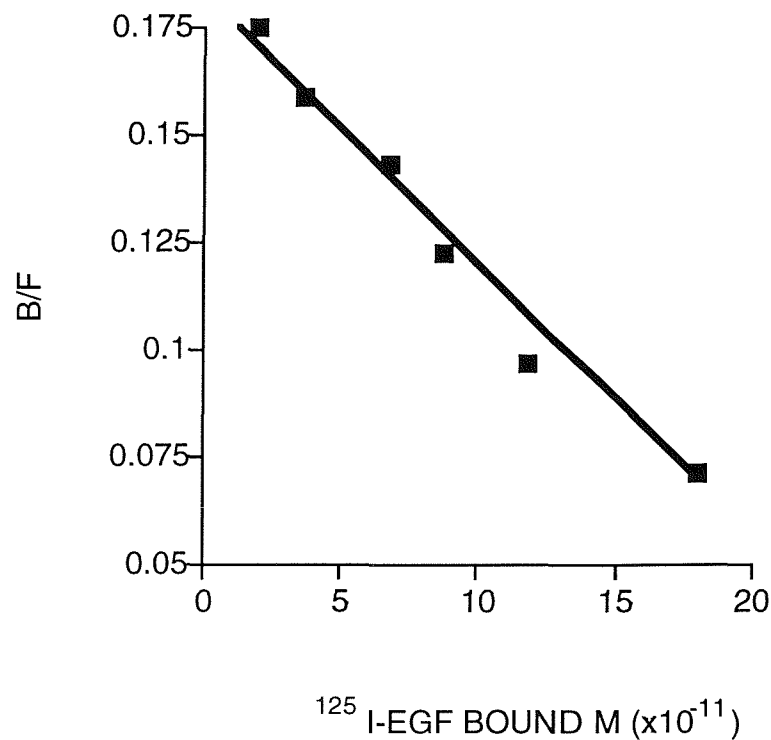


Figure 5.8. Scatchard analysis of $^{125}\text{I-EGF}$ binding data in the presence of PTH (1-34) in JAr cells

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown in the presence of 10 nM PTH (1-34) for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C , 5% CO_2 in air before the start of the binding assay. The cells were incubated for 2h at 4°C , pH 7.6 with increasing concentrations of $^{125}\text{I-EGF}$ (0-3nM) and cell associated radioactivity measured as outlined in section 2.10. Data are from a representative experiment calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

5.4.5. The effect of PTHrP (1-34) upon ^{125}I -EGF binding to its receptor in JAr cells.

To examine the effects of PTHrP (1-34), cells were grown for 72h, in 24 well plates at 37°C in 10%SFCS (replaced with 2.5%SFCS for the final 24h) in the presence of 10nM PTHrP (1-34). The medium was changed after 24h and fresh PTHrP was added for the final incubation period. The cell-line was subjected to a ligand binding assay as outlined previously i.e. the cells were incubated in a total volume of 250 μl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% (w/v) bovine serum albumin) per well with increasing concentrations of labelled ^{125}I -EGF for 2h. Figure 5.9 shows the Specific, and Non-specific binding of ^{125}I -EGF bound to its receptor in the JAr cell line in the presence of 10nM PTHrP (1-34).

The data shown is expressed as a percentage of total binding and indicates that total binding was high (up to 85%), specific binding was saturable (at 1.5-2 nM) and at all concentrations non-specific binding remained lower than specific binding.

The data were transformed into Bound/Free ratios to perform Scatchard analysis in order to obtain Kd and BMax values (Fig 5.10). Data are from a representative experiments (of 3). A curvilinear plot was obtained, similar to that observed in control experiments, suggesting that more than one binding site was present. A high affinity site ($3.6 \pm 0.07 \times 10^{10} \text{M}^{-1}$) and a low affinity site ($58.5 \pm 2.88 \times 10^9 \text{M}^{-1}$) with 23,172 and 49,158 receptors per cell respectively were determined.

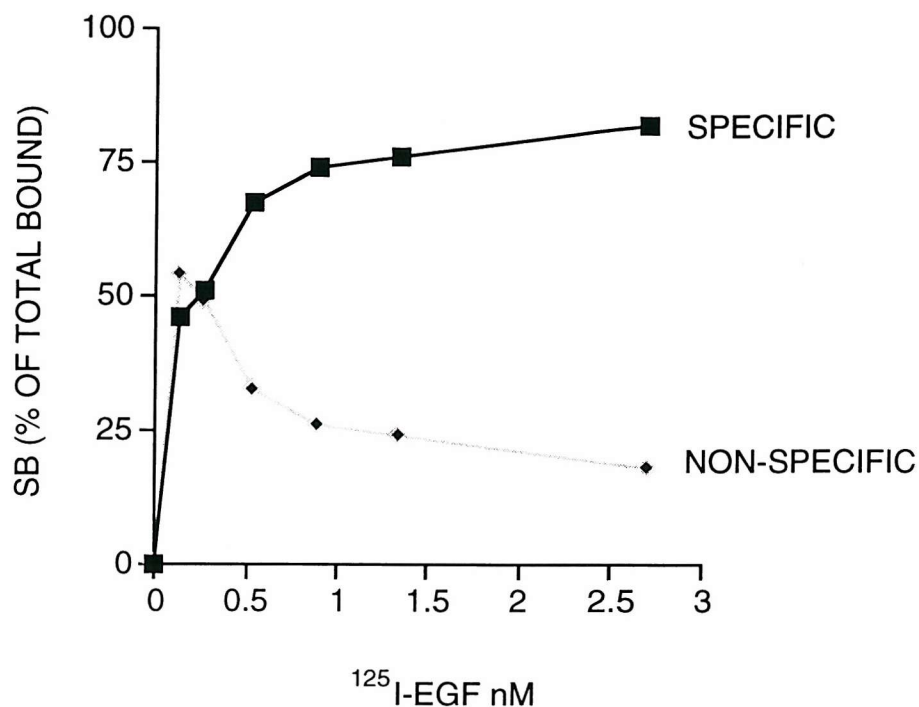


Figure 5.9. Effect of PTHrP (1-34) on binding of $^{125}\text{I-EGF}$ to JAr choriocarcinoma cells.

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay in the presence of 10nM PTHrP (1-34). The assay was carried out as described in section 2.11. The cells were incubated with various concentrations of $^{125}\text{I-EGF}$ over the range (0-3nM) to yield total binding. Data shown are representative of one experiment, which was repeated in triplicate. Non-specific binding was calculated by addition of an excess of unlabelled EGF (200nM) and subtracted from the total binding to obtain specific binding (SB).

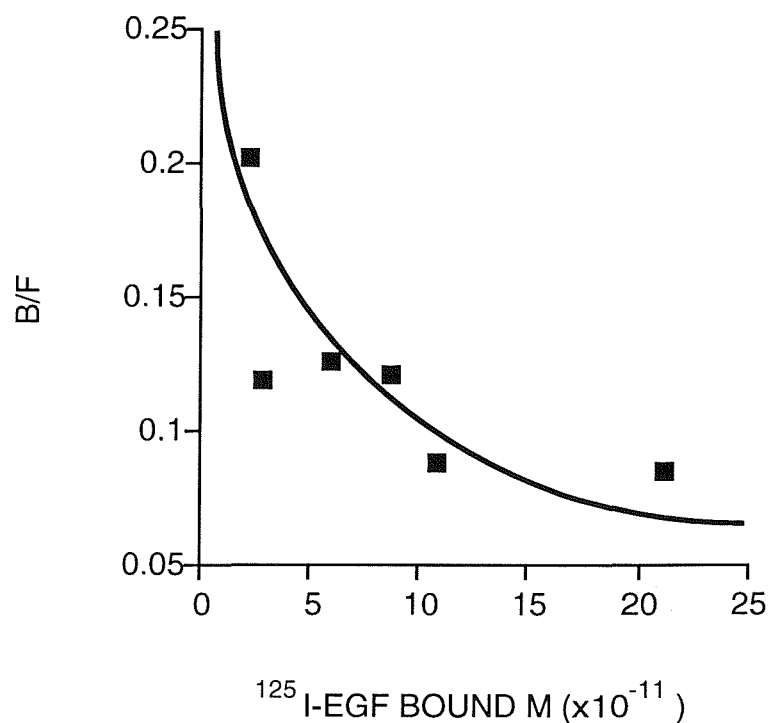


Figure 5.10. Scatchard analysis of $^{125}\text{I-EGF}$ binding data in the presence of PTHrP (1-34) in JAr cells

JAr cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown in the presence of 10 nM PTHrP (1-34) for 72h (in 10% FCS for 48h and 2.5% SFCS for the latter 24h) at 37°C , 5% CO_2 in air before the start of the binding assay. The cells were incubated for 2h at 4°C , pH 7.6 with increasing concentrations of $^{125}\text{I-EGF}$ ($0\text{--}3\text{ nM}$) and cell associated radioactivity measured as outlined in section 2.11. Data are from a representative experiment calculated using the LIGAND program (McPherson, 1985). B/F, bound/free

	Kd ($\times 10^{10} \text{M}^{-1}$)	Kd ($\times 10^9 \text{M}^{-1}$)	Receptors per cell	Receptors per cell
CONTROL	3.0 ± 0.12	59.0 ± 1.4	21,231	40,280
PTH	1.5 ± 0.09	-	41,800	
PTHrP	3.6 ± 0.07	58.5 ± 2.88	23,172	49,158

Table 5.1. Comparison of Kd and Bmax for ^{125}I -EGF binding to its receptor in JAr cells following prior incubation with PTH (1-34) and PTHrP (1-34).

5.5. Discussion.

Since hormone-receptor interactions obey the laws of mass action, the sensitivity of the cell to the hormone can be determined, in part, by the number of receptors present. However, the observed effects can also be influenced by receptor affinity or location. These mechanisms can be controlled by the natural ligand itself, *homologous regulation*, or by hormones which do not bind to the receptor, *heterologous regulation* (Bolander, 1989).

Homologous regulation: Epidermal Growth Factor decreases the number of its receptors by as much as 90%. However, it also stimulates the production of EGF receptor mRNA five-fold and this mRNA accumulation leads to increased receptor synthesis (Clark *et al*, 1985)(Earp *et al*, 1986) (Bjorge *et al*, 1987). Therefore EGF stimulates both synthesis and degradation of its receptor and the net effect is determined by the balance between these two processes. For example, A431 cells possess 1.5×10^6 EGFR/cell and this has been attributed to a high rate of receptor synthesis (12 times faster than that of human fibroblast cells) and a low rate of receptor inactivation (Krupp *et al*, 1982).

Heterologous regulation: Toxic agents such as benzopyrene decrease EGFR expression (Guyda *et al*, 1990). Retinoic acid decreases EGF binding capacity with a specific reduction in high affinity receptor number probably via a transcriptional mechanism (Roulier *et al*, 1994). Interleukin- 1α and interleukin 1 β and TNF α have been shown to stimulate EGFR expression (Steller *et al*, 1994), conversely others have shown a decrease in EGF-EGFR affinity in the presence of TNF α (Donato *et al*, 1989) (Bird *et al*, 1989).

In human breast cancer cells, oestrogen transiently induces EGFR mRNA levels 2-3 fold with a subsequent down-regulation. (Yarden. R. *et al*, 1996). The sequence of the EGFR promoter region reveals putative oestrogen-responsive elements capable of binding oestrodiol receptors and this is the direct evidence that the EGFR gene can be regulated directly by oestrogen (Yarden R. *et al*, 1996).

Therefore it is apparent that the number of available EGFR on the cell surface can be modulated by a wide range of hormones. In this chapter, I investigated the effects of PTH (1-34) and PTHrP (1-34) upon EGFR expression in the JAr cell-line

Following exposure to PTH, the amount of ^{125}I -EGF bound to its receptor decreased. Conversely the response to PTHrP was an increase in receptor associated radioactivity when analysed by mini SDS-PAGE. However, it was unclear whether binding had been affected in both the monomeric and dimeric forms of the receptor - therefore a larger gradient gel (5%-10%) was cast and the samples run out over a wider range to obtain better separation. It was apparent that radiolabelled EGF was binding to both single and aggregated receptors due to the location of bands corresponding to 170kDa and 340kDa. In addition, binding was specific for the EGFR because in the presence of an excess of unlabelled EGF(200nM), the radiolabelled EGF was totally displaced. Scanning the bands using a GS300 Hoeffler transmittance reflectance scanning densitometer revealed that PTH decreased monomeric ^{125}I -EGF-EGFR binding by 9%; Radioactive ligand association with the dimer was also decreased albeit by 1%. In comparison, PTHrP caused an increase in ^{125}I -EGF binding in both the 170 kDa and the 340 kDa species by 53% and 29%, respectively.

Again, in the presence of an excess of cold unlabelled EGF (200nM) binding of the radiolabelled peptide was completely abolished, confirming that the ^{125}I -EGF was binding specifically to its receptor.

However, I did not know if these changes in receptor associated radioactivity were due to an alteration in ^{125}I -EGF affinity for its receptor or whether the actual number of receptors had altered. In order to distinguish between these possibilities it was necessary to repeat the binding kinetic experiments of the previous chapter but this time with prior exposure of the cells to PTH/PTHrP.

In control experiments, a curvilinear Scatchard plot was obtained which was consistent with the existence of two ^{125}I -EGF binding sites - a high affinity site $K_d = 0.3\text{nM}$ and a low affinity site $K_d = 59\text{nM}$ with 21,231 and 40,280 receptors per cell respectively. Non-specific binding in the presence of an excess of unlabelled EGF (200nM) remained low throughout.

The addition of PTHrP (1-34) to the culture conditions prior to ligand binding analysis again generated a curvilinear Scatchard plot, with no significant change in receptor affinity ($K_d = 0.36\text{nM}$ and $K_d = 58.5\text{nM}$). However, the B_{max} for both sites had increased to 23,172 and 49,158 receptors per cell for the high and low affinity states respectively. This clearly shows that the increase in ^{125}I -EGF

receptor association was due entirely to an increase in receptor number without alterations in the affinity of the ligand for its receptor.

The PTH story was less clear cut. From SDS-PAGE analysis it was apparent that in the presence of 10nM PTH (1-34) ¹²⁵I-EGF association with its receptor was reduced. Interestingly, ligand binding studies of EGF to its receptor in the presence of PTH provided a linear Scatchard plot which is indicative of a single binding affinity. The K_d of 1.5 nM of this site suggested it was a high affinity site and that the number of receptors had decreased to approximately 42,000 sites per cell. This was not quite the result I expected since two bands (MW 170 and 340 kDa) were observed on the gradient gel. If PTH truly abolished the existence of the low affinity state then one would have expected to see a single band on the gel since it is widely believed that the two affinity states can be attributed to the monomeric and dimeric species.

PTH has been shown to down-regulate EGFR in clonal osteoblastic mouse calvarial cells, MC 3T3-E1, supposedly via a cAMP-dependent cytoskeleton mechanism (Ohta *et al*, 1989); Lafond *et al* have demonstrated the activation of adenylate cyclase in the basal plasma membrane of syncytiotrophoblast following binding of PTH (Lafond *et al*, 1988). In the placenta, adenylate cyclase is absent from the brush border membrane and since the placenta contains protein kinase C activity (Hamel, 1988) it is feasible that PTH activates PLC and subsequently PKC as observed in the kidney (Coleman, 1990). In addition, the effect of PTH upon EGFR can be abolished in the presence of a PKC inhibitor (Alsat, *et al* 1993b)) These two observations would support my work, since PKC activation has been shown to reduce EGFR affinity by phosphorylation of threonine 654 on the EGFR (Countaway *et al*, 1990). Perhaps this reduction in affinity is sufficient to cause the complete disappearance of the low affinity state yet insufficiently potent to override the formation of high affinity complexes? The two radiolabelled bands (MW 170 and 340k Da) could be accounted for by the presence of internalised EGF-EGFR complexes which would not have been available for binding on the cell surface resulting in the observation of a single, high affinity site in the ligand-binding study.

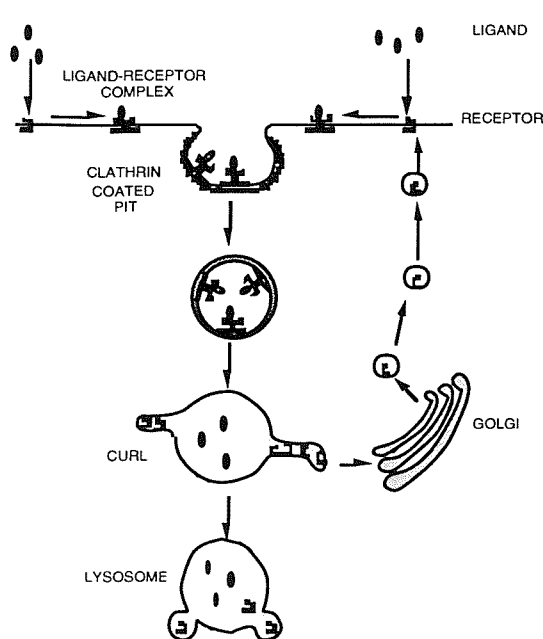
What is clear is that the effects of PTH and PTHrP upon EGFR regulation are different. Alsat *et al*, in 1993, showed that PTH and PTHrP treated cells showed an increase in the number of high affinity sites but no change in their affinity but neither the K_d or B_{max} of the low affinity receptors were affected. In their study, PTH caused a 4-5 fold increase in EGFR and PTHrP a 3-4 fold increase. They

only observed one band at 170kDa when ^{125}I -EGF was affinity cross-linked to its receptor and the relative increases in band density in the presence of the peptides did not correlate well with their binding data! EGFR mRNA transcripts (10.5kb and 5.8kb) were elevated in the presence of both PTH and PTHrP from which, Alsat concluded that these peptides regulate EGFR gene transcription (Alsat, *et al* 1993b). However, mRNA levels could have been enhanced by other processes, for example, inhibition of mRNA degradation. In addition, Alsat's work was conducted in cells from term placenta whereas the JAr cells may afford a better representation of invasive first trimester trophoblast.

PTHrP (1-34) has been shown to alter the cholesterol/phospholipid concentration in the basal plasma membrane (BPM) and brush border membrane (BBM) of syncytiotrophoblast (Lafond *et al*, 1993) and these changes in membrane fluidity could affect the migration of EGFR in the cell membrane thus favouring activation and subsequent dimerisation.

The precise mechanisms controlling receptor regulation remain elusive as there are numerous places whereby receptor expression can be controlled (Diag 5.1). The level of cell-surface receptor can be reduced in at least three ways a) Destruction after endocytosis b) Internalisation after endocytosis and stored in intracellular vesicles c) receptors remain on the cell surface but change so as not to bind ligand. PTH /PTHrP could inhibit any one of these processes resulting in an apparent increase or decrease in receptor number.

Diag 5.1. Model of receptor recycling (adapted from Bolander, 1989)



Following ligand binding, the EGF-EGFR complex aggregates into clathrin coated pits which then bud off and lose their clathrin coats. The endocytotic vesicle develops slender tubules - CURL (Compartment for Uncoupling Receptors and Ligands). Acidification via an ATP dependent mechanism disrupts ligand-receptor binding and some unoccupied receptors migrate into the tubules while the rest, and free ligand, remain in the vesicle. The tubules then detach and return to the plasma membrane where the receptors are recycled. Finally, the vesicles fuse with lysosomes and the receptors and ligand are degraded. The EGF receptor displays a low level of recycling activity, (only 2-3 cycles) but predominantly both the ligand and receptor are degraded by lysosomes (Carpenter, 1987).

Synthesis of new receptors is a slow process that may take more than a day but it is highly likely that the key to controlling receptor number lies in control of gene expression. The EGFR gene has been shown to possess several response elements which are capable of binding E2 and RA (Yarden, R. *et al*, 1996) (Roulier *et al*, 1994).

In order to act as a transcription factor, it would be necessary for PTHrP or PTH to penetrate the nuclear membrane and be capable of binding directly to DNA or to activate nuclear PTHrP/PTH receptors which in turn would activate nuclear transcription factors resulting in an increase in gene processing and a rise in mRNA production. PTHrP is present in the nucleolus of chondrocytic cells (CFK2) implying biological significance (Henderson, *et al* 1995). Mature PTHrP contains a nucleolar target sequence (NTS) which is capable of targeting PTHrP to the nucleolus; the NTS is contained in residues 87-107 comprising an Arginine hinge with an adjacent glutamine residue and is flanked by regions of basic amino acids (Henderson, *et al* 1995). Alternatively, PTHrP may inhibit repressor proteins, the removal of which would allow transcription to occur. However, since the NTS region is found at 87-107 aa it would be absent in PTHrP(1-34) thus ruling out this mechanism.

Perhaps, the mechanism involves a process similar to that of NF κ B - a factor which exists in the cytoplasm of lymphocytes complexed with an inhibitor. A phosphorylation event, which may be triggered by a variety of stimuli, results in dissociation of NF κ B from its inhibitor enabling the free protein to bind to its DNA site resulting in activation of the Ig gene (Darnell *et al*, 1990) (Diag 5.2.).

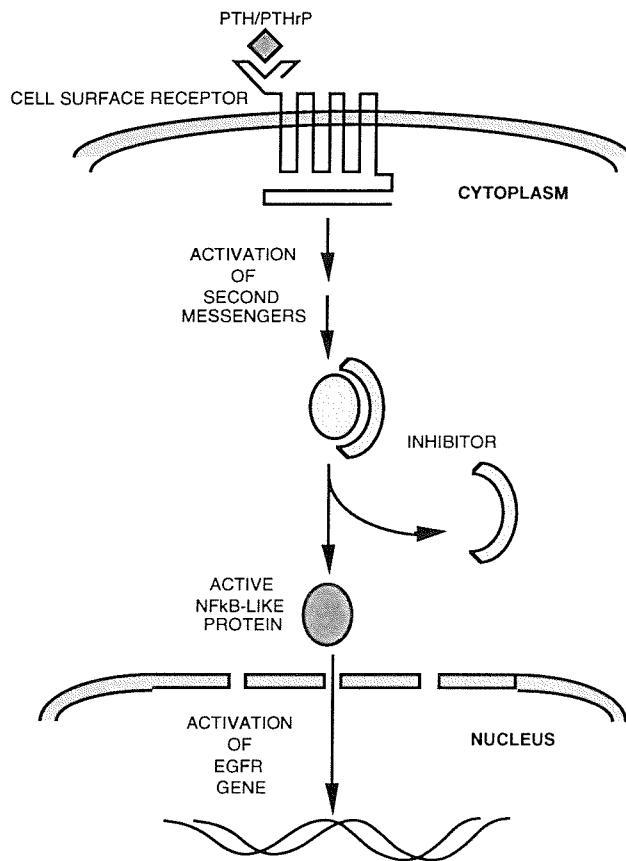


Diagram 5.2. Model of transcription factor activation by cell-surface interaction (modified from Darnell *et al*, 1990).

Inactive cytoplasmic forms of the transcription factor NF κ B can be activated by the removal of an inhibitor. The *in vivo* trigger is thought to involve phosphorylation of NF κ B. There is no evidence that PTHrP activates NF κ B but it may trigger a similar mechanism in the cell.

Differential RNA processing is the second major gene control mechanism but very little is known regarding the importance of translation control in gene expression in eukaryotes.

Recent work on the proliferative effects of PTH and PTHrP on JAr cells by Umar and Peddie (1997), support my results. They found that PTHrP (1-34) potentiated EGF stimulated JAr cell proliferation whereas PTH (1-34) had no significant effect upon EGF induced choriocarcinoma cell proliferation. This could be attributed to the respective increase and decrease in EGFR number determined in my experiments.

EGFR in cultured human trophoblast cells have been shown to spontaneously double in number during the first two days of culture (Alsat *et al*, 1993a) but it is unlikely this occurs in the choriocarcinoma cells since they do not display the morphological differentiation and aggregation observed in isolated cytotrophoblast cells associated with the rise in EGFR number.

In the placenta, several reports are in agreement that both mouse and human placental EGFRs increase in number or in binding capacity from mid- to late gestation (Deal *et al*, 1982). The highest concentrations of EGFR have been identified in very early syncytiotrophoblasts (Maruo *et al*, 1987). These findings support the hypothesis that EGFR respond mitogenically in early gestation while at term they have a predominantly absorptive role.

Sissom *et al* (1987) conducted experiments upon streptozotocin induced diabetic rats and looked at placental weight and EGFR levels, with the view that birth weight of new-borns are directly related to placental weights. In the diabetic rats, the larger placentae did not mature in many respects and there were fewer EGFR present. In particular, there was a deficiency in low-affinity EGFR which appear later, at day 21, in normal rat placentae. Even at term, the large placentae of diabetic rats were increasing in size due to cell proliferation which normally ceases in the last few days of pregnancy. They suggested a lack of mature EGF receptors but did not look at erbB-2 expression which may have been reduced. It might be more realistic to suggest that the diabetic rats failed to express the differentiating component, erbB-2, but I am unaware of any further studies into this area.

New-borns delivered to patients with Yucheng disease (rice oil disease), which occurs many years after exposure to polychlorinated biphenyls (PCBs), are always of low weight, and this has been correlated to a 60% reduction of EGFR stimulated receptor autophosphorylation (Sunahara *et al*, 1987). Whether this decrease in kinase activity is directly proportional to a decrease in EGFR binding kinetics is not certain, however, a decrease in placental EGFR numbers is observed in intrauterine growth retardation (Fujita *et al*, 1991). EGFR function is also altered in the placentae of women who smoke, probably due to benzo(a)pyrene (Wang *et al*, 1988).

Clearly, an understanding of factors contributing to the up or down regulation of the EGFR and indeed erbB-2 receptor in development of the human placenta would better define their precise role not only in normal placental growth but also in disease and fetal growth retardation. I have shown that both PTH and PTHrP

are involved in this process and may serve as useful points in the regulatory mechanism to control EGFR expression. The discrepant effect of PTH and PTHrP upon EGFR expression in JAr cells supports the possibility that the hormones act through related but different classes of receptors. Alternatively, they may bind to the same receptor but in different ways thus triggering different second messenger cascades.

Ligand binding to the PTH receptor results in activation of both adenylate cyclase and phospholipase C (Kronenberg *et al*, 1993; Jueppner *et al*, 1994). This in turn activates two discrete second messenger cascades - the IP3/DAG pathway and the adenylate cyclase/cAMP pathway (section 1.12).

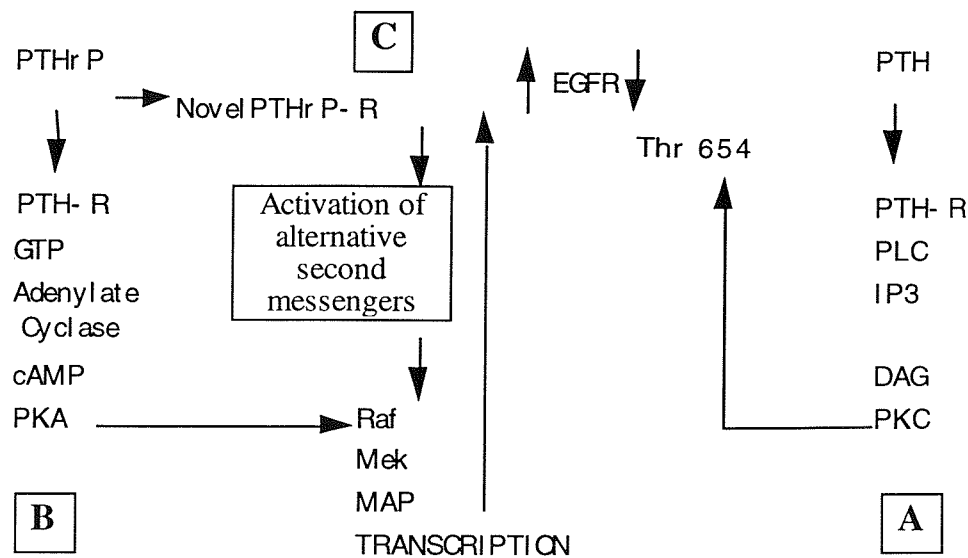
In my work, PTH caused a down-regulation in EGFR expression. Previously (section 1.5.) I highlighted the importance of threonine residue 654 on the juxtamembrane region of the EGFR. Protein kinase C (PKC) phosphorylation of this amino acid has been suggested as a negative feedback mechanism in the EGFR cycle (Hunter and Cooper, 1984). If PTH binding to its receptor activates the IP3/DAG pathway and consequently PKC, then it could result in phosphorylation of threonine 654 and activation of a negative feedback loop ultimately resulting in EGFR down-regulation. (Diag 5.3.).

PTHrP(1-34), on the other hand, caused an increase in EGFR expression which suggests that it is activating a different second messenger cascade. As previously mentioned, the classical PTH/PTHrP R is coupled to both adenylate cyclase and IP3/DAG. Initially, I thought that PTHrP (1-34) could be binding to the same receptor as PTH but to a different site so as to trigger the cAMP pathway. However, in keratinocytes and squamous carcinoma cell-lines PTHrP does not cause an increase in cAMP (Orloff, *et al*, 1995). This is supported by work in our laboratory which showed that addition of cAMP inhibited proliferation of JAr cells whereas addition of PTHrP(1-34) increased JAr cell proliferation (Umar, personal communication). This suggests that PTHrP (1-34) may act via a different receptor and provides further evidence for a unique type II receptor which is not coupled to the adenylate cyclase/cAMP pathway.

A tentative hypothesis for the interactions of PTH/PTHrP with EGF is outlined below (Diag 5.3.) but without further evidence of different receptors this explanation stands alone. Future work looking at the phosphorylation of intracellular substrates in response to the different ligands would clarify the

existence of novel second messenger pathways activated by PTHrP specific receptors.

I wanted to look at the ligand-binding characteristics of PTH and PTHrP to their receptors to see if any parallels could be drawn to the experiments carried out in this chapter. Therefore the next section focuses on the comparative binding of PTH and PTH(1-34) in the JAr cells to determine whether a novel PTHrP-specific receptor is present in the JAr choriocarcinoma cell-line.



Diag 5.3 Hypothesis of how PTH and PTHrP(1-34) may elicit their different effects upon EGF expression.

Following binding to its receptor, PTH (acting via the classical PTH Receptor) stimulates the PLC/IP3 second messenger cascade ultimately increasing intracellular levels of PKC (A). PKC has been shown to inhibit activation of EGFR by acting at Threonine 654. This could therefore account for the decrease in EGFR observed in the presence of PTH. PTHrP may also elicit its effects by association with the classical PTH Receptor. However, since the PTH-R is also able to activate the adenylate cyclase pathway, PTHrP could recruit this alternative set of intracellular substrates which in turn increase EGFR transcription (B). Conversely, PTHrP may be acting via a unique PTHrP Receptor coupled to a different second messenger pathway which leads to phosphorylation of raf, Mek and ultimately MAP kinase (C). MAP kinase could in turn activate transcription factors and thus increase transcription of the EGFR gene resulting in an increased level of EGFR protein on the cell surface.

CHAPTER SIX

INVESTIGATIVE BINDING STUDIES OF PTHrP(1-34), PTHrP(1-86), PTHrP (7-34) and PTH (1-34) IN JAR AND SAOS-2 CELLS.

6.1. Introduction.

As early as 1941, Albright suggested that tumours produced a substance with similar properties but chemically distinct from parathyroid hormone (PTH), to account for the hypercalcaemia observed in certain malignancies (Albright, 1941). In 1966, the first radioimmunoassay results for PTH were published (Berson and Yalow, 1966) although it soon became evident that the radioimmunoassay of PTH presented technical problems and that circulating concentrations of PTH as measured in these assays were extremely low. This, along with several observations in the '70s, cast doubt on the fact that PTH itself was a major contributor to this cancer syndrome and that a PTH-like substance was responsible. Finally, in the late 1980s a PTH-like substance, parathyroid hormone related peptide (PTHrP), was purified, sequenced and cloned from a cultured human lung cancer cell line, BEN. (Mosely, 1987; Suva, 1987).

PTH and PTHrP elicit their biological effects by association with receptors on the cell surface. The classical PTH/PTHrP receptor, in bone and kidney cells, belongs to a 7-membrane spanning guanine nucleotide regulatory binding protein (G-protein) -linked receptor family (Jueppner *et al*, 1991; Abou-Samra *et al* 1992). The PTH/PTHrP receptor is coupled to multiple second messenger signals resulting in a variety of biological responses (Brighurst *et al*, 1993).

Ligand-binding to the type 1 PTH/PTHrP Receptor can elicit two signal transduction mechanisms i) activation of the adenylate cyclase pathway resulting in increased cAMP levels and ii) activation of PLC γ resulting in increased IP3/DAG levels (Kronenberg *et al*, 1993; Jueppner *et al*, 1994). The signalling mechanisms of PTH and PTHrP in some non-classical PTH target tissues, for example squamous cell-lines and keratinocytes, appear to differ from those

observed in the cloned type I receptor (Orloff *et al*, 1995). PTHrP did not cause an increase in cAMP in squamous carcinoma cells although PLC γ and cellular calcium were increased (Orloff *et al*, 1995). It is suggested that multiple receptors mediate these activities and recently a human PTH2 receptor was cloned. The novel receptor has 70% sequence homology with the PTH/PTHrP receptor, yet shows a more limited ligand recognition than the latter (Usdin *et al*, 1995). It is activated by PTH (1-34) and potently antagonised by PTH(7-34) but does not bind PTHrP (1-34) (Usdin *et al*, 1995). Behar *et al* showed that the PTH2 receptor subtype is selectively activated by PTH (1-34) but not PTHrP(1-34); PTH(1-34) stimulated cAMP production and increased calcium levels whereas PTHrP (1-34) elicited no changes in cAMP or calcium accumulation in cells transfected with the PTH type II receptor (Behar *et al*, 1996). In addition, PTHrP (7-34) was shown to be a partial agonist that weakly stimulated both cAMP and increase in calcium.

At the time of writing, little is known regarding the receptors for other PTHrP peptides although a separate class of receptors in bone has been reported to associate with the carboxy terminal of PTHrP (107-139) (Seitz, *et al* 1990). The midregion form of the peptide appears to have its own receptor which mediates placental calcium transport (Abbas, *et al* 1989) and was recently defined by Orloff *et al*, (1997). A type III receptor in the supraoptic nucleus of the brain has recently been described (Yamamoto *et al*, 1997).

The existence of novel PTH/PTHrP receptors in the JAr cell-line is therefore a possibility. In the previous chapter, I demonstrated that PTH(1-34) and PTHrP(1-34) displayed different properties in the regulation of EGFR expression. In this section I wanted to look at the competitive binding of PTH (1-34) and PTHrP(1-34) to see if they were binding at the same site. In addition, I looked at the competitive displacement of ¹²⁵I-PTHrP with PTHrP(7-34) and PTHrP(1-86) since these ligands have been shown to act as partial agonists/antagonists (Usdin *et al*, 1995; Behar *et al*, 1996). PTHrP (7-34) also antagonises the action of PTHrP (1-34) in COS-7 cells (Chorev *et al*, 1990). Comparison between the binding of these compounds with similar structures, could provide information on the nature of their interaction with receptor subtypes.

The osteosarcoma cell line, SaOS-2, was used as a source of the "classic" PTH/PTHrP receptor which is purported to bind both ligands with equal efficacy (Fukayama *et al*, 1988). If the binding properties of the ligands in JAr cells were the same as that observed in SaOS-2 then one could conclude that the native

receptors for PTH/PTHrP in JAr were similar to the classical SaOS-2 receptor. Conversely, if the binding data differed, it would suggest that the JAr cell-line possessed unique receptors for PTHrP (1-34), PTH (1-34) and associated ligands.

6.2. Aims.

The purpose of these experiments was to investigate the binding properties of PTHrP (1-34), PTHrP (7-34), PTHrP (1-86) and PTH (1-34) on JAr and SaOS-2 cells. By incubating cells with a fixed concentration of radiolabelled PTHrP (1-34) I wanted to determine if the aforementioned ligands were binding to the same receptor by competitively displacing the radioactive peptide at concentrations up to 200nM.

I had anticipated conducting saturation experiments so as to gain some idea of receptor number and affinity but unfortunately was unable to complete the study successfully. However, the following experiments provide a preliminary insight into the nature of PTHrP/PTH binding in the JAr choriocarcinoma cell line

6.3. Materials and methods.

As described in section 2.11.2.

6.4. Results.

6.4.1. Competitive binding of ^{125}I -PTHrP (1-34) with PTHrP (1-34) and PTHrP (7-34) in JAr cells.

Prior to conducting ligand binding assays, JAr cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h). The culture medium was discarded, cells washed and 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of PTHrP (1-34) was added to each well in a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours. The process was repeated for PTHrP(7-34).

Figure 6.1. shows the competitive binding curve for PTHrP (1-34) and PTHrP(7-34) for ^{125}I -PTHrP (1-34). For each concentration, cpm (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). In the presence of increasing concentrations of unlabelled PTHrP (1-34), ^{125}I -PTHrP (1-34) was displaced from its receptor by up to 28% suggesting that the unlabelled peptide was competing for the same receptor as it's radiolabelled counterpart. PTHrP (7-34) was less effective at displacing the radiolabelled ligand but did show some competitive actions by reducing receptor associated radioactivity by up to 14%.

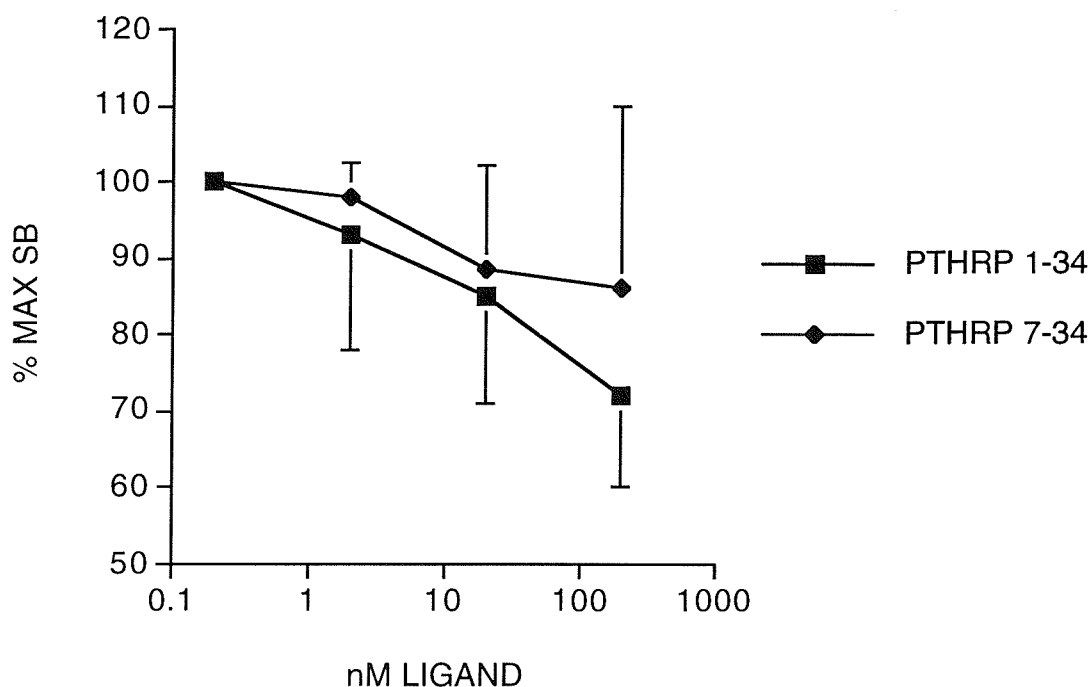


Figure 6.1 Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTHrP (7-34) in Jar cells.

JAr cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or PTHrP (7-34) in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTHrP (7-34) are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.2. Competitive binding of ^{125}I -PTHrP (1-34) and PTHrP (1-86) in JAr cells.

JAr cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h) prior to conducting ligand binding assays. 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of PTHrP (1-86) was added to each well, to a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.2. shows the competitive binding curve for PTHrP (1-86) for ^{125}I -PTHrP (1-34) over the range 0.2nM-200nM. (The displacement curve for PTHrP (1-34) is shown as a comparison).

At each concentration of competing “cold” peptide, counts per minute (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). In the presence of increasing concentrations of unlabelled PTHrP (1-86), ^{125}I -PTHrP (1-34) was not displaced from its receptor. In fact, binding appeared to be enhanced by 6% but the errors observed are large enough for this phenomenon to be ignored. What is clear, especially in comparison to the behaviour of PTHrP (1-34), is that PTHrP (1-86) is not showing the same binding properties as its smaller counterpart PTHrP (1-34). This would suggest that the two peptides are either a) binding to the same receptor but at different sites or b) binding to distinctly separate receptors.

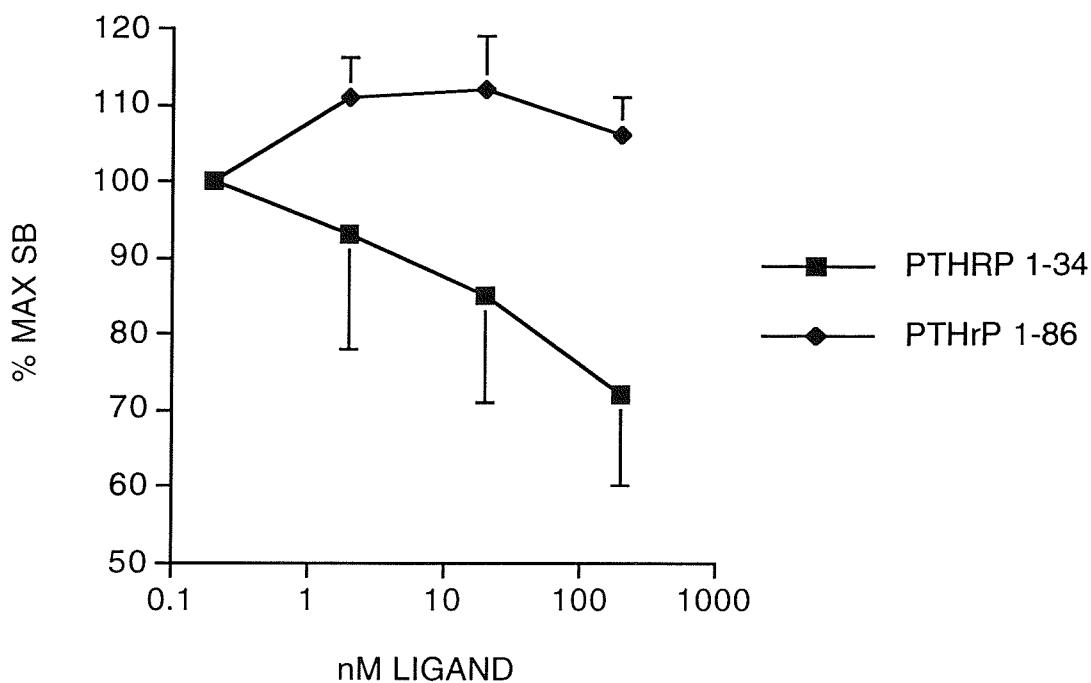


Figure 6.2 Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTHrP (1-86) in JAr cells.

JAr cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or PTHrP (1-86) in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTHrP (1-86) are indicated by the squares and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.3. Competitive binding of ^{125}I -PTHrP (1-34) and PTH (1-34) in JAr cells.

The protocol followed was the same as previously outlined above, with the results for PTHrP (1-34) shown as a comparison. JAr cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h) prior to conducting ligand binding assays. 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of PTH (1-34) was added to each well in a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.3. shows the competitive binding curve for PTH (1-34) and ^{125}I -PTHrP (1-34). (The displacement curve for PTHrP (1-34) is shown as a comparison). At each concentration of competing “cold” peptide, counts per minute (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). Increasing concentrations of unlabelled PTH (1-34) did not result in a reduction of receptor associated radioactivity thus indicating that ^{125}I -PTHrP (1-34) was not being competitively displaced from its receptor by PTH (1-34). This was similar to the result observed for PTHrP (1-34)/PTHrP (1-86) and suggests that ^{125}I -PTHrP 1-34 and PTH 1-34 are not competing for the same binding site.

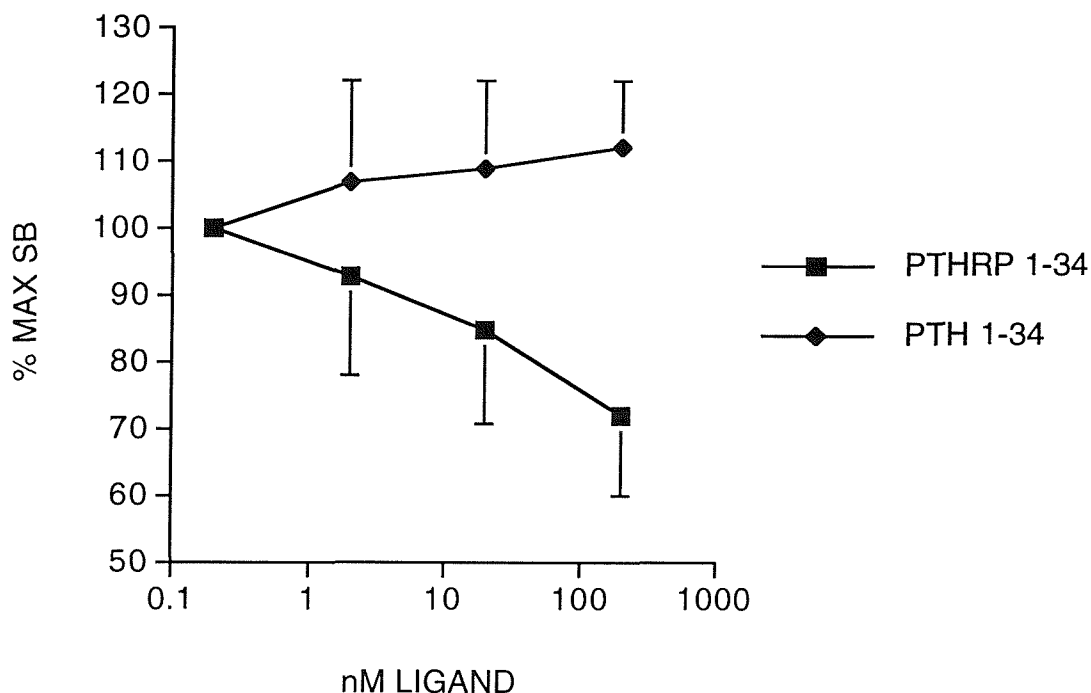


Figure 6.3. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTH (1-34) in JAr cells.

JAr cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or PTH (1-34) in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTH (1-34) are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.4. Competitive binding of ^{125}I -PTHrP (1-34) and EGF in JAr cells.

To ensure that any alterations in receptor associated radioactivity were due to competitive binding for ^{125}I -PTHrP (1-34), cells were incubated with EGF as an internal control. The protocol followed was the same as previously outlined above, with the results for PTHrP (1-34) shown as a comparison. JAr cells were plated out at a concentration of 0.5 μg DNA per well and grown for 72h in CO_2 in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h), washed and subjected to ligand binding assay. 50 μl of 10nM radiolabelled PTHrP (1-34) and 50 μl increasing concentrations of EGF was added to each well in a final volume of 250 μl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled EGF was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.4. shows the competitive binding curve for PTHrP (1-34) and EGF for ^{125}I -PTHrP (1-34). (The displacement curve for PTHrP (1-34) is shown as a comparison). At each concentration of “competing” EGF, counts per minute (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). Not surprisingly, increasing concentrations of unlabelled EGF did not result in a reduction of receptor associated radioactivity, thus confirming that ^{125}I -PTHrP (1-34) was not being competitively displaced from its receptor by EGF. This was the expected result since PTHrP (1-34) and EGF bind to totally different receptors and should show no cross-talk whatsoever!

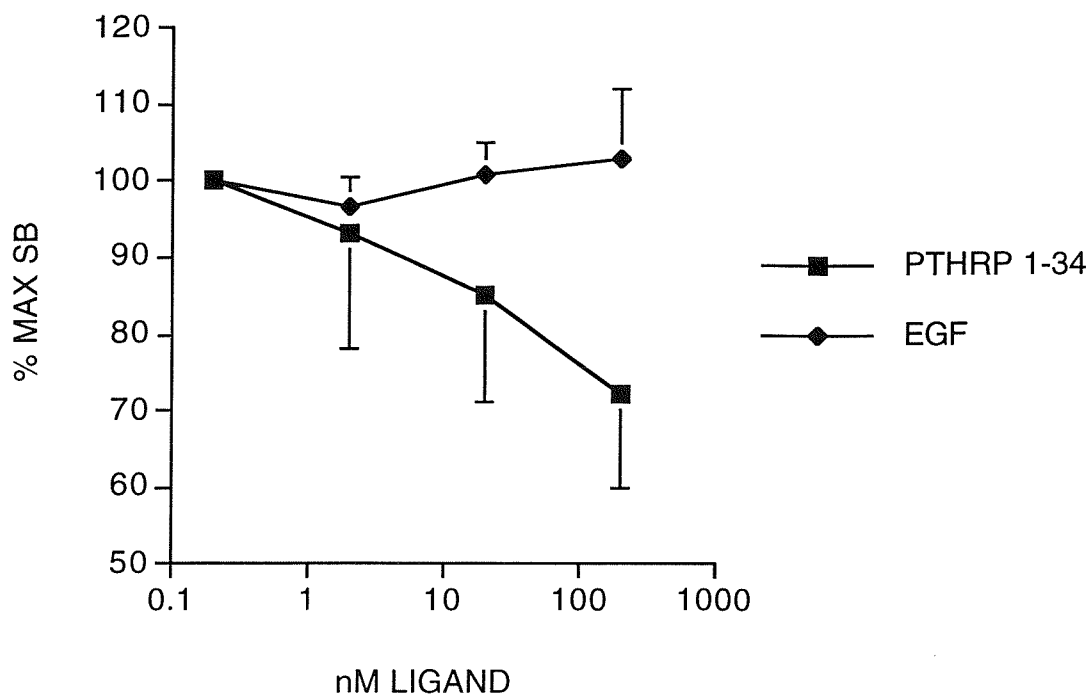


Figure 6.4. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled EGF in JAr cells.

JAr cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or EGF in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and EGF are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.5. Competitive binding of ^{125}I -PTHrP (1-34) with PTHrP (1-34) and PTHrP (7-34) in SaOs cells.

Prior to conducting ligand binding assays, SaOS-2 cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h). 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of PTHrP (1-34) was added to each well in a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours. The process was repeated for PTHrP (7-34).

Figure 6.5. shows the competitive binding curve for PTHrP (1-34) and PTHrP (7-34) for ^{125}I -PTHrP (1-34) in the SaOS-2 cell-line. For each concentration, cpm (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). In the presence of increasing concentrations of unlabelled PTHrP (1-34), ^{125}I -PTHrP (1-34) was displaced from its receptor by up to 20% suggesting that the unlabelled peptide was competing for the same binding site as it's radiolabelled counterpart. PTHrP (7-34) did not displace the radiolabelled ligand thus suggesting that the shorter peptide possessed dissimilar binding properties to its larger counterpart, PTHrP (1-34).

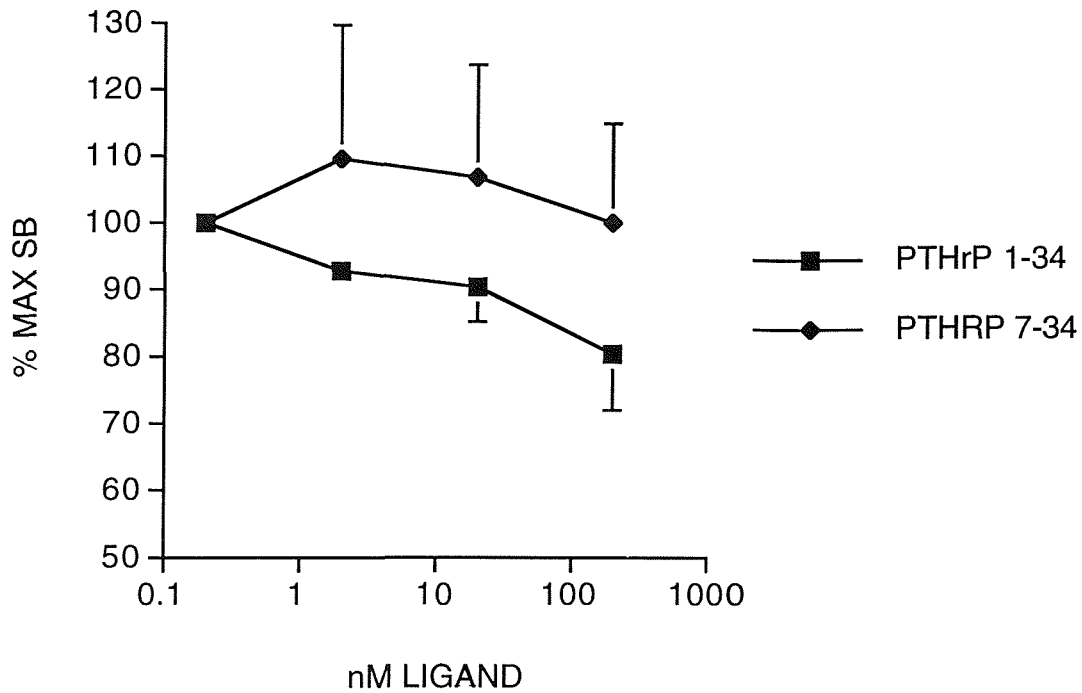


Figure 6.5. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTHrP (7-34) in SaOS-2 cells.

SaOS-2 cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or PTHrP (7-34) in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTHrP (7-34) are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.6. Competitive binding of ^{125}I -PTHrP (1-34) with PTHrP (1-86) in SaOS-2 cells.

SaOS-2 cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h) prior to conducting ligand binding assays. 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of PTHrP (1-86) was added to each well in a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.6. shows the competitive binding curve for PTHrP (1-34) and PTHrP (1-86) for ^{125}I -PTHrP (1-34). For each concentration, cpm (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). The displacement curve for PTHrP (1-34) is shown as a comparison. In the presence of increasing concentrations of unlabelled PTHrP (1-86), ^{125}I -PTHrP (1-34) was only displaced from its receptor at 200nM (by up to 4%) suggesting that the unlabelled peptide may be competing for the same receptor as its radiolabelled counterpart. However, the data were not terribly convincing.

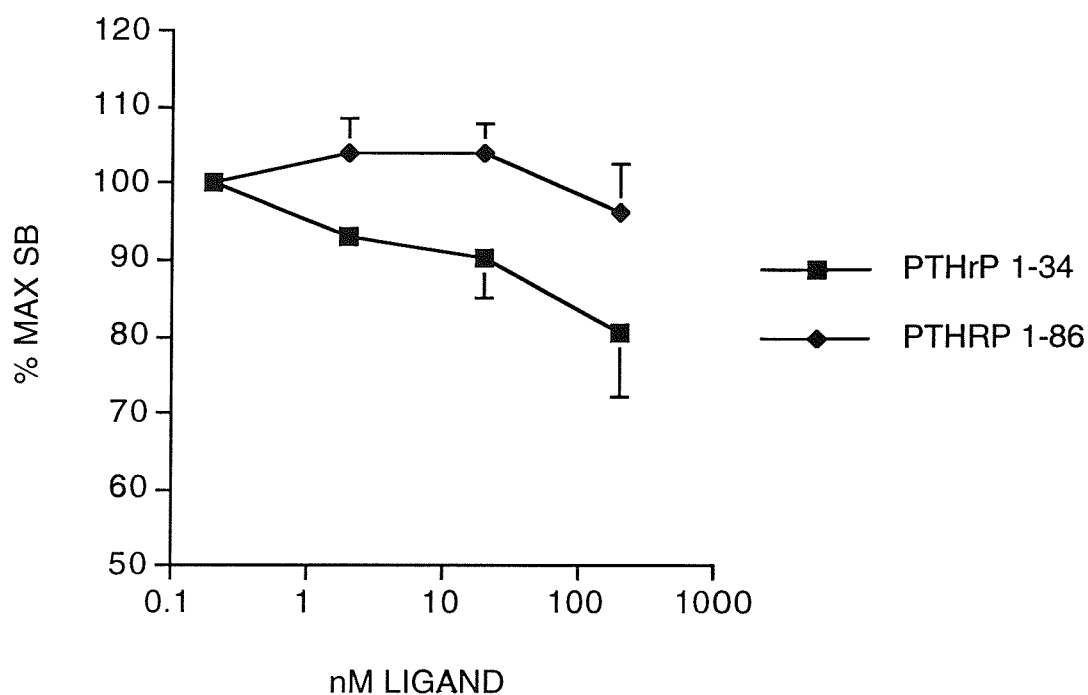


Figure 6.6. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTHrP (1-86) in SaOS-2 cells.

SaOS-2 cells were plated in 24 well culture plates ($0.5\mu\text{g}$ initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C , with $50\mu\text{l}$ of ^{125}I -PTHrP 1-34 (10nM) and $50\mu\text{l}$ of increasing concentrations of unlabelled PTHrP (1-34) or PTHrP (1-86) in the range 0.2-200nM in a final volume of $250\mu\text{l}$ binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTHrP (1-86) are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.7. Competitive binding of ^{125}I -PTHrP (1-34) with PTH (1-34) in SaOS-2 cells.

SaOS-2 cells were plated out at a concentration of 0.5µg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h) prior to conducting ligand binding assays. 50µl of 10nM radiolabelled PTHrP (1-34) and 50µl increasing concentrations of unlabelled PTH (1-34) was added to each well in a final volume of 250µl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.7. shows the competitive binding curve for PTHrP (1-34) and PTH (1-34) for ^{125}I -PTHrP (1-34). For each concentration, cpm (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). In the presence of increasing concentrations of unlabelled PTHrP (1-34), ^{125}I -PTHrP (1-34) was displaced from its receptor by up to 20% suggesting that the unlabelled peptide was competing for the same receptor as it's radiolabelled counterpart. PTH (1-34) also displaced the radiolabelled by reducing receptor associated radioactivity by up to 13%. Since both PTHrP (1-34) and PTH (1-34) were able to displace ^{125}I - PTHrP (1-34) from its receptor one can suggest that they were binding to the same site.

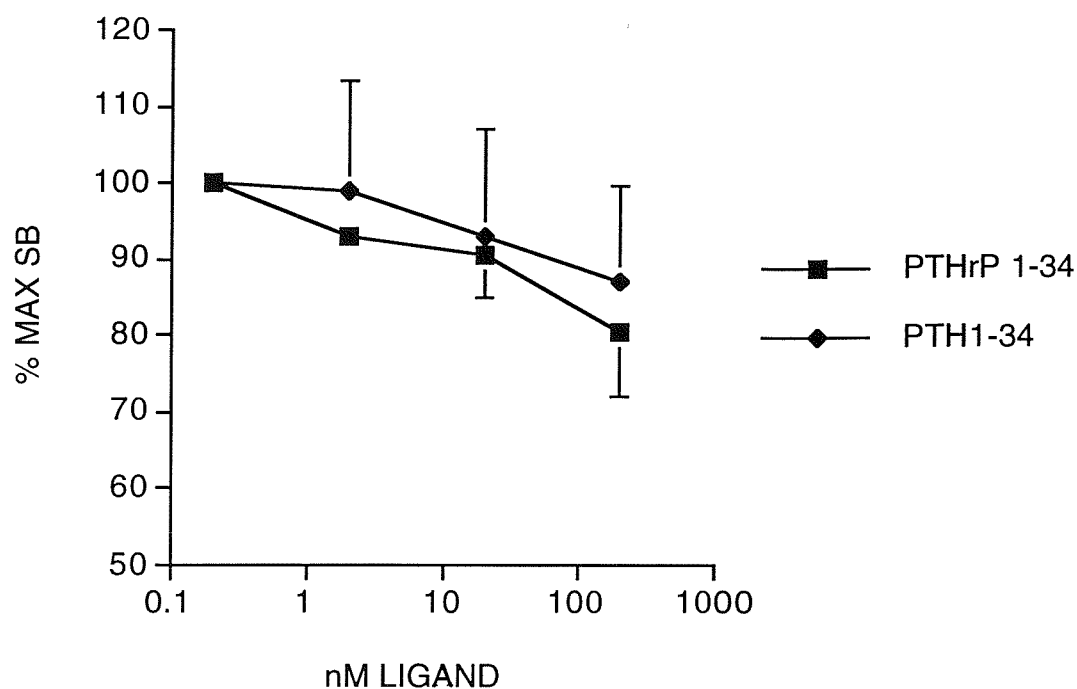


Figure 6.7. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled PTH (1-34) in SaOS-2 cells.

SaOS-2 cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or PTH (1-34) in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and PTH (1-34) are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.8. Competitive binding of ^{125}I -PTHrP (1-34) with EGF in SaOS-2 cells.

SaOS-2 cells were plated out at a concentration of 0.5 μg DNA per well and grown for 72h in CO₂ in air at 37°C (in 10% FCS for the first 48h and 2.5% SFCS for the latter 24h) prior to conducting ligand binding assays. 50 μl of 10nM radiolabelled PTHrP (1-34) and 50 μl increasing concentrations of EGF was added to each well in a final volume of 250 μl binding buffer (25mM sodium phosphate buffer, pH 7.6, containing 150mM NaCl and 0.1% w/v/ bovine serum albumin). The unlabelled ligand was added at final concentrations ranging from 0.2nM to 200nM and cells incubated at 4°C for 2 hours.

Figure 6.8. shows the competitive binding curve for PTHrP (1-34) and EGF for ^{125}I -PTHrP (1-34). For each concentration, cpm (in duplicate) from three experiments were converted to a percentage of the maximum specific binding at 0.2nM unlabelled ligand (100%). In the presence of increasing concentrations of unlabelled PTHrP (1-34), ^{125}I -PTHrP (1-34) was displaced from its receptor by up to 20% suggesting that the unlabelled peptide was competing for the same receptor as it's radiolabelled counterpart. EGF did not displace ^{125}I -PTHrP from its receptor which is not surprising since the two peptides are known to bind to discrete receptors from unrelated families. A marginal increase in ^{125}I -PTHrP (1-34) binding was observed which may have been due in part to an upregulation in its receptor in response to EGF.

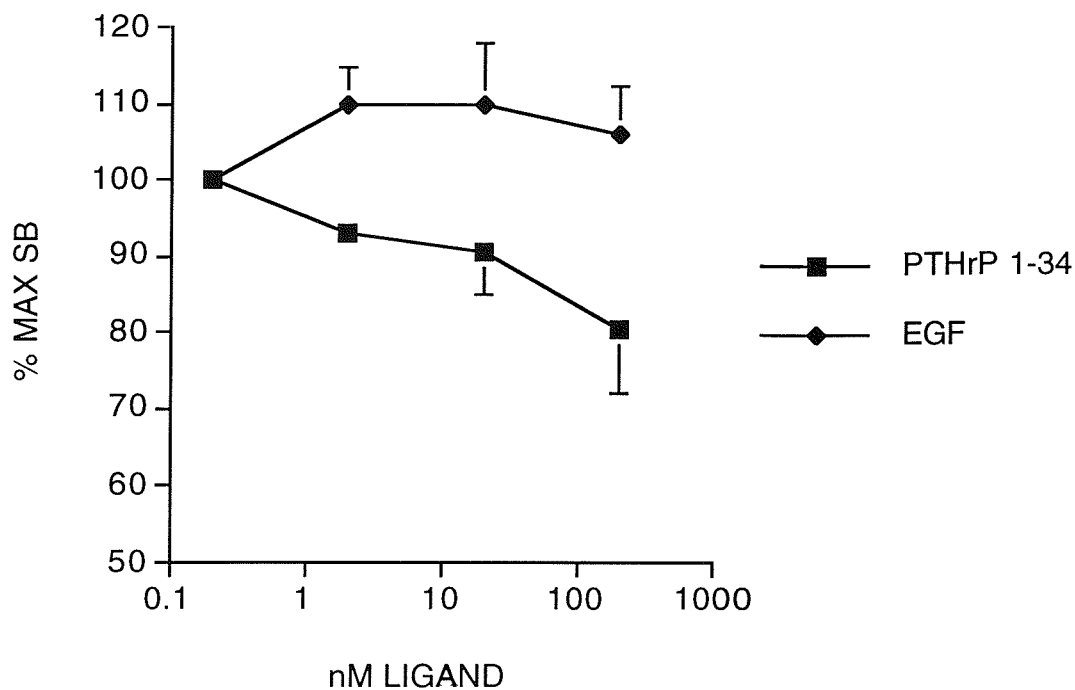


Figure 6.8. Competition for ^{125}I -PTHrP (1-34) by unlabelled PTHrP (1-34) and unlabelled EGF in SaOS-2 cells.

SaOS-2 cells were plated in 24 well culture plates (0.5 μg initial DNA/well) and grown for 72h (in 10% FCS for the first 48h and in 2.5% SFCS for the final 24h) before the start of the binding assay. The assay was carried out as described in section 2.11.2. The cells were incubated for 2h, at 4°C, with 50 μl of ^{125}I -PTHrP 1-34 (10nM) and 50 μl of increasing concentrations of unlabelled PTHrP (1-34) or EGF in the range 0.2-200nM in a final volume of 250 μl binding buffer. Displacement of radiolabelled ligand by PTHrP (1-34) and EGF are indicated by the boxes and diamonds, respectively. Results are shown as a mean percentage of maximum binding \pm s.e. of three experiments repeated in duplicate.

6.4.9. Qualitative analysis of ^{125}I -PTHrP, PTHrP (1-34) and PTH(1-34) binding in JAr and SaOS-2 cells.

JAr and SaOS-2 cells were subcultured in 75ml flasks in the appropriate media and grown for 72h (in 10%FCS for the first 48h and in 2.5% SFCS for the final 24h) at 37°C in 5% CO₂. Cells were harvested and incubated in the dark at 4°C for 1h with 10nM ^{125}I -PTHrP (1-34) and either an excess of 250nM PTHrP (1-34) or PTH (1-34). 2mM BS3 cross-linking reagent was added and the cells incubated for a further 1h under the same conditions. The cross-linking reaction was quenched with 50mM Tris and the cell lysates subjected to 6% mini SDS-PAGE. The gel was dried and autoradiographed for 72h at -70°C. In the JAr cells, an excess of unlabelled PTHrP (1-34) totally displaced the radiolabelled peptide from its receptor resulting in the loss of a band at 80kDa, confirming that both peptides were binding to the same receptor. However, in the presence of an excess of PTH (1-34), a band was observed at 80kDa, suggesting that the radiolabelled PTHrP (1-34) remained bound to its receptor and was not displaced by PTH. A similar observation was seen in the SaOS-2 cells when incubated with both labelled and unlabelled PTHrP(1-34) i.e. the peptides were competing for the same binding site. In the presence of 250nM PTH (1-34) the radioligand-receptor associated band was diminished suggesting that PTH (1-34) was binding to the same site as the ^{125}I -PTHrP (1-34).

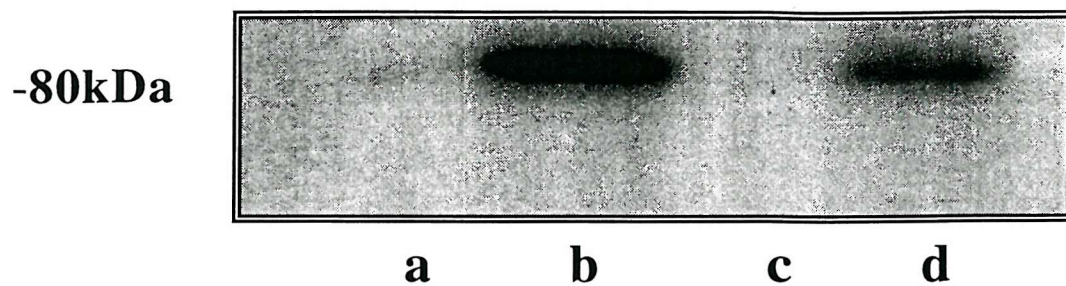


Figure 6.9. SDS -PAGE analysis of binding of PTHrP (1-34) and PTH (1-34) to their receptors in JAr and SaOS-2 cells

Confluent JAr and SaOS-2 cells were incubated in the dark in the presence of competing 250nM PTHrP (1-34) and 250nM PTH (1-34) and 10nM ^{125}I -PTHrP(1-34) for 1h at 4°C. Peptides were cross-linked to receptors using cross-linker reagent BS3 as described. The samples were solubilised and separated by SDS-PAGE on a 6% mini-gel, dried and subjected to autoradiography. Lanes **a** and **b** show the cross-linking of ^{125}I -PTHrP (1-34) to its receptor in JAr in the presence of PTHrP (1-34) and PTH (1-34) respectively. Lanes **c** and **d** show the cross-linking of ^{125}I -PTHrP (1-34) to its receptor in SaOS-2 in the presence of PTHrP (1-34) and PTH (1-34) respectively. The band visible at 80kDa corresponds to the molecular weight of the classical PTH/PTHrP receptor.

6.5. Discussion.

The field of new actions of PTH and PTHrP is expanding rapidly and both the molecular and cellular pathways involved in these actions are being explored thoroughly. Several difficulties exist in studying the binding of PTH and PTHrP to their receptor(s), principally because little is known at the molecular level about the epitopes that constitute the binding interface between peptide and receptor. In this discussion, I will summarise the PTH/PTHrP and PTH/PTHrP receptor structure-activity studies performed to date with the hope that it will cast some light on the nature of binding of these peptides in the JAr cells.

Firstly, let us consider the amino acids in the peptides themselves which have been identified as playing a role in binding and activation.

Comparison of the sequences of PTH (1-34) and PTHrP (1-34) from several species reveals striking sequence homology at the N-terminus (Diag 6.1). Eight out of the first 13 amino acids are identical in humans but in the region 14-34, only 3 out of 21 residues are identical. However, the similarities in their responses suggests that the tertiary conformations of the two peptides are similar. PTH and PTHrP require the first two N-terminal amino acids and the amino acids 25-34 to stimulate adenylate cyclase activity (Rabbani, *et al* 1990) and this limited homology at the amino terminus seems to be sufficient to account for the similar actions of PTH and PTHrP.

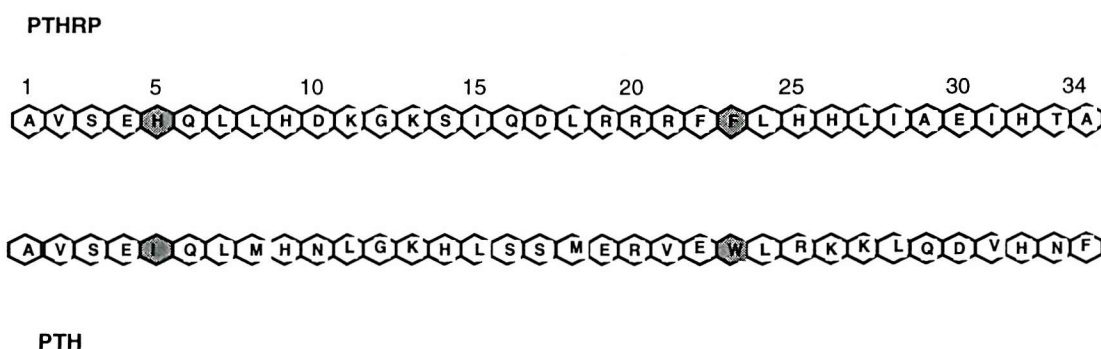


Diagram 6.1. Comparison of the amino acid sequences of PTH and PTHrP (1-34)

PTH is a basic single chain peptide containing no di-sulphide bonds. The residues important in binding to the receptor are the basic sequence at amino acids 25 -27,

although a second binding domain at residue 15 has been suggested (Orloff *et al* , 1989). A further binding region at the carboxy terminus (35-84) has been proposed which recognises a class of low affinity receptors with no known biological function (Orloff *et al* , 1989).

The PTHrP gene can be transcribed into one of three mature mRNAs encoding 139,141 and 173aa peptides. These peptides contain endoprotease motifs which allow further digestion into smaller bioactive fragments which may or may not bind to different receptors.

PTHrP (1-34) has an alpha helical structure between residues 4-14 and there are seven hydrophobic residues within the sequence 5-31. This would result in a fairly compact structure with the C-terminal residues 30-34 “sticking out” from the main body of the molecule. Removal of residues 26-29 results in a total loss of activity probably due to disruption of the second turn between residues 17-27. In addition, removal of amino acids 30-34 could destabilise the alpha helix confirming that the conformation of the peptide is essential for biological activity (Barden, *et al* 1989; McFarlane, *et al* 1993).

Recombinant PTHrP (1-141), (1-108) and (1-84) and synthetic PTHrP (1-34) are equipotent in their ability to stimulate cAMP and intracellular calcium pathways on PTH-responsive cells (Hammonds *et al*, 1989, Kemp *et al*, 1987). Truncation of PTHrP or PTH to (1-29) results in a 90% loss of activity and shorter peptides are inactive altogether (Kemp *et al*, 1987). N-terminal deletions result in PTHrP peptides (PTHrP 7-34) with a greater ability to compete for type 1 receptor binding than their PTH equivalents suggesting it is a partial agonist/antagonist for cAMP production (McKee *et al* , 1988).

The two hormones act via the same receptor in traditional PTH-responsive cells and tissues, supported by substantial evidence (Jueppner *et al*, 1988; Jueppner *et al*, 1991; Abou Samra *et al*, 1992).

To date, three PTH/PTHrP type I receptors have been cloned (rat, opossum and human) and each have a moderately large N-terminal extracellular domain which is thought to participate in hormone binding (Jueppner *et al*, 1991; Abou Samra *et al*, 1992; Schipani *et al*, 1993 & Adams *et al*, 1993). There is 91% homology across species and these receptors afford an opportunity to gain an insight into the interaction between PTH and PTHrP and their common receptor.

As mentioned in section 1.12 substitution of an Arg for Val at position 2 in the PTH peptide results in increased binding to the rat receptor with a decrease in adenylate cyclase activity whereas the opposite effect is seen with the opossum receptor - a reduction in affinity yet a three fold increase in agonist potency (Gardella, *et al*, 1991). Thus it seems that the synthetic peptide Arg2 PTH(1-34) can distinguish between two related PTH receptors. Gardella later employed this peptide to examine potential binding sites of PTH to its receptor. He suggests that residues in or near the transmembrane region of the PTH receptor contribute directly, or indirectly, to interactions with Arg2PTH(1-34) - in particular Val 371 and Leu 427 (Gardella, 1994). A single Serine residue at position 370 was responsible for activation of cAMP and no other residues appeared to be involved.

I have already mentioned that PTHrP binding to its receptor can activate the phospholipase C second messenger cascade and the amino acids involved in this association have been identified by Iida-Klein and co-workers. They synthesised rat PTH/PTHrP receptors with random mutations in the second cytoplasmic loop and assessed their properties. It was found that replacement of amino acids 317-320 (EKKY) with DSEL constituted receptors retaining full capacity to bind ligand and to generate cAMP but had little or no PTH-stimulated PLC activity. Subsequent individual mutations of these four amino acids elucidated to a critical role for Lys 319 in receptor G-protein coupling (Iida-Klein, *et al* 1997).

The role of the extracellular regions of the receptor in ligand binding have been extensively studied by Lee and co workers (Lee *et al*, 1994). They found that a series of deletions on the extracellular portion of the receptor did not affect peptide binding and thus reported that over a quarter of the EC residues were not involved in ligand binding. However, they did discover that residues at the amino terminus (31-47) and loop 3 were essential for ligand-receptor interaction. In addition, disruption of extracellular cysteine residues resulted in a decrease in receptor function. The cysteines are strictly conserved between PTHrP, calcitonin and secretin receptors suggesting that the extracellular topology of these receptors may be similar.

The afore mentioned studies, looking at differences in both the peptide and the PTH/PTHrP receptor, have focused on PTH and PTHrP's association with the classical type 1 receptor. The existence of a PTH2 receptor in non-classical tissues which is activated by PTH(1-34) but not PTHrP (1-34) is now established (Usdin *et al*, 1995).

The PTH2 receptor displays unusual ligand selectivity compared to the hPTH/PTHrP type 1 receptor - it recognises PTH (1-34), PTH(7-34) and PTHrP (7-34) but not PTHrP(1-34) (Behar *et al*, 1996a). Affinity cross-linking identified a 80-90 kDa cell membrane doublet with a similar molecular mass to the hPTH/PTHrP type 1 receptor (Behar, *et al*, 1996a; Adams *et al*, 1995). Competitive binding of PTH(1-34), PTHrP(1-34) and PTHrP(7-34) for the potent PTH agonist K13 (a PTH(1-34) analogue linked to photo-reactive benzophenone) showed that 10^{-6} M PTH (1-34) and PTH (1-84) inhibited doublet formation. However, the equivalent concentration of PTHrP (1-34) did not compete with K13 whereas PTH (7-34) was able to effectively inhibit cross-linking to the PTH2R (Behar *et al*, 1996a). Furthermore, PTH(1-34) but not PTHrP(1-34) was able to stimulate cAMP and intracellular calcium pathways whereas the truncated peptide, PTHrP (7-34), acted as a weak agonist (Behar *et al*, 1996a).

Thus it appears that the presence of the six N-terminal residues of PTHrP, or some structural feature included in it, interfere with binding to the PTH2 receptor. Comparisons in the 1-6 region of hPTHrP with the currently known mammalian PTH sequences reveals considerable amino acid identity with histidine 5 being the only non-conserved amino acid. It was thought that the side chain of histidine 5 was detrimental to the recognition of PTHrP (1-34) by the PTH2 receptor. This was confirmed by Behar *et al* (1996b) who demonstrated that the substitution of His 5 by Ile in PTHrP (1-34) could convert PTHrP (1-34) from an inactive ligand to an agonist for the PTH2 receptor. In addition, Gardella performed similar experiments with phenylalanine at position 23 in PTHrP and showed that this residue was crucial in ligand-receptor binding. Substitution of Phe 23 with the tryptophan of PTH (Trp) increased the binding of PTHrP (1-34) to the PTH2 receptor 71-fold suggesting it is important in maintaining ligand structure and modulating receptor-binding interactions.

It is intriguing that, despite the high sequence homology in the 1-6 regions of PTHrP(1-34) and PTH(1-34), PTHrP(1-34) does not interact with PTH2R whereas PTH (1-34) does. In addition the 7-34 regions of PTH and PTHrP contain markedly reduced homology yet both bind the PTH2 receptor!

In 1988, a Harvard research group led by Fukayama looked at the comparative biological activities of human PTH and PTHrP on SaOS-2 cells. Measurements of cAMP accumulation in the SaOS-2 cell culture medium, in response to both peptides, elicited similar dose response curves. When SaOS-2 cells were

pre-incubated with either PTH or PTHrP, and subsequently challenged with either peptide, a decrease in response was observed. This could be due to desensitisation of the receptor and is a phenomenon that has been observed in a number of hormone target cell systems in which receptors are coupled to adenylate cyclase. They concluded that PTH (1-34) and PTHrP (1-34) bind to and activate the same receptor on bone cells (Fukayama *et al*, 1988). Orloff *et al* reported that PTHrP had an affinity constant of 1.6nM for its receptor in SaOS-2 cells and that there were 30,000 sites/cell.

My work is in agreement with this, since both PTHrP (1-34) and PTH (1-34) were able to displace ^{125}I -PTHrP (1-34) from the SaOS-2 cells with similar competitive binding curves. This would suggest that the two unlabelled peptides were competing for the same binding site as their radiolabelled counterpart. One can assume that the binding site is found on the same receptor i.e. the "traditional" PTH/PTHrP type 1 receptor alluded to by Fukayama. In addition, N-terminal fragments of human PTH and PTHrP are equipotent in stimulating cAMP in SaOS-2 cells (Blind *et al*, 1993).

PTHrP (7-34) and PTHrP (1-86) only displaced ^{125}I -PTHrP (1-34) from its receptor at the highest concentration in SaOS-2 (200nM) and did not display the same properties as either PTHrP (1-34) or PTH (1-34). Both peptides may be acting as partial agonists and if I had been able to conduct binding studies with higher concentrations of unlabelled competitor I may have observed a further decrease in radioligand-receptor association. PTHrP (7-34) is a partial agonist in brain and pancreatic cells (Usdin *et al*, 1995). In SaOS-2, PTHrP (1-86) showed a 30% relative reduction in potency compared to cAMP accumulation by PTHrP (1-34) (Blind *et al*, 1993) which could be due to weaker ligand-receptor association.

In the JAr cell-lines, radiolabelled ^{125}I -PTHrP (1-34) was displaced by increasing concentrations (0.2nM to 200nM) of unlabelled PTHrP (1-34) by up to 28%. This indicates that the unlabelled peptide was competing for the same receptor as its radiolabelled counterpart. Cross-linking of ^{125}I -PTHrP to its receptor, using BS³, and subsequent analysis by autoradiography following SDS-PAGE revealed a single radioactive band at 80kDa. This is a similar molecular mass (80-90 kDa) to both the PTH/PTHrP Type I and PTHrP Type II receptors (Behar, *et al*, 1996a; Adams *et al*, 1995). In the presence of excess unlabelled PTHrP (1-34) (250nM) the radioactivity was diminished confirming that PTHrP (1-34) was displacing radioactive ^{125}I -PTHrP (1-34), from the same binding site.

PTHrP (7-34) was less effective at displacing ^{125}I -PTHrP (1-34) but did show some competitive actions by reducing receptor associated radioactivity by up to 14%. PTHrP (7-34) has been shown to act as a partial agonist in brain and pancreatic cells (Usdin *et al*, 1995; Behar *et al*, 1996) and thus it could be binding weakly to the receptor in JAr cells. As mentioned previously, a histidine residue at position 5 is the 'switch' between specificity of PTH (1-34) and PTHrP (1-34) when binding to the PTH2 receptor. The shorter carboxylic fragment, PTHrP (7-34), lacks this residue which would suggest an inability to bind to the receptor. However, PTH (7-34) does contain the phenylalanine at position 23 which is crucial in ligand-receptor binding (Gardella *et al*, 1996). Thus, the presence of Phe 23, despite the absence of His 5, enables PTHrP (7-34) to associate with the receptor albeit weaker.

PTHrP (1-86) does contain the necessary amino acids at position 5 and 23 to facilitate binding but it did not displace ^{125}I -PTHrP (1-34) from its receptor in JAr cells. In MCF-7 cells PTHrP (1-34) stimulates cell proliferation while PTHrP (1-86) does not (Safadi and Peddie, 1995). In rat osteoblast-like cells (UMR 106), C-terminal extension of PTHrP (1-34) to PTHrP (1-86) resulted in a reduced relative potency to stimulate cAMP accumulation from 100% to 13% (Blind *et al*, 1993). Further evidence for discrepant effects of PTHrP (1-34) and PTHrP (1-86) was reported by Inomata *et al*. They showed that, in a leukaemia cell-line, PTHrP (1-86) has little or no effect on cell proliferation compared with PTHrP(1-34) (Inomata *et al*, 1995). Thus it appears that despite the presence of the necessary residues at position 5 and 23, the tertiary structure of PTHrP (1-86) is inhibiting its binding to the same site as PTHrP (1-34). This could be due to protein folding, whereby the amino acids important in binding are hidden within the peptide, or the long 'tail' of the PTHrP (1-86) peptide could, literally, be getting in the way and inhibiting the peptide-receptor interface.

Both PTHrP (1-34) and PTHrP (1-86) significantly increased cell proliferation in JAr (Umar, personal communication). However, there was no evidence that the similarities in proliferation were due to PTHrP (1-34) and PTHrP (1-86) binding to the same receptor. Indeed, a specific receptor has been defined for the mid-molecular region of PTHrP (Orloff *et al*, 1995) which could account for the effects observed by Umar.

Due to the financial constraints it was not possible to conduct displacement experiments at higher concentrations of peptides. Had I reduced the concentration

of radiolabelled ligand, the counts would have been too low to have observed any major differences in receptor binding. In addition, the amount of unlabelled ligand used to displace had to be 5 fold the final concentration, so in fact stock solutions of up to 1000nM were used so as to give a final concentration of 200nM in the incubation wells. Realistically a 20 fold excess of “cold” peptide, should be sufficient to displace its “hot” counterpart from the receptor. Looking through the literature, I have noticed that the majority of PTH/PTHrP competitive binding studies have used an unreasonably large excess of competitor up to 10^4 nM (Orloff, 1992) and 10^{-2} M (Rosenblatt *et al*, 1980). The concentration of peptide required is considerably higher for receptor binding than circulatory levels and proved to be too expensive to allow studies to be conducted at this concentration. It could be questioned that if such high concentrations of unlabelled ligand are required then the binding is non-specific since the binding system would be “swamped” with unlabelled peptide and the binding affinity of unlabelled peptide would be too low to warrant being called a “specific binding site”. It is important to bear in mind the physiological significance of an experiment and it would seem rather fantastic to suggest that PTHrP or PTH must be present in the Molar range to elicit their effects!

It is evident from the experiments in this chapter that competitive binding of PTHrP (1-34) and its associated ligands differs between the JAr and SaOS-2 cells. Binding of PTHrP (1-34) to the PTH/PTHrP type I Receptor has been well documented in SaOS-2 (Blind, *et al* 1993) and it is apparent from my work that the receptor binding in JAr is not of the same nature. Therefore my work provides evidence that a novel receptor for PTHrP (1-34) exists in the JAr choriocarcinoma cell-line.

CHAPTER SEVEN

GENERAL DISCUSSION.

The major reasons for undertaking this research was to study the growth factors and their receptors found on trophoblast cells at the site of implantation. At the time of experimentation very little was known about the communication and interactions at the maternal-fetal interface but in recent years it has become apparent that correct implantation of the trophoblast, subsequent invasion of the maternal decidua and correct placentation at early stages of gestation are crucial in determining the well-being of the fetus, mother and indeed long-term morbidity of the adult (Brown, 1999; Barker, 1996; Barnea *et al*, 1993).

In this discussion I will summarise the pathophysiological problems associated with dysfunctional trophoblast proliferation, migration and invasion, the rationale behind approaching this study and the reasons for using choriocarcinoma cell-lines as a model. I will summarise the existing data on EGFR and erbB-2 expression in early placenta and how my proliferation assays and ligand-binding data are in agreement with this. I will discuss my observations of the effects of PTH and PTHrP on expression of the EGFR and how their divergent responses could be explained by the existence of a novel PTHrP receptor. Finally, I will present my hypothesis of how the PTHrP system impacts on the expression of EGFR and erbB-2 throughout the early stages of trophoblast implantation.

Eighty five percent of couples who receive IVF treatment and viable pre-embryos fail to achieve term pregnancy. These depressing statistics may in some part be due to failure of the embryo to implant or to complications of pregnancy including, intra uterine growth retardation (IUGR) and pre-eclampsia. IUGR occurs in 3-5% of all pregnancies and preeclampsia affects 7-10% of all pregnancies (Redman, 1993; Fondacci, *et al*, 1994). Preeclampsia is a complex disorder which results in increasing maternal blood pressure and renal dysfunction, but also has a profound impact on the fetus resulting in intrauterine growth retardation and perinatal mortality. There is no cure for the disease other than termination of pregnancy. Clearly an understanding of the causes of preeclampsia will have wide-reaching implications. The placenta, rather than the fetus, is the source of the disorder and faulty trophoblast invasion may be the principal reason behind this. In normal

pregnancy, up to 60% of very early pregnancies are lost before pregnancy is perceived (Redman, 1993). This highlights that determining the controlling factors in these early processes is a crucial area of reproductive research with widespread implications for the health of both mother and fetus.

In order to continue further discussion it is necessary to recap on some of the previous ideas I have presented. As discussed in the introduction to this work, implantation involves apposition and adhesion of the blastocyst followed by invasion by fetally derived trophoblast cells. These extraembryonic trophoblast cells undergo what appears to be well regulated sequential development involving proliferation, migration and differentiation. These events appear to be critically regulated in time and space and it may be these early events which become asynchronous in the pathologically abnormal placentae seen in IUGR and preeclampsia. Normally, the cytotrophoblast cells underlying the villous syncytium migrate from the basement membrane of the chorionic villi in large cell columns to form the placental shell. Individual extravillous trophoblasts (EVT) penetrate further into the maternal uterus, as far as the inner third of the myometrium, as isolated interstitial cells before they differentiate into placental bed giant cells. In pathological pregnancies mentioned previously, normal invasion is impaired (Johnson *et al*, 1993; Meekins *et al*, 1994) and in preeclampsia trophoblast invasion is abnormally shallow suggesting that some component of the invasion process is altered. A key element of my work was to study some aspects of the control systems involved in the proliferation and invasion mechanisms which may help to determine a better understanding of abnormal placentation.

One of the difficulties in approaching this research was the lack of adequate experimental models. No animal species shows a similar pattern of trophoblast invasion and pathological changes in pregnancy to the human hence an animal model does not exist. Naturally there are ethical constraints in conducting experiments in humans and as I was interested in the early stages of pregnancy term placentae did not provide an appropriate model. First trimester trophoblast cells have been isolated and cultured (Fisher, *et al*, 1989) but there is a high risk of contamination from other cell populations such as placental fibroblasts.

I decided to utilise transformed choriocarcinoma cell-lines as an experimental model for the reasons mentioned in chapter three, but mostly because they represent an homogenous population of pure trophoblast which can be manipulated in vitro. I appreciate that any interpretation of the data may be constrained by use

of these models, however, I think my work has brought some interesting comparisons.

It is well published that paracrine interactions between trophoblast and uterine cells are an important part of development and it is likely that a proportion of these effects are mediated by growth factors. Since human placenta is a rich source of Epidermal Growth Factor Receptor it seemed logical for me to suggest a role for EGF and its related ligands (O'Keefe *et al*, 1974), although data regarding the gestational age-related changes in the localisation of the receptor and its ligand in humans are conflicting (Ladines-Llave *et al*, 1991; Bissonnette *et al*, 1992; Maruo *et al* 1987; Hofmann *et al*, 1992; Huot *et al*, 1981; Brown *et al*, 1987; Chen *et al*, 1988; Miyazawa *et al*, 1992).

In mature human placental cultures and in Jeg-3 choriocarcinoma cells of placental origin, EGF increases progesterone, hCG and hPL production suggesting that Jeg-3 may represent a differentiated stage of trophoblast development. (Bahn *et al*, 1980; Lai *et al*, 1984; Benveniste *et al*, 1978). Other workers demonstrated that EGF caused proliferation in term placental cell cultures (Truman and Ford, 1986) illustrating that EGF can have diverse effects. EGF also increases placental permeability (Mimouni *et al*, 1991) and enhances the uptake of amino acids and glucose by trophoblast cells (Guyda *et al*, 1991; Lai *et al*, 1986). EGF has also been shown to initiate implantation in the uterus of hypophysectomised, progesterone-primed, delayed-implanting rats providing that uterine trauma preceded addition of EGF (Johnson and Chatterjee, 1993)

Clearly EGF can confer a wide range of biological responses not only between tissues but also within the same system. My focus has been on the effects of EGF on proliferation and JAr cells were studied in some detail. As discussed previously, EGF causes cell proliferation through its actions on EGF-R. However, the role of the dimer formation with erbB-2 is less clear.

Although EGFR and erbB-2 have been detected in the placenta, data are sparse concerning their precise temporal and spatial localisation, particularly at the implantation site. The work of Muhlhauser on the expression of EGFR and erbB-2 provided the basis for my work as it was apparent that the spatial distribution and presumed proliferative/migratory activity of the trophoblast cells was reflected in the type of receptor present. Muhlhauser *et al* (1993), using immunohistochemistry, looked at the patterns of expression of EGFR and erbB-2 in first and third trimester placental tissue.

In first trimester specimens, EGFR staining was evident along the entire plasma membrane of the cytotrophoblast with weaker staining along the apical plasma membrane of the syncytiotrophoblast. However, erbB-2 protein product was not found in the villous trophoblast but staining was observed along the apical plasma membrane of the syncytiotrophoblast. In extravillous cytotrophoblast, at the tip of the large villi, EGFR immunoreactivity decreased with increasing distance from the adherent villi whereas the inverse was observed for erbB-2 (Muhlhauser *et al*, 1993). Thus EGFR was associated with the early proliferative stages and final stages of adhesion.

Additional work by Jokhi *et al* (1994) supports this: in early placentae, as villous cytotrophoblasts migrate off their basement membrane to form cell columns, their EGF-R expression is diminished and the cells express erbB-2 instead. This pattern is maintained in Extra Villous Trophoblast (EVT) with positive erbB-2 immunoreactivity and completely negative EGFR expression. EVT invading and destroying the maternal spiral arteries were also strongly erbB-2 positive and EGFR negative. EGFR immunoreactivity was reacquired following terminal differentiation into sessile placental bed giant cells (Jokhi *et al*, 1994). Thus erbB-2 expression was associated primarily with the migratory phenotype.

The balance between proliferation, migration and differentiation determines the structure and function of the developing placenta. EGFR expression by cytotrophoblast is only seen in the proliferative cell populations and is lost with increasing differentiative and invasive ability. Both Muhlhauser *et al* (1993) and Jokhi *et al* (1994) suggest that EGFR activation results in proliferation in cytotrophoblasts whereas the erbB-2 gene product is involved in trophoblast invasion and differentiation. In JAr choriocarcinoma cell-lines, cytopspin preparations showed strong positive staining for EGFR expression but weaker reactivity for erbB-2 (Jokhi *et al*, 1994) suggesting that this cell-line displays similar properties to the proliferating cytotrophoblast cells of first trimester placentae.

The idea that EGF causes proliferation of trophoblast was evident in my work in chapter three. EGF caused proliferation of the JAr choriocarcinoma cell line, and to some extent in the BeWo, but appeared to have no proliferative effect in Jeg-3. This led me to believe that the JAr cells displayed similar characteristics to the highly proliferative cytotrophoblast cells in line with Jokhi *et al's* thinking and that

these cells would in turn express a high number of EGF receptors (this was later confirmed in chapter four).

Perhaps BeWo and Jeg-3 cells represent the migrating extravillous or hormone-producing syncytiotrophoblast populations? EGF increases hCG production by Jeg-3 (Wimalasena *et al*, 1994) and it would be of value to compare the changes, if any, of hormone production following treatment with EGF in all three cell lines. If each cell-line could be clearly classified in line with differing stages of the trophoblast cells it would provide an excellent research tool to look at the precise switching mechanisms between populations.

EGF mediates its effects by binding to cell surface receptors and initiating intracellular signalling pathways (section 1.5 to 1.9). My main focus was on the EGFR rather than erbB-2, erbB-3 and erbB-4 since at the time of my experiments specific ligands for each receptor had not been discovered. Now six ligands have been characterised that bind EGFR (erbB-1) : EGF, TGF α , amphiregulin, HB-EGF, betacellulin and epiregulin and two families of Neuregulins which can bind erbB-3 and erbB-4 (reviewed in Alroy & Yarden, 1997).

My binding studies results in chapter four can be combined with the proliferation work to try and explain the differences in proliferation of each cell-line in response to EGF. I found that the JAr cell line, which had the greatest growth response to EGF, have the most EGF receptors per cell (57,000 in total). Since the response of EGF is determined by the number of occupied receptors, it was no surprise to me that the magnitude of response in JAr was greater than that seen in the other two cell lines.

Similarly, the affinity of EGF for its receptor in the JAr cell line was greater than that observed in the other two cell lines, occupation of which may lead to an amplified response. I could speculate that this high affinity was due to a preponderance of homodimer formation. I concluded therefore that in JAr cells, ligand binding results in a high proportion of EGFR-EGFR homodimers which activate intracellular events ultimately resulting in increased cell proliferation.

Despite the fact that the Jeg-3 cells possessed more receptors than the BeWo cells, they did not respond in a proliferative manner following EGF treatment. I suggest that this is due to the existence of a higher proportion of heterodimers as opposed to EGFR-EGFR homodimers. The EGF Receptor, or erbB, signalling network is reviewed in my introduction and recently by Alroy & Yarden (1997) and it is

feasible that in Jeg-3, binding of EGF to its receptor EGFR results in coupling with not just EGFR (erbB-1) but with erbB-2 or erbB-3. Since a number of combinations are possible it is likely that each dimer combination can elicit its actions through a unique set of signalling pathways intracellularly. At this time I am not aware of any comparison of erbB-2 (or erbB-3 and erbB-4) expression on these cell lines, nor any direct measurement of alternative dimer formations in response to the several ligands.

It is the general consensus that EGF binds to its receptor with more than one affinity and currently it is accepted that there is a low affinity and a high affinity site. My work on the choriocarcinoma cell-lines is in agreement with this in terms of both affinity constants and the fact that there are proportionally more low affinity sites than high. In addition to the homodimer versus heterodimer hypothesis, it is thought that the occupancy of different affinity sites results in differing responses. Brabyn *et al* (1995), put forward the idea that occupancy of the high affinity site (i.e. at low EGF concentrations) resulted in cell proliferation in T51B rat epithelial liver cells whereas occupancy of the low affinity sites led to hyperproliferation but this is in conflict with the proposals of Wollenberg *et al* (1989) who stated that, in rat hepatocytes, the high affinity sites did not elicit a proliferative response to EGF. Some authors have even gone so far as to suggest that the high affinity site could be involved in differentiation whereas the low affinity binding site is involved in mitogenesis (Gladhaug *et al*, 1992).

One possibility is that the low and high affinity sites are natural receptors for different ligands since TGF α is capable of binding to the EGFR with equal affinity as EGF (Massague *et al*, 1983). With more and more members of the EGF family being discovered it could be that amphiregulin, cripto, B-cellulin, HB-EGF, or epiregulin have increased affinity for one of the sites in preference to EGF. Further ligand binding studies with EGF homologues would shed more light on this with competitive binding assays giving a clear indication provided that the EGFR could be isolated to prevent interference from, perhaps, unidentified specific receptors for the other ligands. This might be best achieved using transfected cells.

Given the time and resource I would suggest future experiments looking at the binding of not just EGF homologues but also of the Neuregulins on erB-1, erbB-2, erbB-3 and erbB-4 receptors in choriocarcinoma cells. It would be of tremendous value to determine what influences the formation of the heterodimers

since at least nine potential combinations exist and it is apparent that the trophoblast phenotype is associated with changes in EGFR/erbB-2 expression. Preference for affiliation with erbB-2 appears to be top of the hierarchy but whether this is due to behaviour of the ligand, phosphorylation of the receptors or simply due to abundance of receptors remains unclear but what is clear is that the potential for diversification is immense.

If one can determine the factors controlling receptor expression then it goes some way to offering explanations for abnormally shallow trophoblast invasion. That is, in the first trimester, an imbalance in EGFR or erbB-2 expression could account for a less invasive trophoblast population.

To some extent, Fondacci *et al* in 1994 examined this by looking at EGFR expression in term placentae in cases of intrauterine growth retardation. They looked at the placental levels of EGFR in fourteen cases of 'small for age babies' and showed that twelve of the fourteen cases showed reduced or absent EGFR phosphorylation which in six cases was due to a decrease in EGF binding sites (Fondacci, *et al*, 1994). Fujita had previously shown that there was a marked decrease in EGFR and EGFR mRNA in eight IUGR placentas (Fujita, *et al* 1991).

These findings confirm my thinking that additional factors could be influencing the levels of EGF or erbB-2 receptor expression in the choriocarcinoma cell-line JAr.

As outlined in chapter five, the rate of transcription of the EGFR gene can be regulated by several factors (Clark, 1985) including PTH and PTHrP (1-34), which increases EGF binding to its receptor in cultured human trophoblast cells from early and term placenta (Alsat *et al*, 1991; Alsat *et al*, 1993). Alsat had already shown that PTH (1-34) and PTHrP (1-34) increase EGFR and EGFR mRNA expression in human trophoblast cells and that the number of EGFR was increased with no change in receptor-ligand affinity (Alsat, *et al*, 1993). I wanted to see if similar effects were observed in my choriocarcinoma cell-line model. The JAr cells were selected since they had shown the greatest proliferation in response to EGF and also the highest number of EGFR in my binding studies.

PTHrP and the PTH/PTHrP receptor is expressed in the placenta (Lafond, *et al*, 1988; Rodda *et al*, 1988) but its precise role is unclear although it is one of the earliest peptides to be expressed in the mouse embryo and has been implicated in differentiation of the parietal endoderm (Behrendsten, *et al*, 1995). Messenger RNA for PTHrP has been isolated from tissues of the human utero-placental unit

(Ferguson, *et al*, 1992) and the peptide has been localised by immunohistochemistry in first trimester placenta (Dunne, *et al*, 1994). Changes in PTHrP expression in the fetus throughout gestation suggest that it may have roles in growth and differentiation (Moseley, *et al*, 1991).

The mechanism by which PTHrP interacts with the EGF stimulated response causing proliferation, differentiation or migration of trophoblast in the early stages of pregnancy is not fully understood. In Chapter five, I looked at the effect of PTH (1-34) and PTHrP (1-34) upon EGFR expression in the JAr choriocarcinoma cell-line to determine if either peptide could be involved in altering levels of EGF receptor and hence influence cell proliferation.

Since the work of Alsat, new research has suggested that PTH and PTHrP may act via different receptors (Inomata, *et al*, 1995; Behar, *et al*, 1996) which in turn could account for differing effects upon the cell. In my work, radiolabelled EGF bound to its receptor in both the monomeric (170 kDa) and dimeric (340 kDa) forms. Prior treatment of the JAr cells with PTH (1-34) decreased receptor associated radioactivity suggesting either a reduction in EGFR number or a loss in ligand receptor affinity. On the other hand, PTHrP (1-34) caused an increase in radiolabelled EGF-EGFR association suggesting that receptor affinity or number had in fact increased. Further quantitative binding studies showed that the changes in EGF-EGFR association were due to alterations in EGF receptor number rather than changes in the affinity of the ligand for its receptor. In summary, PTH (1-34) downregulated EGFR expression whereas PTHrP (1-34) upregulated expression of the receptor.

The differences in PTHrP and PTH (1-34) elicited responses implies that these peptides act in differing ways but at first glance, how this is achieved is unclear. It could be that both peptides bind to the same receptor but at unique sites therefore eliciting alternative intracellular second messenger cascades. Alternatively they may act through related but different classes of receptors.

Increasing evidence supports the hypothesis that PTH and PTHrP can bind separate and distinct receptors.

The classic type 1 PTH/PTHrP receptor is a well characterised 7 TMSR, G-protein linked glycoprotein cloned from a rat osteoblast cell-line, UMR-106 (Abou-Samra, *et al*, 1992) and kidney (Schipani, *et al*, 1993). The type 1 PTH/PTHrP receptor

can trigger both the PLC γ /IP3/DAG and adenylate cyclase/cAMP pathways (Jueppner, *et al* 1994) so at first glance, the discrepant effects of PTH and PTHrP upon EGFR regulation seen in my results could be accounted for in this way. That is, binding of PTH to its receptor results in activation of Protein Kinase C, via the IP3/DAG pathway resulting in phosphorylation of Threonine 654 of the EGFR and subsequent downregulation of the EGFR. One could then assume that PTHrP is acting via the adenylate cyclase/cAMP route. This however is misleading as there is evidence to suggest that PTHrP does not activate the cAMP route. In some non-classical PTH target tissues PTHrP does not cause an increase in cAMP although PLC γ and cellular calcium increase (Orloff, *et al*, 1995). In our laboratory it was shown that cAMP inhibited proliferation of JAr whereas PTHrP (1-34) increased cell proliferation (Umar, personal communication). This supports my work and provides circumstantial evidence for the existence of a novel PTHrP receptor.

Multiple PTH/PTHrP receptors may well exist. A PTH specific PTH2 receptor in non-classical tissues is now established, which is activated by PTH (1-34) but not PTHrP (1-34) (Usdin *et al*, 1995). The type 2 receptor is expressed at high levels in pancreas and brain and is activated by PTH (1-34), antagonised by PTH (7-34) but does not bind or respond to PTHrP (1-34) (Usdin, *et al*, 1995). Therefore it is reasonable to suggest that a novel Type 3 or 4 receptor may exist in JAr cells which specifically binds PTHrP (1-34).

Although a specific PTHrP receptor has not been cloned there is evidence that one may exist in the supraoptic nucleus (Yamamoto, *et al*, 1997). In support of the multiple receptor theory, we already know that an additional, yet uncloned, receptor for the midregion of PTHrP is involved in placental calcium transport (Rodda, *et al*, 1988; Abbas, *et al*, 1989; Kronenberg, *et al*, 1999). Until this has been cloned we will not be able to readily study its localisation in the early trophoblast. However, one could investigate some aspects of its functional activity and pharmacological properties in the early trophoblast models.

This evidence led me to assume that trophoblast or JAr cells may possess alternative PTH/PTHrP receptors, which may account for the differences in response to PTH (1-34) and PTHrP (1-34). In chapter six I conducted a series of competitive binding assays in both JAr trophoblast cells and in SaOS-2 osteoblastic cells. SaOS-2 express the classic type 1 PTH/PTHrP receptor (Orloff, *et al*, 1995) and therefore if the binding of PTH (1-34) and PTHrP (1-34) in JAr cells proved to be

comparable to SaOS-2 it suggests that the receptors in both cell-lines are similar. In my experiments, both PTH (1-34) and PTHrP (1-34) were able to displace radiolabelled PTHrP (1-34) from its receptor in SaOS-2 cells confirming that both peptides were competing for the same binding site on the classical type 1 PTH/PTHrP receptor.

The type three receptor is purported to exist in the supraoptic nucleus of the brain, which when activated by PTHrP (1-34) increases vasopressin secretion; PTH (1-34) elicits no effect (Yamamoto, *et al* , 1997). Yamamoto also showed that radiolabelled PTHrP (1-34) was displaced from this receptor by 'cold' PTHrP (1-34) but not PTH(1-34). My results were in agreement with these hypotheses because in the JAr cell line, unlabelled PTH (1-34) did not displace radiolabelled PTHrP (1-34) whereas unlabelled PTHrP (1-34) did compete for the same binding site as its radioactive counterpart. This suggests that PTHrP receptors in the JAr cell-line differ from the traditional type 1 receptors observed in SaOS-2 and it is unlikely that the receptor in JAr is the type II receptor since it was capable of binding PTHrP (1-34). No other receptors for PTHrP have been cloned but it is probable that further receptors will be characterised and my work suggests that a PTHrP (1-34) specific receptor is present in the choriocarcinoma cell-line JAr, which may also exist in early trophoblast.

One question that puzzled me was where is the PTHrP produced and what physiological role does it play? We know that PTHrP is present in the first trimester human trophoblast (Dunne, *et al* 1994). Recent work by Verheijen *et al* has implicated a paracrine interaction of PTHrP and the type 1 PTH/PTHrP receptor in Parietal Endoderm formation in mice (Verheijen, *et al* 1999) and we already know that PTHrP is one of the earliest peptides secreted by the future trophoblast cells in mice (Hayman, 1993) suggesting that it may contribute to the initial outgrowth of the trophoblast. However, PTH and PTHrP could also originate from the mother at early stages. If the production is local, how are the effects of PTHrP (1-34) being controlled?

If PTHrP is such a key player in trophoblast growth and differentiation then how is its production regulated? There is little known of the genomic regulators of PTHrP production but parallels can be drawn from work in chondrocytes which have identified an upstream regulatory protein, Ihh (Indian hedgehog - a secreted protein expressed in the vertebral growth plate) (Bitgood and McMahon, 1995). Ihh stimulates PTHrP mRNA synthesis in chick chondrocytes and is controlled through a negative feedback loop but there are likely to be a number of additional

intermediates in the cascade which have not yet been identified (Kronenberg *et al*, 1998). However, it is a starting point and it would be of interest to look at the levels of *Ihh* in JAr choriocarcinoma cells to see if PTHrP mRNA production is controlled in the same way.

From my results, I propose the following hypothesis which is outlined in Diag 7.1. In the proliferating cytotrophoblast cells, *Ihh* increases transcription of PTHrP which is secreted by the cell and acts in an autocrine/paracrine fashion/ upon a novel PTHrP specific receptor expressed on the cell surface. Binding of PTHrP to its receptor triggers intracellular second messenger pathways which upregulate expression of the EGFR on the cell surface. EGF, TGF α or indeed any one of the locally produced EGFR ligands then bind to monomeric and homodimeric forms of the EGFR, triggering phosphorylation processes internally which lead to cell proliferation. As the proliferating cells become more distal from a high concentration of locally produced PTHrP, they begin to come in contact with a increasing maternal PTH gradient. Maternal PTH binds to type 2 PTH specific receptors on the cell surface and subsequent intracellular mechanisms lead to a down-regulation of EGFR expression. This now leaves erbB-2 as the abundant population of receptors on the cell surface. The subsequent binding of EGF or related ligands to EGFR will the formation of heterodimers composed of EGFR/erbB-2 as opposed to the previous EGFR/EGFR complex. As the PTH gradient increases the formation of erbB-2/erbB-3, erbB-2/erbB-4, erbB-3/erbB-4 complexes may become more prevalent with EGFR expression being depleted and the cells differentiate into the highly invasive, non-proliferative, extravillous phenotype.

If one could repeat the Muhlhauser experiments but look at the expression of differing PTHrP and PTH receptors instead of comparing EGFR and erbB-2 expression, then it would shed some light as to whether my theory is plausible.

Whatever the intricate mechanisms eventually turn out to be, I have shown that the sensitivity of trophoblast cells to EGF, PTH, PTHrP and associated ligands acting via differing receptors could contribute to proliferation, migration and differentiation and hence the structure of the placenta. However, much needs to be done to extend these studies and investigations with the trophoblast cell lines could give valuable insight into differences in PTH/PTHrP receptor expression during the early stages of trophoblast differentiation.

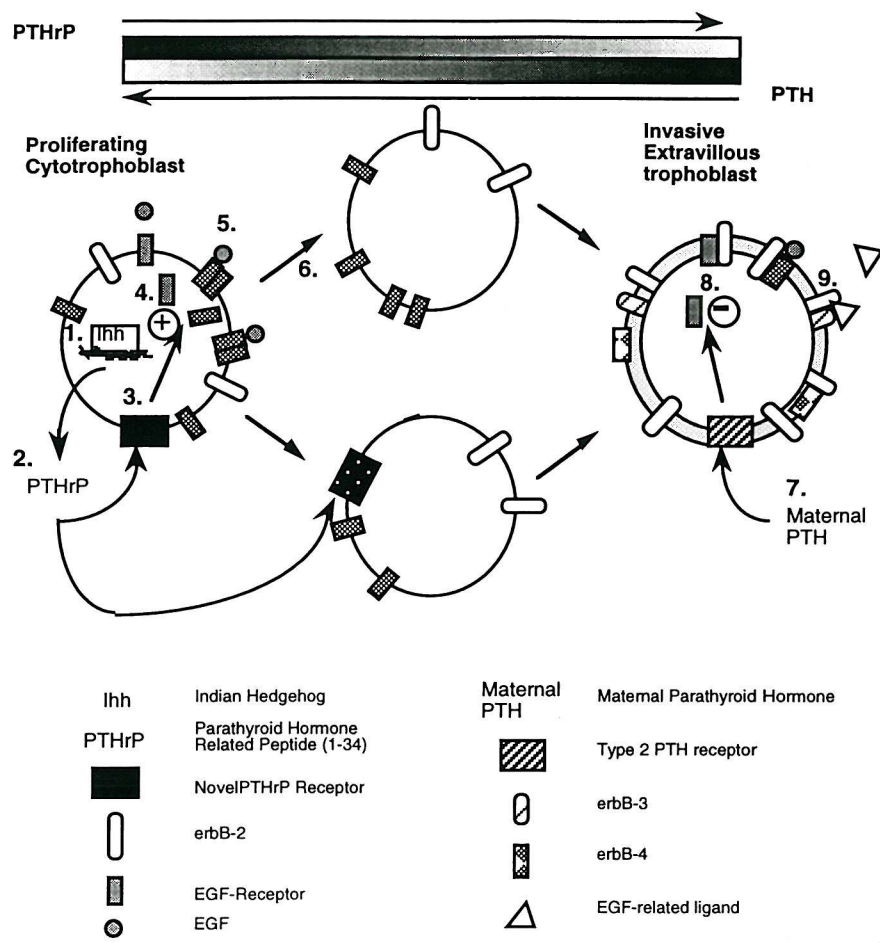


Diagram 7.1. Proposed model of PTHrP/EGF interaction

Indian hedgehog increases production of PTHrP (1.) PTHrP acting in an autocrine or paracrine fashion (2.) binds to the novel PTHrP receptor (3.). Activation of the novel PTHrP receptor triggers intracellular mechanisms causing an upregulation in EGFR expression on the cell surface (4.). EGF association with the EGFR and subsequent formation of EGFR/EGFR homodimers (5.) results in cell proliferation (6.). As the cells come into closer contact with maternal PTH the PTH type 2 receptor is activated (7.) which causes a down regulation in the expression of EGFR (8.) and the formation of erbB-2 heterodimers is favoured (9.). The changes in concentration of PTHrP and PTH are shown at the top of the diagram where dark shading indicates an abundance of peptide and lighter shading shows a reduction in peptide levels.

CONCLUSION

Although the regulated and orderly growth of placental proliferation and differentiation may seem disparate from the growth of choriocarcinoma cells, the common expression of certain growth factors, protooncogenes and cytokines is striking. This work provides insight into the effects and binding of EGF to its receptor and the effects of PTHrP in this system. I demonstrated:

- a) EGF increased cell proliferation in the JAr choriocarcinoma cell-line and to a lesser extent in BeWo cells. Jeg-3 did not proliferate in response to EGF.
- b) Characterisation of the EGF receptor in JAr, BeWo and Jeg-3 supported the above work by highlighting differences in both EGF ligand's affinity for the EGF receptor and in EGFR number between the three cell-lines.
- c) PTHrP (1-34) upregulated EGFR expression in the JAr choriocarcinoma cell-line, whereas PTH (1-34) decreased receptor number.
- d) PTHrP (1-34) and PTH (1-34) elicit their effects on EGFR expression in JAr cells by acting on different receptors.
- e) Finally, a novel PTHrP receptor exists in the JAr choriocarcinoma cell-line accounting for the differing actions of PTHrP (1-34) and PTH (1-34).

Little is known of the precise mechanism by which PTHrP interacts with EGF/EGFR responses to bring about proliferation, migration or differentiation during trophoblast invasion. My work assists in elucidating EGF/PTHrP driven mechanisms regulating cell growth in normal early pregnancy. In addition, competitive agonist/antagonist treatment through PTH/PTHrP receptors is a possible approach to which could be utilised in the treatment of defects in placental development.

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