GROWTH REGULATION

IN

BREAST CANCER

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN THE

FACULTY OF MEDICINE, UNIVERSITY OF GLASGOW.

©JUDITH LILIAN GODDEN (BSc MSc)

CRC DEPARTMENT OF MEDICAL ONCOLOGY

UNIVERSITY OF GLASGOW

November, 1992

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To Murray

Acknowledgements

I would like to thank my supervisor, Professor David Kerr for all the useful discussions and encouragement over the past three years. I am also grateful to Dr Robin Leake in the Dept.of Biochemistry, for his advice and help throughout.

In particular I wish to acknowledge the help of Dr Frank Rinaldi and members of the Dept.of Biochemistry for ER and EGF receptor measurements, Dr Jane Plumb for helpful advice on using the MTT assay, Dr Gerry Graham of the Beatson Institute for partial purification of conditioned medium (FPLC analysis), and Dr Nicol Keith for practical help and advice with western blotting experiments and his enthusiastic interest in all the phosphotyrosine work.

Finally, I would like to thank all my friends and colleagues in Medical Oncology, who have helped me enormously over the last three years.

CONTENTS

TABLES

FIGURES

LIST OF ABBRIEVIATIONS

Adr	adriamycin
APS	ammonium persulphate
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CM	conditioned medium
CSS	charcoal stripped serum
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRC	dose response curve
E_2	oestradiol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ER	oestrogen receptor
FCS	foetal calf serum
FITC	fluorescein isothiocynate
[^{3}H]	tritiated label
HEPES	4-(2)-hydroxyethyl-1-piperazine-ethanesulphonic acid
Hrs	hours
HT-CSS	heat treated charcoal stripped serum
IgG	immunoglobulin G
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II

IGF-1r	insulin-like growth factor-I receptor
kDa	kilodaltons
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PgR	progesterone receptor
PI	phosphatase inhibitor
prf	phenol red free
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
TBST	tris buffered saline tween
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF-α	transforming growth factor alpha
TGF-ß	transforming growth factor beta
WT	wild type
w/v	weight per volume
v/v	volume per volume

ABSTRACT

For the successful treatment of breast cancer it is important to understand the underlying mechanisms of growth regulation in breast tumours. This study has examined breast tumour cell growth at three different levels; growth factor regulation of cellular proliferation, growth factor effects on signal transduction pathways and fibroblast derived paracrine regulation of growth. Four different breast cancer cell lines were selected to cover a wide range of breast tumour characteristics. MCF-7WT and ZR-75-1 are two oestrogen and progesterone receptor positive cell lines which show oestrogen regulated growth, whilst MCF-7Adr and MDA-MB-231 are both oestrogen receptor (ER) negative but have increased levels of the epidermal growth factor receptor.

Growth responses to oestradiol, the antiestrogen tamoxifen and a range of growth factors were examined in each of the cell lines using an MTT cell growth assay and cellular uptake of tritiated thymidine. Growth regulation by each of these factors was clearly seen in the two ER positive cell lines, although the effect was much smaller in the ZR-75-1 line. The ER negative cell lines produced no growth response to exogenously applied factors, although they did show a much higher level of growth in basal medium conditions.

Given the importance of tyrosine kinase activity in signal transduction, patterns of tyrosine phosphorylation were compared in each of the cell lines when grown in low stimulatory growth conditions. It was interesting to note that overall

tyrosine phosphorylation was much higher in the MCF-7Adr line than its parent MCF-7WT line, suggesting increased phosphorylation activity may partly explain why these cells have escaped external growth control. Evidence points to altered regulation of the *ras* GTPase activating (GAP) protein as one possible factor involved in the autonomous growth of the ER negative MCF-7Adr cell line. Tyrosine phosphorylation response to growth factor stimulation was also examined in the ER positive MCF-7WT and the ER negative MCF-7Adr cell lines. This generally resulted in phosphorylation of proteins specific to the growth factor, as well as phosphorylation of a number of substrate proteins which were common to each of the growth factors tested, suggesting different growth factors share the same signal transduction pathways.

Finally, growth regulation of each of the breast cancer cell lines was examined within the broader context of a multicellular tumour environment. Fibroblast cell lines derived from breast tumour stromal tissue were examined for their capacity to regulate tumour cell growth through paracrine mechanisms. Breast cancer cell lines were exposed to conditioned medium from the fibroblasts and the growth response measured in an MTT assay. The two ER positive cell lines MCF-7WT and ZR-75-1 responded to fibroblast conditioned medium, again the affect was greater in the MCF-7WT line. The ER negative cell lines showing autonomous growth were unaffected by exposure to the conditioned medium. The conditioned medium was found to synergise with oestradiol to produce very large increases in the growth of MCF-7WT cells.

This study suggests paracrine influences on tumour cell growth may be important in the progression of hormonally dependent breast tumours. In

contrast, hormonally responsive or hormonally independent breast tumours show the capacity for self-regulated growth therefore appear to be less influenced by external factors. It is by defining the major growth influences affecting both hormone dependent and hormone independent tumours that new and important therapeutic targets will be identified.

CHAPTER 1

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1 BREAST CANCER-EPIDEMIOLOGY

Breast cancer is one of the most common forms of cancer amongst women in the western world affecting nearly 10% of the female population, although recently lung cancer has taken over as the commonest cancer affecting this population. The incidence of breast cancer has been rising steadily over the last century with 18% of all cancer deaths amongst women due to cancer of the breast.

It has long been known, due to clinical observation, that oestrogens play an important role in the growth and development of breast cancer. An example of this can be seen in women suffering from primary ovarian failure where the incidence of breast cancer is reduced to 1% of that seen within the normal female population. (Lippman *et al.*,1986) Sir George Beatson first noted the relationship which existed between the functioning ovaries and the progression of malignant breast disease after successfully healing a locally recurrent cancer of the breast following ovariectomy (Beatson,1896). Subsequently, clinical trials suggested that such a treatment was successful in approximately one third of all cases of premenopausal breast cancer. (Hamm & Allegra, 1991)

Through time, the oestrogens were discovered and their role in the promotion of breast cancer confirmed. It was not until radioactive steroids were produced that a receptor for oestrogen was found to be present. The oestrogen

receptor (ER) was able to be detected in 60-80% of all human breast cancers (McGuire *et al.*,1975) and the concentration of the receptor showed close associations with age, menstrual status and the degree of tumour differentiation.

1.1.1 Endocrine Treatment

Many endocrine treatments are available which exploit the presence of a functional ER in the tumour cells of advanced breast cancer patients. These treatments work by two different mechanisms, either they lower the circulating levels of oestradiol, or they block the action of oestradiol at the receptor. Ablation of the hormone secreting organs, as in a ovariectomy removes the source of direct oestrogen synthesis, and adrenalectomy removes the source of all precursor steroids which are converted to oestrogen by aromatase in peripheral tissues, especially important in the case of postmenopausal patients.

Aromatase inhibitors play an important role in the reducing the level of circulating oestrogens, by inhibiting the peripheral conversion of androgens to oestrogens, of particular concern in post menopausal women where this is the primary source of oestrogens. The antiestrogens work at the ER receptor site. They can competively bind to the ER so bringing about an inhibition of steroid induced mitogenesis. Tamoxifen is the most widely used of all the non-steroidal antiestrogens and it has become an extremely important drug in the management of breast cancer today, although its actions are thought to be more complex than a simple antiestrogenic effect (reviewed in Jordan, 1990).

1.1.2 Malignant Progression

When factors which are involved in the early and late stages of malignancy in human breast cancer are examined, the high levels of ER expression in a tumour and its responsiveness to oestrogen and antiestrogen treatment associates closely with early stages of the disease. The presence of ER in a high percentage of breast tumours implies that the ER is expressed in the parental stem cells. It would seem these parental cells eventually give rise to a population of cells resistant to endocrine treatment which have lost ER expression or express an ER no longer able to bind E_2 (reviewed in Horwitz, 1992). Since many tumours are heterogenous in the expression of functional ER, endocrine treatment of the disease will often select out subpopulations of cells which are unresponsive to oestrogens. (Isaacs, 1988)

(Clarke *et al.*,1990)

1.2 OESTROGEN RECEPTOR

The human ER has been mapped to human chromosome 6 (Evans *et al.*, 1988). The ER molecule appears to exist as a dimer which is bound to a dimer of hsp-90 (heat shock protein 90, Baulieu, 1987) within the nucleus of the cell. It only becomes active in the presence of oestradiol (E_2) which is able to move freely into the cell. One molecule of oestradiol will bind each receptor causing the hsp-90 dimer to dissociate and allow the ER complex to bind to DNA and activate target genes.

Much of the work responsible for our understanding of oestrogen regulated

cell growth and gene regulation has been carried out in cell lines, particularly the MCF-7 cell line. This line has proved particularly useful for study because of its response to oestrogen, showing induction of the progesterone receptor (PgR), increased growth and the transcription of a number of mRNAs.

1.2.1 Progesterone Receptor Induction

It has been shown that levels of PgR are increased in the MCF-7 human breast cancer cell line upon treatment with E_2 (Horwitz *et al.*,1978). In both the MCF-7 cell line (Katzenellenbogen *et al.*, 1987) and the ZR-75-1 cell line (Glover *et al.*, 1988) transfer to oestrogen free medium resulted in a decreased growth and loss of the PgR. Therefore the presence of the PgR in human breast cancer cells was a further indicator of a hormone responsive tumour.

1.2.2 Oestrogen Induced Growth Regulation

Oestrogens have been shown consistently to increase the growth rate of a number of ER positive human breast cancer cell lines particularly MCF-7 (Lippman, 1981) and ZR-75-1 (Dabre *et al.*, 1983). Whether E_2 exerts a direct or indirect growth stimulus on the cell remains unclear but there is evidence to support both hypotheses. It has been proposed that E_2 induces proliferation directly by the induction of the c-*fos* proto-oncogene which together with insulin-like growth factor induction of c-*jun*, stimulates proliferation (van der Burg *et al.*, 1991). E_2 acting indirectly to increase the stimulation of cell growth has been

more extensively investigated. Experimental evidence has shown that a number of growth factors are secreted by ER positive breast cancer cells under oestrogen regulation and it has been proposed that these act in an autostimulatory manner toward the cell (autocrine factor). Transforming growth factor-α (TGF-α) and to a lesser extent insulin-like growth factor-I (IGF-I) are released by MCF-7 cells upon oestrogen stimulation and are able to partially replace E_2 as a tumorigenic stimulus in nude mice tumour models (Dickson *et al*1986[a]).Other growth factors such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) are also secreted by human breast cancer cells, although not all are under direct oestrogen stimulation. All of these growth factors are able to affect tumour growth either by autocrine or paracrine stimulation. (Peres *et al.*,1987)

Also of interest in breast cancer is the protease cathepsin-D. In breast cancer cell lines the precursor, pro-cathepsin-D is overexpressed when compared to normal mammary epithelial cells and shows oestrogen regulation *in vitro*. In ER negative cell lines there is a constitutive high production of cathepsin-D mRNA (Rochefort *et al.*,1989). This protease is suspected of having a number of important tumour promoting functions including promotion of cellular proliferation by activation of the latent form of growth factors and promoting tumour invasion and metastasis through degradation of extracellular matrix by its own proteolytic actvity and its ability to activate other proteases (Montcourrier *et al.*,1990). In primary human breast cancer, high levels of cathepsin-D in the cytosol of tumours has been correlated with increased frequency of relapse and metastasis within a five to six year period after surgery (reviewed in Rochefort,

1990).

Plasminogen activator (PA) is a serine protease which activates plasminogen to plasmin. It has been found at high levels in human breast cancers and correlates with poor prognosis (Duffy *et al.*,1990). Using organ culture techniques, PA shows oestrogen modulation through activation of the ER (Mira-y-Lopez *et al.*,1991), but *in vitro* studies into breast tumour invasiveness found no correlation with PA secretion (Madsen *et al.*,1990), although it may play a role in tumour stroma formation (section 1.6.1). A model representing all of these cellular effects is detailed in Figure 1.

1.2.3 Oestrogen Regulated mRNAs

Finally a number of oestrogen regulated RNAs have been identified in MCF-7 cells; pS2 (Masizkowski *et al.*, 1982) or pNR-2 (May *et al.*, 1988), c-*myc* and elevated p53 levels (Thomson *et al.*, 1990). In a study of 172 primary breast cancers using immunohistochemical staining of pNR-2/pS2, there was found to be a significant positive association between pNR-2 expression and response to endocrine therapy upon relapse. This makes expression of pNR-2/pS2 a useful predictor of hormonal response (Henry *et al.*, 1991).

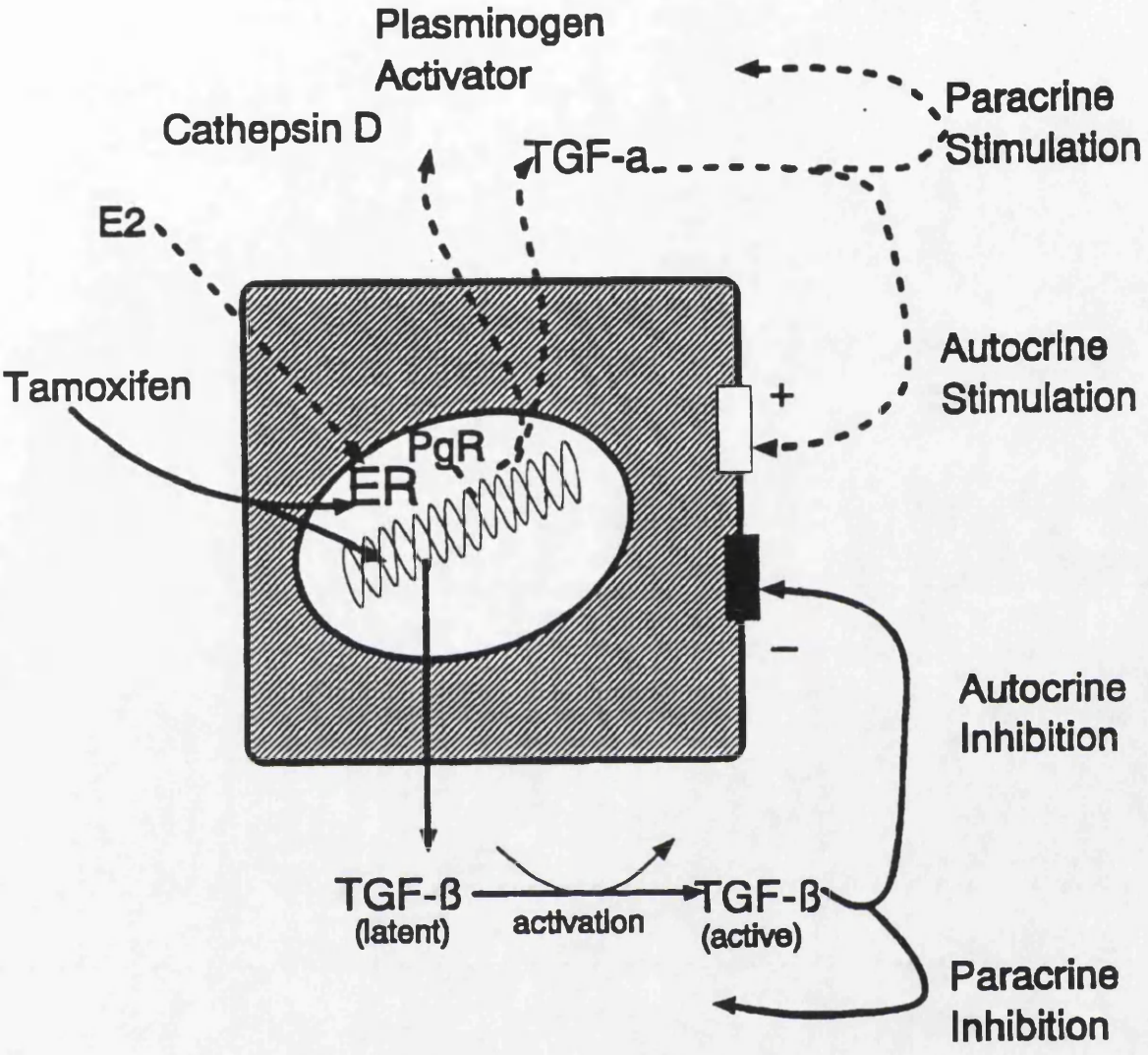
1.2.4 Loss of Oestrogen Receptor

As breast tumours become progressively more malignant, they reach intermediate stages between responsive ER positive tumours and the hormone unresponsive, ER negative tumours. Two types of ER variants have been found

to occur in human breast tumour specimens. In patients who are found to be ER positive and PgR negative, a truncated ER receptor frequently occurs which is unable to bind to DNA and induce normal ER function (Faqua *et al.*, 1992). On the other hand, the small percentage of tumours found to be ER negative but PgR positive show defective oestrogen binding but functional oestrogen responsive genes. These tumours will not respond to oestrogens or endocrine therapy. They seem to have escaped hormone control and are able constitutively to activate target gene expression (Faqua *et al.*, 1991). There are many tumour cell line models of variant ER which mimic some of these clinical situations. A number of subclones of the T47D cell line show intermediate stages of hormone resistance although they tend to be unstable and display a large amount of heterogeneity within each clone. It was suggested subpopulations found to be hypertetraploid contain a number of ER alleles and therefore may express a mixture of wild type and mutant receptors allowing the existence of ER positive oestrogen resistant cells (Graham *et al.*, 1990).

Katzenellenbogen *et al,* (1987) found MCF-7 cells which were grown in the absence of oestrogens considerably reduced their growth rate but this eventually increased to a near normal rate over a period of months, during which time the cells lost the ability to respond to oestrogens and adapted to another form of growth regulation as described in section 1.2.5.

Figure 1-1 Oestrogen and Tamoxifen Growth Regulation



Model for oestrogen and tamoxifen growth regulation in an ER positive breast cancer cell.

E_2 increases synthesis of : PgR, proteases;plasminogen activator, cathepsin D, and TGF-α. TGF-α can directly stimulate growth through the EGF receptor. Tamoxifen competitively inhibits E_2 at the ER and down-regulates oestrogen regulated pathways. Tamoxifen increases production of TGF-ß both in latent and active form which can inhibit the growth of the cell through specific TGF-ß receptors.

1.2.5 Loss of Response to Oestrogens

For human breast tumours to survive endocrine treatments loss of oestrogen dependence is a critical step in malignant progression. Most studies examining this malignant progression have compared ER positive with ER negative human breast cancer cell lines. T47D human breast cancer cell line deprived of steroid during long term culture, progressed to exhibit a degree of steroid autonomy accompanied by a number of changes in growth factor gene expression, these included upregulation of TGF-α, TGF-β_1 and TGF-β_2 mRNA (Daly *et al.*, 1990). Loss of steroid sensitivity appears to relate to changes in the levels of growth factor activity, with an increase in autocrine growth stimulatory pathways and a possible decrease in autocrine growth inhibitory pathways. It follows, if oestrogens cause an ER positive breast cancer cell to increase growth factor production, the ER negative cell lines showing equivalent autonomous levels of growth factor production have escaped oestrogen regulation.

There are many points at which a cell may escape oestrogen regulation, it may increase growth factor production, increase growth factor receptor levels and alter the level of ligand affinity. Alternatively changes in the cellular signal transduction pathway such as lack of specific receptor phosphatases will allow the receptor or its substrates to remain phosphorylated and signalling uncontrolled, also affecting cellular growth regulation. There is little evidence yet available, to substantiate some of these theories but it is known that the epidermal growth factor receptor (EGFr) numbers are generally found to correlate negatively with the ER in breast cancer cell lines (Koenders *et al.*, 1991). Growth factors and

growth factor receptors known to play a role in breast cancer will be reviewed in detail further on in this chapter.

1.3 ENDOCRINE TREATMENT OF BREAST CANCER

Of all the treatments mentioned earlier in this chapter, tamoxifen, a nonsteroidal antiestrogen, has gained widespread acceptance as the treatment of choice for patients with locally advanced and metastatic breast cancer. The drug is able to inhibit specifically the effect of oestrogens on target ER positive cells, measured as inhibition of oestrogen-regulated growth of cells in culture. This was characterised by an increased accumulation of cells in the G_0-G_1 phase of the cell cycle (Taylor *et al.*, 1983, Sutherland *et al.*, 1983 & Osborne *et al.*, 1984), an effect which was reversed by increased E_2 concentrations (Osborne et el., 1984) implying that the drug acts as a cytostatic agent rather than a cytotoxic agent. However it is important to acknowledge that tamoxifen is able to stimulate growth when applied to oestrogen resposive cells in the absence of oestrogens, this is examined in chapter 3 of the thesis.

1.3.1 Cellular Mechanisms of Tamoxifen

Generally, tamoxifen inhibition of oestrogen growth regulation is thought to be due to the drug binding to the ER and acting as a competitive inhibitor of oestrogen. More recently, it has been suspected that tamoxifen must have additional effects on the cell lines, because of the range of responses seen between species, target organs and differential gene and growth factor responses. (Wakeling, 1987)

At high tamoxifen concentrations growth inhibition was no longer able to be reversed by oestrogen in the MCF-7 human breast cancer cell line suggesting there was a secondary non-oestrogen regulated drug effect (Sutherland *et al.*, 1987). The concentrations in the micromolar range at which this effect was seen are cytotoxic and it is thought most of this inhibitory effect is due to cellular toxicity, the mechanism of which is unknown. The differing effects of oestrogens and antiestrogens on cellular production of growth factors and growth regulatory enzymes also points to alternative mechanisms for tamoxifen action. From studies in the MCF-7 cell line it has been postulated that tamoxifen association with the ER inhibits the oestrogen driven autocrine stimulation of the cell by growth factors such as TGF-α and IGF-I and increases production of an inhibitory growth factor TGF-ß (Knabbe *et al.*, 1987). TGF-ß is able to inhibit the growth of both ER positive and negative epithelial cells (Roberts *et al.*, 1985) although it also acts as a mitogen on other cell types, such as fibroblasts and endothelial cells. This means that tamoxifen's cellular effects may reach beyond specific ER inhibition and cause the cell to increase production of a negative growth regulator inhibiting its growth in an autocrine manner. It has also been suggested (Knabbe *et al.*, 1987) that tamoxifen's effects through TGF-ß production may reach beyond the target cell inhibiting neighbouring ER negative cells by a paracrine mechanism. Possible tamoxifen inhibitory mechanisms are illustrated in Figure 1.

A major metabolite of tamoxifen, 4-hydroxytamoxifen is produced and binds to the ER in a number of species (Borgna *et al.*, 1981). This metabolite has

a hundred fold greater affinity for the ER than the parent drug (Sutherland *et al.*, 1986) although both display partial agonist activity on the expression of the progesterone receptor. *In vivo*, the metabolite 4-hydroxytamoxifen is rapidly metabolized and eliminated therefore its importance clinically may not be as great as tamoxifen itself. (Rochefort *et al.*,1983)

1.3.2 Clinical Effects of Tamoxifen

Response rates to all forms of first line endocrine treatment including tamoxifen are found to be approximately one third, in an unselected population of breast cancer patients. In patients whose tumours are found to be ER positive the response rate to tamoxifen treatment increases to around 50% (Howell *et al.*,1990).

Originally tamoxifen was introduced for the treatment of advanced breast cancer in postmenopausal women where it has been used both as a palliative therapy for advanced disease and as adjuvant therapy in primary breast cancer. Adjuvant therapy with tamoxifen confers a survival advantage in node positive postmenopausal women which is increased when the primary tumour was found to be ER positive (Early Breast Cancer Trialists Collaborative Group, 1988). In this study a tamoxifen treatment duration of 1-2 years was employed. Also to benefit from adjuvant tamoxifen treatment have been patients with ER positive node negative breast cancer (Fisher *et al.*,1989).

Tamoxifen treatment is now continued for much longer periods, usually five years since the drug has proved to be very well tolerated by patients with few

side effects. Tamoxifen has also been found to be a useful treatment for premenopausal patients. In a study by Sawka *et al.*,(1986), tamoxifen was used as the initial hormone therapy in a group of premenopausal breast cancer patients with metastatic disease. A complete or partial response was found in 27% of patients or 43% when a stable no change response was included. A response to tamoxifen was found to strongly predict the response to ovarian ablation.

1.3.3 Pure Antiestrogens

New pure antiestrogens have been developed which show no partial agonist activities at any concentration. Such a drug is ICI 164,384 which is able to successfully compete with oestradiol to bind to the ER producing no oestrogen like cellular responses. This has the advantage over tamoxifen in that all ERs can be completely blocked from all available sources of oestrogens. It still competitively inhibits the binding of E_2 but with a much greater affinity than the partial agonist, tamoxifen (Wakeling, 1989). It is also less likely to bind to other sites within the cell such as the antiestrogen binding sites. Upon binding to the ER the pure antagonist ICI 164,384 ER complex has lost the ability to recognise DNA (Wilson *et al.*, 1990) and is unable to activate any oestrogen responsive genes (Weaver *et al.*, 1988, Wiseman *et al.*, 1989). As yet, this drug still awaits an outcome in clinical trials, but scientific evidence points to a longer lasting and higher percentage response in ER positive breast cancer. It may only fall down by its effect outside the tumour where premature onset of osteoporosis and atherosclerosis could be a problem. Tamoxifen has been shown to have a

beneficial oestrogenic effect on bone density (Jordan *et al.*, 1987) and shows a significant decrease in LDL cholesterol levels in women during tamoxifen treatment (Love *et al.*, 1989), effects which may be attributable to its partial agonist activities.

1.4 AUTOCRINE AND PARACRINE GROWTH MECHANISMS

Autocrine secretion is thought to be one of the main mechanisms responsible for the ability shown by malignant cells for autonomous growth. Polypeptide growth factors released by tumour cells can act on the cell through functional specific receptors (Sporn & Todaro, 1980). TGF-α and PDGF have both been recognised as growth factors involved in positive autonomous growth of cancer cells (Todaro *et al.*,1980; Richmond *et al.*, 1983; Heldin *et al.*,1980; Nister *et al.*, 1984). TGF-α has already been mentioned as a growth factor involved in the E_2 regulated growth response of ER positive breast cancer cells (Section 1.2.2) where it increases cellular growth through an autocrine mechanism. TGF-ß can act as a negative autocrine growth factor in many epithelial lines and it is thought to be one of a number of mechanisms responsible for the growth inhibitory effects of the antioestrogen tamoxifen.

Paracrine growth control occurs when a cell responds to a growth factor synthesised and secreted by a neighbouring cell (Sporn & Todaro, 1980). The cell synthesising the growth factor may or may not possess the specific receptors to respond to that growth factor but the target neighbouring cells do, although they may lack the ability to synthesise the growth factor. The peptide growth factor

will travel by local diffusion through the extracellular space to its target receptor but does not travel through the bloodstream. Often the two cells involved may be of different cell types as in stromal control of tumour growth (1.6). In breast cancer, a number of growth factors have already been identified for their autocrine and paracrine regulation of cell growth. The list includes TGF-α, IGF-I, IGF-II, PDGF, TGFß and the FGF's. Each one will be described in detail in the next section. The list of growth factors involved in autocrine and paracrine growth regulation in breast cancer is by no means complete and many more remain to be identified.

1.4.1 Epidermal Growth Factor (EGF)

Transforming Growth Factor alpha (TGF-α)

In 1962 Stanley Cohen first discovered EGF by its ability to accelerate eyelid opening and the eruption of teeth in new born mice. Later, it was found that a protein purified from human urine which had the ability to inhibit gastric acid secretion, was the same peptide (Starkey *et al.*, 1975). TGF-α was purified from sarcoma growth factor (SGF) present in the conditioned medium of transformed murine 3T3 fibroblasts (De Larco *et al.*, 1978). SGF was able to stimulate the clonal growth of normal rat kidney cells in suspension due to the presence of two distinct growth factors TGF-α and TGF-ß. TGF-α was characterized by its ability to bind and activate the EGF receptor. Both EGF, a 6kDa polypeptide of 53 amino acids, and TGF-α, a 5.6kDa species consisting of

50 amino acids, are cleaved from large membrane bound precursor molecules. They have a variety of biological functions which are often concentration dependent causing cellular proliferation at low levels and differentiation at high levels (Yoshida *et al.*, 1987).

The tissue distribution of EGF and TGF-α varies somewhat. EGF appears late in animal development (Popliker *et al.*, 1987) whilst TGF-α is synthesized during embryonic development. TGF-α is found at high levels in self-renewing epithelial tissues such as skin and the gastro-intestinal tract (Malden *et al.*, 1989). Most importantly, TGF-α has been closely associated with neoplastic transformation and its synthesis is most frequently found in tumour cells. Neither EGF or its precursor are produced by transformed cells, although EGF is a useful tool for mimicking the effects of TGF-α in an experimental situation. Human breast cancer cell lines are known to contain the EGFr and many respond to EGF with increased growth, further investigation has found these cells to produce TGF-α. In the MCF-7 cell line, secretion of TGF-α is regulated by oestrogen *in vitro* (Fig.1), with levels of TGF-α mRNA increasing upon exposure to oestrogen. A decline in TGF-α mRNA levels was found *in vivo* upon oestrogen withdrawal (Bates *et al.*, 1988). These data suggest TGF-α acts as a mitogenic growth factor and is a possible factor involved in mediating the mitogenic effects of oestrogens in ER positive cells. When TGF-α was constitutively expressed in MCF-7 cells using a TGF-α cDNA expression vector in an attempt to override oestrogen regulated cell growth, little change in the growth rate was seen from the parent line either *in vitro* or *in vivo* (Clarke *et al.*,1989), this suggested TGF-α alone was

(Burgess
1989)

(Ennis *et a*
1989)

not responsible for the mitogenic activity of oestrogen. TGF-α mRNA expression has also been reported in the ER negative human breast cancer cell line MDA-MB-231 at a level equivalent to that seen in oestrogen stimulated ER positive lines (Bates *et al.*,1988).

When TGF-α was measured in pleural effusions from human breast cancer patients and normal control subjects, it was found to be present in both groups but the level was significantly higher in the cancer patients (Ciardiello *et al.*, 1989). In a separate investigation no differences were found between breast cancer and control groups but within the breast cancer patients tamoxifen treatment notably reduced the levels of detectable TGF-α (Gregory *et al.*,1989).

1.4.2 EGF Receptor

Both EGF and TGF-α are known to bind with high affinity to the cell surface EGF receptor. The 170kDa EGF receptor is a member of the tyrosine kinase receptor family and contains three main components, an extracellular ligand binding domain, a hydrophobic transmembrane region and a cytoplasmic (Merlino 1990) domain containing the tyrosine kinase catalytic domain. Many types of tyrosine kinase receptors exist; distinction between different structural characteristics divides these into a number of subclasses. The EGF receptor belongs to subclass I (Fig.2). Upon ligand binding, the extracellular domain of the receptor undergoes dimerization allowing transmission of signal to the cytoplasmic domain and autophosphorylation of the receptor tyrosine residues (Schlesinger, 1988).

Phosphorylation of receptor tyrosine kinases mediates the mitogenic signal brought about by EGF or TGF-α and is also responsible for downregulation of the receptor by internalization and lysosomal degradation.

EGF recptor was found to exist in membrane preparations from primary human breast carcinomas (Sainsbury *et al.*,1985, Perez *et al.*,1984) and in surrounding normal tissue at lower expression levels (Barker *et al.*, 1989). As already mentioned, (section1.2.5) levels of EGFreceptor negatively correlate with ER and overexpression of the EGF receptor has been shown to be a marker for poor prognosis in patients with operable breast cancer (Sainsbury *et al.*,1987, Rios *et al.*,1988, Nicholson *et al.*,1991).

Studies in breast tumour cell lines have consistently found the EGF receptor to be present, although expression levels vary (Fitzpatrick *et al.*,1984, Davidson *et al.*,1987). Again an inverse relationship exists between the ER and the EGF receptor leading to the suspicion that one may be able to regulate the other, no direct evidence for this exists as yet. Addition of EGF to cultures of breast tumour cell lines only brought about an increase in cell growth when there was low expression of EGF receptor as in the MCF-7 cell line, whilst the cell line MDA-MB-231 expressing high levels of receptors showed growth inhibition by EGF (Davidson *et al.*,1987) an effect thought to be due to ligand binding to low affinity EGF receptors (see section 4.3.1). Evidence for the existence of an autonomous mitogenic pathway is to be found in the breast cell line MDA-468 where the addition of antibody to the EGF receptor resulted in inhibition of growth. This was not common to all cell lines expressing high levels of EGF

receptors, where other mechanisms may have a greater influence on cell growth regulation (Ennis *et al.*,1989).

1.4.3 The c-erbB-2 Protein

The gene product of human c-*erb*B-2 is closely related to the EGF receptor with 40% homology on the extracellular domain and 82% homology on the tyrosine kinase domain, the transmembrane region being the only area showing significant structural differences (Gullick,1988). It has a molecular weight of 185kDa and also belongs to subclass I of the tyrosine kinase receptors. Overexpression of the c-*erb*B-2 gene occurs frequently in breast tumour cell lines (Kraus *et al.*,1987). Its expression has been found to be regulated by oestrogen in two ER positive breast cancer cell lines, T47D and MCF-7. The c-*erb*B-2 oncogene expression was increased in the absence of oestrogen and decreased in the presence of oestrogen (Dati *et al.*,1990).

As yet, a ligand responsible for phosphorylation of the c-*erb*B-2 gene product has not been characterised but its existence was verified by Segatto et al (1992) who created a chimeric molecule encompassing the EGF receptor extracellular domain and the c-*erb*B-2 gene product transmembrane and intracellular domains. Upon expression in NR6 fibroblasts autophosphorylation of the EGF/c-*erb*B-2 chimera required the addition of EGF. Two ligands to the c-*erb*B-2 receptor with molecular weights of 30 and 75kDa have been identified which are secreted by ER positive breast cancer cell lines (Lippman,1991).

In clinical breast cancer overexpression of c-*erb*B-2 occurs in approximately

20% of cases. A combined study found c-*erb*B-2 overexpression to be an independent risk factor for overall survival and poor relapse free interval (Gullick *et al.*,1991). A similar finding was made by Winstanley et al (1991) in a single study of 465 breast cancer patients.

1.4.4 The Insulin-like Growth Factors

Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are polypeptide growth factors with molecular weights of 7600 and 7500 respectively. They both show a large degree of structural homology to pro-insulin and together with relaxin and nerve growth factor form a family of related polypeptides. (Yee *et al.*,1991)

IGF-I has been shown to stimulate proliferation in a number of breast cancer cell lines, MDA-MB-231, ZR-75-1 and Hs578T (Huff *et al.*,1986). IGF-II is also mitogenic to the breast cancer cell lines MCF-7 and T47D (Yee *et al.*,1988), although less potent than IGF-I. These results suggest both IGFs play an important role in neoplastic cell growth and their possible role as autocrine growth regulators has been widely investigated. The mRNA expression of IGF-I was not found upon examination of a number of human breast cancer cell lines (Yee *et al.*,1989) although a number of homologous nucleotide sequences were detected which may represent an unidentified IGF related peptide. Similarly, expression of IGF-II mRNA, has not been found in the majority of breast cancer cell lines except T47-D where expression is under oestrogen control (Yee *et al.*,1988). These results suggested IGF-I and IGF-II were not acting as autocrine

regulators of cell growth, whereas studies into IGF mRNA expression in breast tumour tissue suggested both were present in the tumour (Yee *et al.*,1988, Yee *et al.*,1989). The major difference being the inclusion of nonmalignant cells in breast tumour samples such as stromal and endothelial cells. More detailed examinations in human tissues have pinpointed IGF-I mRNA expression to the normal breast ductules and particularly the normal stroma, with no expression detected in the malignant epithelial cells (Yee *et al.*,1989). IGF-II mRNA expression is uniformly distributed between stromal and malignant cells and appears to be perfectly capable of acting as an autocrine factor. Both show evidence of being important paracrine growth regulators in breast cancer because of their expression in the normal tissue adjacent to malignant cells which contain specific IGF receptors.

1.4.5 Insulin and IGF Receptor

The IGFs are known to bind to specific membrane receptors and separate receptors have been identified for IGF-I and IGF-II. The IGF-I receptor shows (Czech 1982) remarkable similarity to the insulin receptor and belongs to the type II subclass of tyrosine kinase receptors (Fig1.2). Upon ligand binding to the insulin or IGF-I receptor a cascade of phosphorylation events occurs starting with autophosphorylation of the receptor and a number of cytoplasmic substrate proteins, The IGF-I receptor has a 130kDa binding subunit and comprises a heterotetrameric structure of two α and two ß subunits joined by disulphide bridges. IGF-I receptors were found to be present in a number of breast cancer

cell lines (Furlanetto *et al.*,1984) which showed increased proliferation upon stimulation by the ligand IGF-I. It was also recognised that the mitogenic effects of insulin were being mediated through the IGF-I receptor due to the higher concentrations of insulin required to illicit a response. Histo-autoradiographic analysis of the IGF-I receptor found it was localised to the proliferative epithelial component in human breast tumours and expressed at significantly higher levels than in benign or normal breast tissue (Jammes *et al.*,1992).

The IGF-II receptor shows very different characteristics, it consists of a single polypeptide chain which lacks any tyrosine kinase activity. It does not elicit a mitogenic response upon binding of its ligand and its functional role is uncertain (Furlanetto *et al.*,1987). It is unlikely that this receptor is important as an autocrine or paracrine regulator of growth in breast cancer. On the other hand, the ligand IGF-II along with IGF-I and insulin are all able to bind to the IGF-I receptor with differing affinities and bring about a mitogenic response. Most breast cancer cell lines show expression of all three receptors for insulin, IGF-I and IGF-II (Cullen *et al.*,1990).

The IGFs are further regulated by a family of IGF binding proteins (Baxter *et al.*,1989) which bind to the IGFs with high affinity altering their interaction with the receptor.

In summary, the IGF system seems to play a predominantly paracrine role in breast tumour regulation, although IGF-II is capable of stimulating autocrine control. There is three major components of regulation, the growth factors, their receptors and specific binding proteins, interactions between each of these brings

about the growth effect.

1.4.6 IGF Binding Proteins

Human breast cancer cell lines have been found to express several species of IGF binding proteins. Many of these binding proteins are selectively produced by different cell lines. A 24kDa protein was common to all the breast cancer cell lines studied by Favoni *et al*, (1989). The function of the IGF binding proteins is not well understood, certainly they will play a regulatory role in the interaction between the IGF ligands and receptors and should not be overlooked as important modulators of IGF autocrine, paracrine and endocrine function.

1.4.7 The Fibroblast Growth Factor Family

The heparin binding growth factor family of fibroblast growth factors (FGF) represent a group of mitogenic growth factors with diverse functions in a large variety of cells. Besides mitogenesis, they participate in angiogenesis, differentiation, cell migration, neuronal maintenance and embryonic development. (Baird & Klagsburn, 1991) The family presently contains seven members which show significant areas of sequence homology to one another, these are; acidic and basic FGF (aFGF and bFGF respectively), *hst*-1/K-FGF, *hst*-2/FGF-6 and *int*-2 proteins, FGF-5 and keratinocyte growth factor (KGF). bFGF is one of the more highly characterised members of the family. It is a single chain polypeptide which has been shown to promote angiogenesis and wound healing *in vivo* (Davidson *et al.*,1985). Expression of the bFGF gene was not found in a number of mammary tumour

cell lines which included, BT474, MCF-7 and T47D although it was found in cultured normal human mammary epithelial cells (Li *et al.*,1991). In malignant biopsies bFGF was found to exist in the benign areas within the tumour (Gomm *et al.*,1991) suggesting bFGF is not important in breast cancer. bFGF has been shown to stimulate growth of cultured cells from both normal and malignant breast biopsies *in vitro*, with a higher response in the malignant cell lines (Takahashi *et al.*,1989). Since the breast tumour cells appear to respond to bFGF *in vitro* perhaps another family member activates the receptor *in vivo*. The *hst*-1 gene product is known to share the same receptor (Moscatelli *et al.*,1989) and amplification of this gene together with *int*-2 is associated with poor prognosis in primary hormone dependent breast cancer (Borg *et al.*,1991) both are located in the same chromosome band (11q13). Amplification of five different FGF genes was examined in 238 breast carcinomas, the list included; bFGF, *int*-2, *hst*-1, *hst*-2 and FGF-5. Only *hst*-1 and *int*-2 were amplified in a significant number of tumours (17%), the *hst*-1 gene mRNA was expressed only on gene amplification whilst *int*-2 mRNA expression showed no relationship to its amplification (Theillet *et al.*,1989). Lidereau et al (1988) found a close association between *int*-2 amplification in primary human breast tumours and subsequent disease recurrence locally or a distant metastatic sites, therefore the *hst*-1/*int*-2 co-amplification shows these proteins could be involved in breast cancer growth regulation.

Another member of the family, KGF, shows specificity as a growth factor for epithelial cells and was first discovered in the conditioned medium of embryonic fibroblast cells (Rubin *et al.*,1989). KGF expression has been found

to be specific for stromal cells with no expression in epithelial tissue (Finch *et al.*,1989). It would appear this member of the family has an important role to play as a paracrine regulator of cell growth. Its importance in breast cancer has yet to be investigated.

1.4.8 FGF Receptors

The FGF receptors show tyrosine kinase activity and fit into subclass IV of this family (Fig.2). The extracellular domain of the FGF receptors vary from that of EGF and IGF-I receptors by having an immunoglobulin-like structure. The members of the FGF receptor family, *bek* and *flg* will bind acidic and basic FGF with high affinity (Dionne *et al.*, 1990) and *flg* tyrosine kinase can be activated by *hst*-1 (Mansukhani *et al.*,1990). Amplification of *bek* and *flg* were looked for in a large sample of breast tumours and was found in 11.5 and 12.7% respectively. Interestingly in this set of data amplification of *flg* significantly correlated with the amplification of the *hst*-1/*int*-2/*bcl*1 cluster on chromosome band (11q13) (Adriane *et al.*,1991). Since *hst*-1 is able to activate the tyrosine kinase activity of *flg*, an autocrine FGF network could be suggested in this group of breast tumours.

Eisemann et al,(1991) found five different isoforms of the human bFGF receptor (*flg*) which differ in the number of immunoglobulin-like structures on the extracellular domain, all generated from a single gene. Each isoform may show specificity for different FGFs thereby differentiating the function of each FGF and the tissue specificity. One isoform encodes a truncated form of the receptor

which lacks a transmembrane region, its function is unknown.

1.4.9 PDGF and the PDGF Receptor

Human platelet derived growth factor (PDGF) is a 30 to 34kDa protein existing as a heterodimer of two polypeptide chains (A & B) which are disulphide linked. PDGF is synthesised by human breast cancer cell lines and is under oestrogen regulation in the hormone dependent cell line MCF-7 (Bronzert *et al.*,1987). Since epithelial cells do not posses PDGF receptors or respond to exogenous PDGF (Heldin *et al.*,1981) the purpose of PDGF production must be toward paracrine growth regulation. PDGF is known to be a major mitogen for fibroblast cells and PDGF receptors are present in the breast stromal tissue. The PDGF receptor is also a tyrosine kinase receptor (subclass III, Fig.2) and will elicit a mitogenic response through ligand binding. PDGF is known to be a potent stimulant of IGF-I production in fibroblast cells and this could account for the self perpetuating paracrine system between the malignant cells and their surrounding stroma (Clemmons *et al.*,1981).

(Heldin 1992)

1.4.10 TGF-beta

Transforming growth factor beta (TGF-ß) was discovered alongside TGF-α and shown to be quite distinct from TGF-α, binding to its own separate receptors. TGF-ß in its active form is a 25kDa disulphide linked homodimer and exists as a number of isoforms TGF-$ß_1$, $ß_2$, $ß_3$ and $ß_4$, although most investigation has been focused on TGF$ß_1$. TGF-ß shows a high degree of conservation between species (Sporn *et al.*,1986)

and plays a multifunctional role in cell proliferation, differentiation and is important for development and tissue repair. Platelets form the major storage site for this growth factor in the body (Assoian *et al.*,1983). TGF-ß is biosynthesized as a protein-bound, latent inactive form of growth factor and requires to be purified under acid conditions to its active component. Activation of latent TGF-ß *in vivo* may act as a regulatory step in controlling TGF-ß function although the mechanism of cleavage is unknown. The proteases, plasmin and cathepsin D, have been shown capable of activating TGF-ß (Keski-Oja *et al.*,1987) and they make good candidates for this physiological control. Exposure to acidic microenviroments which are known to exist in tumours and during wound healing have also been suggested as an *in vivo* mechanism for activation (Sporn *et al.*,1987).

Since TGFß was originally discovered as a growth promoter of fibroblasts it was termed a growth factor. TGF-ß is known to enhance proliferation in many cells of mesenchymal origin but of particular interest in breast cancer is TGF-ß's inhibitory function. TGF-ß has been shown to have potent antiproliferative effects in a number of cell types *in vitro* but particularly epithelial cells (Moses *et al.*, 1985). Most tumour cells express TGF-ß mRNA and many secrete TGF-ß protein (Derynck *et al.*,1987, Salomon *et al.*,1984). In hormonally responsive breast cancer cell lines the expression of TGF-ß is regulated by oestrogen (Arrick *et al.*,1990). Oestrogen stimulation of MCF-7 and T-47D cells brought about a decrease in TGF-ß production of up to 50% in both cases (Dickson *et al.*,1986[b]). Under tamoxifen treatment TGF-ß secretion is induced up to 27 fold in the MCF-7 cell line (Knabbe *et al.*,1987) and the authors proposed this to be a

mechanism mediating tamoxifen inhibition (Fig.1), they also suggest TGF-ß secreted by ER positive cells can inhibit the growth of neighbouring ER negative cells by paracrine regulation. The hormone independent line MDA-MB-231 expressed a high level of TGF-ß activity (Dickson *et al.*,1986) and is strongly growth inhibited by TGF-ß (Arteaga *et al.*,1988) this indicates its activity as an autocrine growth regulator.

In general *in vivo* experiments have been in agreement e.g., MCF-7 xenograft models showed suppression of TGF-ß mRNA upon oestrogen stimulation in thymectomised mice (Thompson *et al.*,1990). The same system found a sustained high level of TGF-$ß_1$ mRNA expression upon tamoxifen treatment which resulted in a reduction in tumour size although these effects were not found in a group of breast cancer patients studied where TGF-ß mRNA levels were increased in tamoxifen resistant tumours (Thompson *et al.*,1991). In mouse models TGFß treatment was found to reversibly inhibit mammary gland growth (Silberstein *et al.*, 1987).

1.4.11 TGF-ß Receptor

TGF-ß binds to a specific cell membrane receptor consisting of two disulphide linked subunits with no intrinsic tyrosine kinase activity. It appears to be present to varying extents in all cell types tested (Wakefield *et al.*,1987), in epithelial cells it brings about growth inhibition by reducing c-*myc* transcription essential to proliferation (Moses *et al.*, 1990). The mitogenic action of TGF-ß is also indirect and is mediated by PDGF which causes autocrine stimulation of stromal and endothelial cells.

In summary, TGF-ß should be regarded as an important negative regulator of breast tumour cell growth. It is regulated at a number of points, synthesis of the latent peptide, cleavage of precursor to activate TGF-ß and presence of receptors to bring about cellular response. In ER positive cells TGFß action is hormonally regulated.

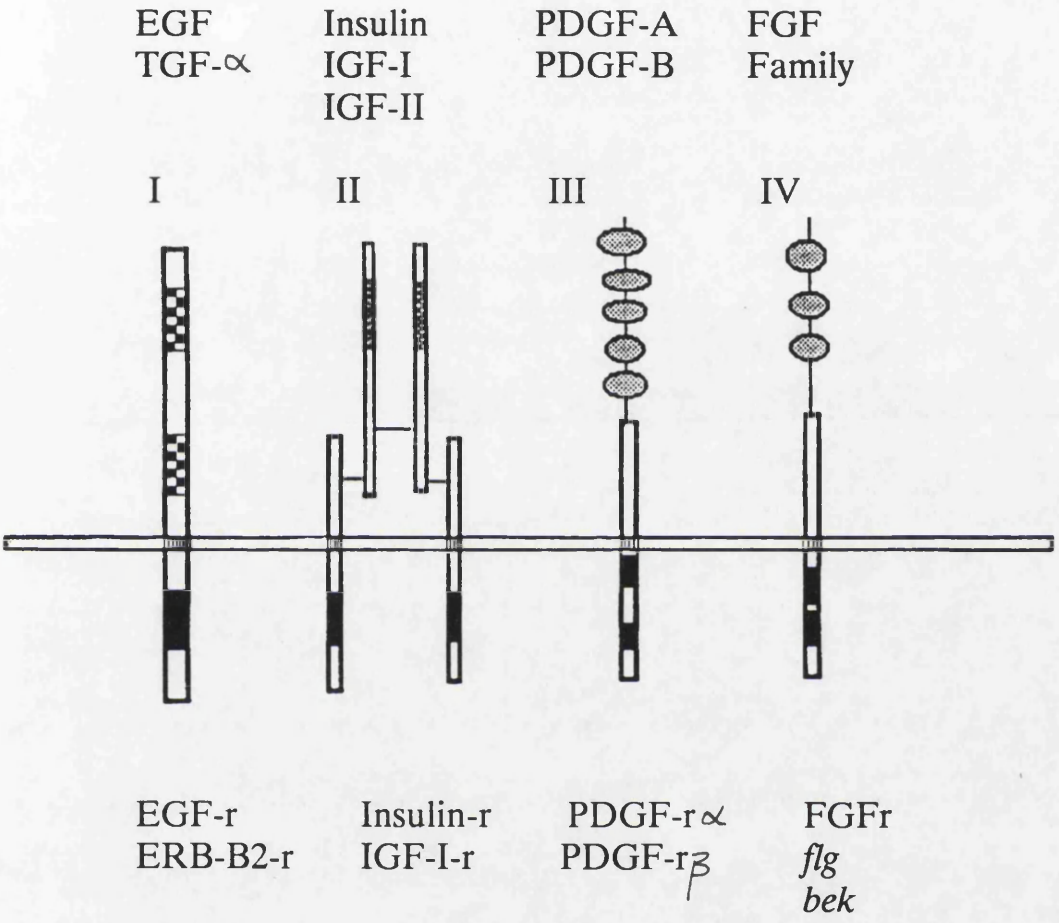
1.4.12 Other Growth Inhibitors

Mammostatin, consisting of polypeptides of 47 and 65KDa is also an inhibitor of mammary epithelial cell proliferation and was found to be produced in the normal mammary cells, but much lower levels of production were seen in transformed lines suggesting this route of inhibition may be lost upon transformation (Ervin *et al.*,1989).

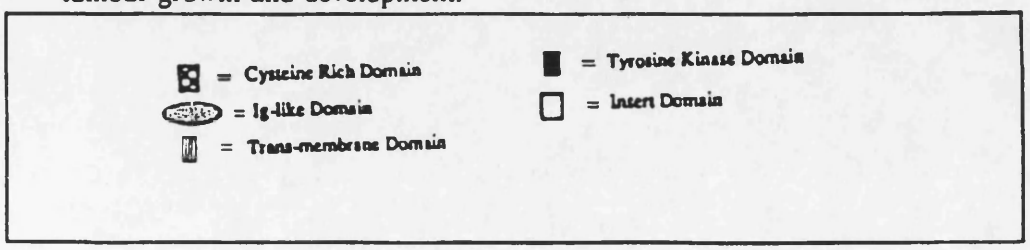
1.4.13 Interleukin-6

Proliferation of breast cancer cell lines is inhibited by the cytokine interleukin-6 (IL-6) (Chen *et al.*,1991) which is often detected in neoplastic tissue of cancer patients. Independent of this inhibitory growth effect, IL-6 brings about distinct morphological changes in breast cancer cells, altering cell shape and motility with cells converting to fibroblastoid characteristics (Tamm *et al.*,1991). This property of IL-6 suggests it may have an important role to play in tumour cell scattering and movement within the body.

Figure 1-2 Growth Factor Receptors with Tyrosine Kinase Activity



Structural features of tyrosine kinase receptors known to be important in breast tumour growth and development.



1.5 GROWTH FACTOR -SIGNAL TRANSDUCTION MECHANISMS

Considerable structural diversity exists between members of the protein tyrosine kinase group of growth factor receptors, which are illustrated in Figure 1.2. Despite this, they all perform basically similar functions which involve recognition of a specific ligand and initiation of a signal eventually leading to a specific cellular response such as, proliferation or increased receptor synthesis. Each receptor can be divided into three main parts, the extracellular ligand binding domain which shows growth factor specificity within each receptor subclass, a transmembrane region which acts as an anchor holding the receptor in the membrane, and finally the intracellular tyrosine kinase domain which is indispensible for induction of mitogenic stimulation by growth factors (Chen *et al.*,1987). This region shows the greatest conservation between receptors. For general review see, (Ullrich & Schlessinger, 1990)

1.5.1 Phosphorylation of Substrate Proteins

After receptor activation, associated tyrosine residues become rapidly phosphorylated. The major site of phosphorylation is the receptor itself, and tyrosine phosphorylation at a number of specific points on each receptor induce binding of cytoplasmic proteins. *Src* homology regions called SH2 domains are contained in many cytoplasmic signalling proteins and are responsible for high affinity binding to phosphorylated receptor tyrosine kinases (Rozengurt, 1992). After the initial receptor phosphorylation, an array of cellular substrates are phosphorylated which will eventually lead to the cellular response. This chain of

events is only partially understood but a number of cytosolic proteins recruited by activated protein tyrosine kinase receptors have been identified. Receptor activation leads to physical association with four main substrate proteins; phospholipase C-γ(140kDa), GTPase-activating protein (120kDa), phosphatidylinositol 3' kinase (85kDa) and Raf-1(74kDa).

Tyrosine phosphorylation of phospholipase C-γ (PLC-γ) was induced by both the EGF and PDGF receptor and showed association with activated receptor kinases (Margolis *et al.*,1989, Meisenhelder *et al.*,1989) through an SH2 domain (Stahl *et al.*,1988). PLC-γ causes the production of diacylglycerol (DAG) leading to activation of PKC and inositol-(1,3,4)-tris-phosphate both important cellular second messengers.

Another cytosolic substrate phosphorylated by receptor activation is *ras* GTPase-activating protein (*ras* GAP) (Kaplan *et al.*,1990). GAP acts as a negative regulator of *ras* function which is a critical component of intracellular mitogenic signalling pathways. GAP contains two adjacent SH2 domains indicating its ability to interact with tyrosine kinases. In fibroblasts stimulated with EGF, GAP becomes rapidly phosphorylated along with two co-precipitating proteins p62 and p190 (Ellis *et al.*,1990). The importance of GAP seems to be in linking receptor tyrosine kinases with $p21^{ras}$, GAP regulates the $p21^{ras}$GTPase activity. The GTPase activity of ras proteins are enhanced by 120kDa GAP which is thought to alter the balance between GTP and GDP of the ras protein (M^{c}Cormick,1989).

Phosphatidylinositol 3' kinase (PI-3)kinase is known to associate with a

number of activated tyrosine kinases. The EGF receptor shows limited association with this substrate (Margolis *et al.*,1989) whilst it associates more consistently with the PDGF and insulin receptor.

The protein product of c-*raf* is also activated by a number of growth factors (Li *et al.*,1991) this protein Raf-1, is mostly phosphorylated on serine although some tyrosine phosphorylation has been identified. Insulin along with other growth factors causes an increase in serine phosphorylation of the Raf-1 74kDa protein (Blackshear *et al.*,1990) although its method of receptor association is unknown, since it contains no SH-2 domains. Raf-1 can enhance transcription indepently of PKC (Kaibuchi *et al.*,1989) and shows areas of sequence homology to PKC (Li *et al.*,1991). Recent work has identified the mitogen-activated protein (MAP) kinases as being involved in the same phosphorylation cascade as Raf-1. A 50kDa MAP kinase-kinase has been identified as a physiological substrate for Raf-1 (Kyriakis *et al.*,1992).

A number of other substrate proteins have been identified to associate with growth factor receptors, whose functions are not well characterised, these include the calpactins or annexins and ezrin. It is thought they may play a role in the rearrangement of actin filaments which are important for a number of cellular functions, including membrane ruffling and cell rounding.

Ezrin is a 80kDa cytoskeletal protein which phosphorylates on tyrosine and serine in A431 cells after treatment with EGF. Ezrin becomes associated into cellular microvillar structures at a time corresponding to these physiological changes in the cell and may play a role in formation of cell surface projections

(Bretsher,1989).

The family of proteins which include the annexins, lipocortins, calpactins and calcimedins, contain at least eight members. Many are phosphorylated as substrate proteins to a number of growth factor receptors. Their physiological roles include calcium transport, inositol phospholipid signalling and regulation of phospholipase A_2. Each member of the family contains a characteristic amino acid sequence motif of 70 amino acids which is repeated a number of times in each annexin (Glenney *et al.*,1987).

1.5.2 Protein-tyrosine Phosphatases

It is important for normal cellular function that phosphorylated proteins are able to return to their resting inactive state. This function is tightly regulated by specific protein phosphatases, these proteins are themselves regulated through ligand receptor binding. Both receptor and nonreceptor protein tyrosine phosphatases exist which are thought to differ in specificity. Cell signalling through protein tyrosine kinase phosphorylation is positively regulated by ligand binding of the protein tyrosine kinase receptor and negatively regulated by activated protein tyrosine phosphatases. Loss of a specific phosphatase may result in unregulated tyrosine phosphorylation and cellular transformation (for review see, Hunter,1989).

1.5.3 Growth Factor Receptor Transmodulation

A small region of the growth factor receptor contained between the

transmembrane region and the tyrosine kinase domain called the juxtamembrane sequence appears to be involved in receptor transmodulation. PKC activation by a growth factor can phosphorylate a threonine 654 residue located on the juxtamembrane region (Yarden *et al.*,1988[a]).A phosphorylation event which is thought to reduce receptor binding affinity allowing growth factor induced PKC to lower the affinity of other growth factor receptors. For the EGF receptor phosphorylation of Thr 654 seems to be involved in the control of receptor mitogenic signalling (Livneh *et al.*,1988).

1.5.4 Signalling Targets for Drug Development

As knowledge of the signalling pathways for mitogenesis become better characterised, differences can be identified between normal and transformed cells such as overexpression of certain growth factor receptors or constitutive activation of a signalling pathway. This opens new areas for drug targetting with the possibility of higher specificity and greater therapeutic potential.

One group of drugs, the tyrphostins, are synthetic compounds able to inhibit tyrosine kinases specifically. These can be designed to be highly site specific, able to block EGF dependent cell proliferation (Akiyama *et al.*,1987, Yaish *et al.*,1988) whilst the indole tyrphostins have proved to be selective inhibitors of PDGF receptor activation (reviewed in, Levitzki *et al.*,1991), others again show selective inhibition toward the insulin family of growth factor receptors.

1.6 STROMAL INTERACTIONS IN BREAST CANCER

It is important when examining the regulation of breast cancer growth that all cellular components of the tumour are taken into account. A breast tumour consists of many different cell types, including fibroblasts, endothelial cells, macrophages, lymphocytes as well as the carcinoma cells. It is the interactions between all these different cells which will ultimately regulate tumour growth and may even be important for tumour invasiveness and metastatic ability. The regulation of tumour stroma formation by carcinoma cells and the influence of the stromal cells over carcinoma cell growth will both be examined in some detail. In breast tumours there are often very strong stromal elements present, an example of this is the scirrhous type of tumours commonly found in breast cancer. They exhibit a high proportion of collagenous stroma, and it is important the growth supporting role of this tissue is not overlooked.

1.6.1 Matrix Development by Tumour Cells

A solid tumour consisting solely of carcinoma cells would very quickly lose the ability to grow due to limiting factors such as, oxygen diffusion and nutrient supplies. To overcome these difficulties, tumours are able to equip themselves with a blood supply and a protective extracellular matrix (ECM), allowing them to grow parasitically within the body tissues.

For a solid tumour to proliferate it needs to contain a strong stromal component with a good blood supply. In the first instance this appears to be due

to properties of the tumour cell, which cause extravasation and coagulation of plasma derived fibrinogen (Dvorak *et al.*,1983). A so called vascular permeability factor, secreted by tumour cells seems to be responsible for the leakage of plasma proteins, amongst them fibrinogen, from normal blood vessels (Senger *et al.*,1983). Tumour cell derived procoagulants, coagulate fibrinogen to form a cross-linked fibrin deposition. The fibrin gel is transformed over time into the tumour stroma containing vascular and collagenous components. This again is under tumour cell regulation. Protease plasminogen activator (section 1.2.2) is synthesised by breast tumour cells and converts vascular derived plasminogen to plasmin, which is an active fibrinolytic protease (Dano *et al.*,1985). A number of other enzymes may also be involved in this process. The cycle of fibrin accumulation and fibrinolysis is a dynamic process. To accomodate the changeover from fibrin gel matrix to tumour stroma, macrophages,endothelial cells and fibroblasts migrate into the tumour. This may be a chemotactic function of fibrin itself (Dvorak *et al.*,1979). Migration eventually leads to the growth of tumour blood vessels, fibroblast proliferation and formation of mature collagenous ECM. TGF-ß also has an important stromal function, in chick embryo fibroblasts it causes an increased expression of the ECM components, fibronectin and collagen (Ignotz *et al.*,1986). Matrix components from human breast cancer cell lines have been shown to be mitogenic for normal fibroblasts which upon contact increase their growth and lose contact inhibition (Kao *et al.*,1984). This may be a direct effect of the ECM or indirect through the presence of growth factors in the ECM. The ability of breast cancer cells to synthesize growth factors has already been discussed

(section 1.4), many of these factors are mitogenic toward cellular components of the breast stroma. PDGF, FGF, IGF-II and TGF-α all are mitogenic toward fibroblasts.

1.6.2 Tumour Vascularisation and Angiogenesis

Solid tumour growth is dependent on neovascularization whereby new blood vessels are formed allowing the delivery of oxygen and nutrients to the tumour cells. The formation of new blood vessels is dependent upon the migration of endothelial cells into the tumour and their subsequent proliferation and differentiation. Much of this process seems to be under the control of tumour cell derived angiogenesis factors, these include the heparin binding growth factors, aFGF and bFGF, angiogenin, TGF-α and TGF-ß (reveiwed by Folkman and Klagsburn, 1987). The extent of capillary infiltration in human breast cancer has been found to correlate with the occurrence of metastases (Weidner *et al.*,1991), therefore the ability of a tumour to vascularise not only allows it to grow beyond a very limited size but also gives it an escape route into the systemic circulation from where it can gain access to other target organs.

1.6.3 Paracrine Growth Effects of Fibroblasts

Interactions between the stromal and epithelial components of breast tumours are currently receiving a lot of attention and it is clear the breast tumour stroma play a more active role in the growth of the tumour cells than simple supportive tissue. Normal fibroblast cells were found to increase tumour take

rates in nude mice if included in the inoculum of tumour cells (Horgan *et al.*,1987). The authors concluded that the fibroblasts were having a stimulatory effect on the growth and development of the xenograft.

Oestrogen induced proliferation of epithelial tissue shows regulation by associated stromal tissue (Cunha *et al.*,1992). Human breast fibroblasts were found to secrete a factor able to stimulate 17ß-estradiol dehydrogenase (E2DH). This reduces available oestrogens to biologically active oestradiol (E_2)(Adams *et al.*,1988), this activity was significantly higher in the fibroblasts collected from benign and malignant lesions rather than those collected from normal breast tissue. This is not the only evidence for differences between the activity of fibroblasts derived from normal and malignant tissue.

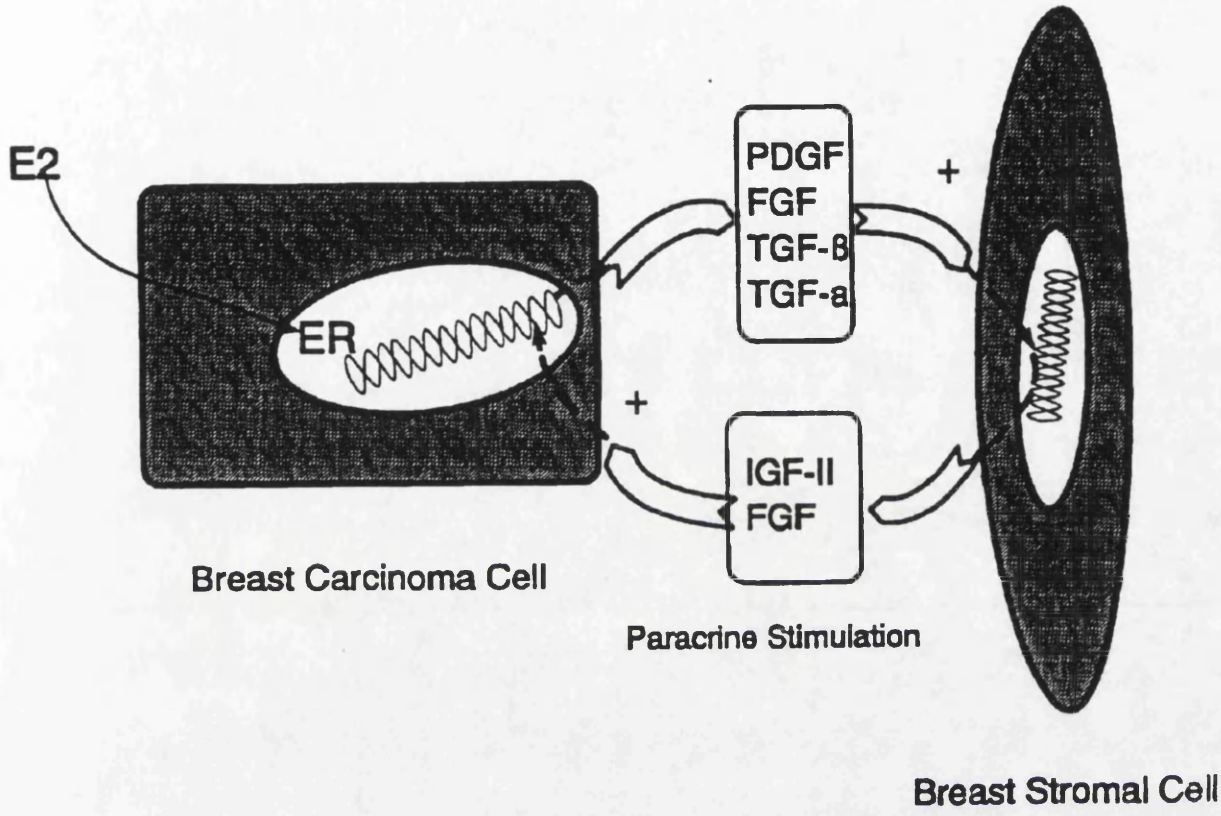
On examination of a number of growth factor mRNAs, fibroblasts derived from both benign and malignant breast tumours were found to express PDGF A chain, bFGF, FGF-5, TGF-$ß_1$, whilst IGF-I and IGF-II mRNAs were differentially expressed. IGF-I mRNA was found most frequently in fibroblasts of benign origin, whilst IGF-II mRNA was more closely associated with the malignant derived fibroblasts (Cullen *et al.*,1991). This work highlights the complexity of paracrine control in breast cancer growth and also points to a growth control mechanism unique to malignant tumours which may ultimately be under tumour cell control through an epithelial-stromal paracrine effect.

Basset *et al*, (1990) found specific expression of stromolysin-3 a metalloproteinase enzyme in the stromal cells surrounding only the invasive component of breast carcinomas and suggested this plays an important role in the

progression of epithelial tumour through degradation of the ECM. An abnormal or 'activated' population of fibroblasts in breast cancer patients has been the subject of intense study by Schor. He found that fibroblasts derived from breast cancer patients display a foetal-like migratory behaviour not seen in normal human fibroblasts. This was thought to be due to the production of migration-stimulating factor unique to fibroblasts of breast cancer patients and foetal tissue (Schor *et al.*,1988, Grey *et al.*,1989). The foetal-like behaviour was also associated with fibroblasts originated from close relatives of breast cancer patients and may prove a useful predictive factor for breast cancer (Haggie *et al.*,1987).

It is becoming clear that breast tumours are under the control of an integrated network of growth factors acting in both a paracrine and autocrine manner. Different cellular components of the tumour all play a part in the overall growth of the tumour. PDGF production by breast tumour epithelial cells can induce neighbouring fibroblasts to proliferate but also induce IGF production which has the ability to act mitogenically on the carcinoma cell where further PDGF upregulation may occur (Cullen *et al.*,1991). This is one example of interactive control, many more may exist and a number of possibilities have been illustrated in Figure1.3.

Figure 1-3 Stromal-Epithelial Interactions in Breast Cancer



Possible breast tumour, stromal-epithelial interactions. Breast tumour epithelial cells synthesise: PDGF, FGF, and TGF-α. All can increase fibroblast growth through specific receptor interactions. TGF-ß can indirectly increase the growth of fibroblast cells.

IGF-II and FGF are synthesised by breast fibroblast cells and each can stimulate the growth of breast epithelial cells.

1.7 AIMS OF THESIS

This thesis explores a number of aspects of breast cancer cell growth regulation in a range of breast cancer cell lines with the ultimate aim of finding new target areas of therapeutic value. The cell lines under study have been selected to represent different stages of malignant progression through variable expression of the ER and EGF receptor. At each stage of the study growth regulation of ER positive and ER negative cell lines have been compared at three different levels.

1. Characterisation of the growth effects of oestradiol and the antiestrogen tamoxifen in defined culture medium conditions.

2. Examination of the mitogenic response to specific peptide growth factors and the effects of conditioned medium derived from breast tumour fibroblasts.

3. Determination of cellular tyrosine phosphorylation pathways following growth factor stimulation with defined mitogens and breast fibroblast conditioned medium.

CHAPTER 2

MATERIALS & METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 SUPPLIERS

All chemicals supplied by **BDH Chemicals Ltd.**, Poole, Dorset unless otherwise stated.

Affiniti Research Products Ltd.,

10, Cossal Industrial Estate, Ilkenston, Derbyshire DE7 5UG

ATCC American Type Culture Collection,

12301 Parklawn Drive, Rockville, MD 20852 USA

Amersham International plc,

White Lion Rd., Aylesbury, Bucks HP20 2TP

Bio-rad Laboratories Ltd.,

27, Homesdale Road, Bromley, Kent

Boehringer Mannheim UK,

Bell Lane, Lewes, East Sussex BN7 ILG

BRL-Gibco,

PO Box 35, Trident House, Renfrew Rd., Paisley PA3 4EF

Canberra Packard (Packard),

Pangbourne, Berks

Corning Ltd.,

Stone, Staffordshire, ST15 OBG

Flow Laboratories,

Woodcock Hill, Harefield Rd.,Rickmansworth, Hertfordshire, WD3 IPQ

ICI Parmaceuticals,

Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG

John Stainiar & Co.,

Manchester Wire Works, 34 Stanley Rd., Whitefield, Manchester

Kodak Ltd.,

PO Box 66, Kodak House, Station Rd., Hemel Hempstead, Herts

Millipore UK Ltd.,

Millipore House, 11-15, Peterborough Rd., Harrow, Middlesex

Nalgene Labware Dept.,

PO Box 20365, Rochester, NY 14602, USA

National Diagnosics,

1013-1017, Kennedy Blvd., Manville, New Jersey 08835, USA

Nunclon c/o BRL-Gibco, Paisley

Oncogene Science c/o Cambridge Bioscience,

25 Signet Court, Stourbridge Common Business Centre, Cambridge

Premier Brands UK Ltd.,

PO Box 171, Birmingham

Severn Biotech Ltd.,

Unit 23, Worcester Rd., Kidderminster

Sigma Chemical Co. Ltd.,

Fancy Rd., Poole, Dorset

Sterilin Ltd.,

Sterilin House, Clock House Lane, Feltham, Middlesex

2.2 CELL CULTURE GENERAL METHODS

2.2.1 Cell Lines

Table 2-1, gives a comprehensive list of all the established cell lines used in this work. It gives the source and major characteristics of each line.

2.2.2 Routine Cell Maintenance

All the cell lines were routinely grown in RPMI (Gibco) containing 23mM sodium bicarbonate (Gibco), 2mM L-glutamine (Gibco) and variable amounts of foetal calf serum (FCS) dependent on the cell line; 5% FCS for all breast cell lines, 10% FCS for A431 and Swiss 3T3 cells and 15% FCS for all tumour derived fibroblast lines.

The breast tumour cell lines were passaged at weekly intervals. They were seeded at an initial density of 10^6 cells per $75cm^2$ flask (Bibby, Corning) and incubated at 37°C in a 5% CO_2 humidified atmosphere. Stock cultures were grown for a maximum of 10 passages from frozen stocks and were checked at monthly intervals for the presence of mycoplasma. Frozen stocks were stored in liquid nitrogen in normal culture medium plus the addition of 10% DMSO. For all experimental work cultures were used at day 3 from passage unless stated otherwise.

Cells were passaged by removal of all medium from the flask then 5ml of PBS containing 1mM EDTA and 0.25% trypsin (Gibco) was added to the flask and removed after 1 min.

TABLE 2-1 SOURCE AND CHARACTERISTICS OF CELL LINES

CELL LINE	SOURCE	CHARACTERISTICS	REFN.
MCF-7(WT)	Human breast adenocarcinoma Pleural effusion	Human epithelia ER positive Cells obtained from K.Cowan, NCI, Bethesda, USA.	H.D.Soule et al., 1973
MCF-7(Adr)	MCF-7 cells selected for resistance against adriamycin	Overexpression of MDR gene ER negative K.Cowan, NCI, Bethesda, USA.	P.P.Vickers et al., 1988
ZR-75-1	Human breast carcinoma Ascitic Effusion	Human epithelia ER positive Cells obtained from ATCC	L.W.Engel et al., 1978
MDA-MB-231	Human breast adenocarcinoma Pleural effusion	Human epithelia ER negative Cells obtained from ATCC	R.Cailleau et al., 1978
A431	Human epidermoid carcinoma	Human epithelial High expression of EGFr. Med.Oncol. stock cultures	D.J.Giard et al., 1973
Swiss 3T3	Mouse embryonic fibroblasts	Fibroblast Confluent monolayers. Med.Oncol. stock cultures	G.Torado 1963

The cells were then incubated for approximately 10minutes to allow the cells to detach from the surface. Finally the cells were collected into 10mls of medium and counted before dilution and plating into fresh tissue culture flasks.

2.2.3 Derivation of Fibroblast Cell Lines

All the human fibroblast lines used in this work were derived from fresh breast tumour biopsy material and were labelled for convenience BF1 to BF12. Immediately after surgery the fresh tumour material was placed in medium [Medium 199 (Gibco) containing 2mM L-glutamine, 15mM Hepes buffer (Gibco), 50 μg/ml penestreptomycin (Gibco), 2 μg/ml amphotericin B (Gibco)] and stored at 4°C until further processing within a 24Hr period.

Dissociation

The biopsy was drained of media and trimmed of excess fat then finely minced using crossed scalpel blades. The tumour pieces were placed in Medium 199 containing BSA (1mg/ml), catalase (100U/ml), collagenase type II (80 μg/ml), hyaluronidase (50 μg/ml) (purchased from Sigma). The material was placed in a sterile tissue culture flask and gently rocked at 37°C to allow digestion to take place. After a period of time varying from 2-12 Hrs dependent upon each individual tumour, fibroblast cells were digested away from the epithelial cellular clusters (organiods) allowing the single cells to be separated out through a 300 μm mesh size filter (John Stainiar & Co.), thereby enriching the fibroblast population considerably. Further purification of the cells was carried out through differential

growth. The single cells were collected and plated onto 6-well tissue culture plates (Nunclon) and cultured in a 37°C humidified atmosphere 5% CO_2 in RPMI medium, 2mM L-glutamine and 15% FCS. Over a period of 7-10 days dense fibroblastic monolayer cultures emerged and were passaged two more times before storage as stock cultures in liquid nitrogen. No experimental work has been carried out in cells grown beyond passage 8.

2.2.4 Mycoplasma Testing

All cell lines were checked on a monthly basis for the presence of mycoplasma. Cells were fixed with 25% glacial acetic acid in methanol (v/v) and then stained with the fluorescent DNA stain Hoescht 33258 at 100ng/ml for 15mins at room temperature. Plates were then examined under a fluorescent microscope for visual evidence of infection. All experiments were carried out on mycoplasma free cells.

2.2.5 Cell Counting

For each experiment cell suspensions were counted using an Improved Neubauer Counting Chamber (haemocytometer). The cell number was counted within a defined area of the haemocytometer chamber $1mm^2$ giving a cell number in 10^{-4}ml. For each preparation the average of two separate counts was taken.

2.2.6 Growth Curves

Cell growth curves were carried out on a number of cell lines under different media conditions. In general, cells were plated onto 24 well tissue culture plates (Nunclon) at 10^4 cells/ml and 1ml per well. The cultures were fed every third day and counted from day 4 of culture onwards.

Cell doubling times were obtained from the cell growth curves. Cell numbers were plotted against time in days and the best fit line was used to calculate an average doubling time for the cells over the period of counting from day 3 to day 7. These cell growth curves are show beside Table 3-2 on p72.

2.3 FIBROBLAST CONDITIONED MEDIA PREPARATION

Fibroblast cell lines were cultured in 175cm^2 tissue culture flasks and grown to a semi-confluent state in RPMI + 15% FCS. The cells were then washed over a 24Hr period in 50mls of RPMI phenol red free (prf) medium (Gibco) to remove all serum from the cells, before addition of 50mls of fresh RPMI(prf) media for conditioning over a 96Hr period at 37°C. The conditioned media (CM) was removed from the cells and centrifuged to remove any cellular debris, finally the CM was stored at -20°C until experimental use.

2.3.1 Oestradiol and Tamoxifen Treated C.M.

In some situations the fibroblast CM has been treated with oestradiol (E_2) (Sigma) or tamoxifen (ICI Pharmaceuticals), at optimal growth stimulatory or growth inhibitory levels in the MCF-7WT cell line (i.e. E_2 10^{-9}M, tamoxifen 10^{-6}M). The hormone or drug was prepared in ethanol and added to the conditioning medium at 0.1% v/v at the beginning of the conditioning period and remained present in the CM throughout the 96Hr incubation and was therefore still present in the CM samples. Treated CM was handled identically to the untreated CM. As controls, sham incubated media containing E_2, tamoxifen or combinations of the two were also set up. These preparations were incubated for 96hrs in the absence of any fibroblast cells.

2.4 PREPARATION OF CHARCOAL STRIPPED SERUM

Foetal calf serum was treated with activated charcoal in order to remove all the endogenous steroids (Leake *et al.*, 1987). Activated charcoal (Sigma) was added to fresh FCS at a concentration of 50mg/ml and stirred for 30 minutes at room temperature. The preparation was then centrifuged for 15 minutes at 3000 rpm at 4°C and the supernatant removed and spun at 12000 rpm for 30 minutes (4°C). The final supernatant was filtered through a Nalgene 22 μM sterile filtration unit and the resultant charcoal stripped serum (CSS) stored at -20°C.

2.4.1 Preparation of Heat-treated Dextran Charcoal Stripped Serum (HT-CSS)

Following the method of Dabre *et al.*,1983, activated charcoal was added to FCS at a concentration of 5mg/ml and dextran (Sigma) at 0.5mg/ml. The preparation was stirred constantly at 60°C for 30 minutes. The serum was then centrifuged and sterile filtered as for CSS and finally stored in aliquots at -20°C.

2.5 TRITIATED THYMIDINE LABELLING OF BREAST CANCER CELL LINES

Uptake of [^{3}H]thymidine by two breast cancer cell lines, MCF-7WT and MCF-7Adr was measured under a range of variable growth conditions.

2.5.1 Culture Conditions

Subconfluent cultures of cells were plated onto 6 well tissue culture dishes in RPMI(prf) medium + 5%CSS at 10^5cells/well in 2ml of medium. The cells were allowed to attach over a 24Hr period at 37°C before the medium was changed to RPMI(prf) with no added serum at 2mls/well. After 24Hrs, growth factor, hormone or drug was added to the cultures at the stated concentrations. At a number of time points which varied from 2-30Hrs cells were labelled with 1 μMCi [^{3}H]thymidine (Amersham, [methyl-^{3}H]thymidine, specific activity = 85Ci/mM) per well for 1Hr. Each experimental condition was set up in triplicate with triplicate control cultures.

2.5.2 Fixation and Counting

Cells were then fixed by the addition of 2mls of methanol:acetic acid (3:1 v/v) for 1Hr. They were then washed twice with 2ml of 80% methanol and incubated at room temperature in the presence of 0.5ml trypsin for 1Hr. Finally 0.5ml of 1% SDS was added to each well to aid solubilization into 9ml of scintillation fluid (Ecoscint A; National Diagnostics).

Overall tritium counts were made using a Canberra Packard scintillation counter with a counting efficiency of 60%. All treated cultures were expressed as a percentage of the control, with control cultures taken to be 100% of activity.

2.6 TETRAZOLIUM BASED MICROTITRATION ASSAY (MTT ASSAY)

This assay was adapted from the original assay described by Mosmann, (1983), to allow measurement of growth regulation by mitogens. Throughout the work a number of adaptations of the assay were used. Each method is described here in detail as MTT Assay Method 1,2 or 3.

2.6.1 MTT Assay Method 1

A longterm growth assay used to examine growth regulation by oestrogens. Subconfluent cultures of breast tumour cell lines were trypsinized and plated onto 96 well tissue culture plates (ICN, Flow Lab.) in RPMI(prf) medium + 5%CSS at a concentration of $5x10^3$ cells/ml in a volume of 200 µl per well. The cultures were incubated for 24Hrs at 37°C to allow cell attachment before medium was aspirated off, and test medium added to the cells at 200 µl/well. Each column of 8 wells constituted a single sample. The first column on each plate was fed medium alone with no cells, this acted as a background control. The second column was cells plus untreated medium and acted as the experimental control. Each plate was repeated in triplicate. The plates were fed specific medium daily for a period of 7 days. On the eighth day medium was removed from the cells and fresh untreated medium RPMI(prf) with no added serum was added to each of the wells plus an additional 50 µl/well of MTT solution [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dissolved in PBS at 5mg/ml. Plates were then incubated at 37°C for 4Hrs in the dark to allow live cells to reduce the

tetrazolium based compound to a blue formazan product. The medium was finally removed from the cells and the formazan crystals were dissolved in 200 µl of DMSO per well and buffered with 25 µl of Sorensen's Glycine Buffer (0.1M glycine,0.1M NaCl, equilibrated to pH 10.5 with 0.1M NaOH). Plates were then read on a Bio-rad microplate reader at an absorbance of 570nm. Results have been expressed as optical density (absorbance reading) or as a percentage of control cultures where control is 100%.

2.6.2 MTT Assay Method 2

A short term growth assay used to measure cellular growth response to a range of mitogens. Subconfluent cultures of breast tumour cells were plated at a concentration of $2.5x10^4$ cells/ml in a volume of 200 µl per well of RPMI(prf) + 5%CSS in 96 well tissue culture plates. After 24Hrs of incubation the medium was replaced with test medium . As in Method 1, column 1 acted as a background control and column 2 as a growth control, other columns were treated with test media. In Method 2 the cultures were incubated for 72Hrs at 37°C without any further change in medium. The medium was then removed from the cells and RPMI(prf) medium + 50 µl/well MTT solution added to each well and plates were read as before.

2.6.3 MTT Assay Method 3

This method was used alongside method 2 as an alternative growth assay with different basal media conditions. Heat treatment of serum will remove

factors other than steroids (Leake *et al.*, 1987). Plates were set up as in Method's 1 & 2, on day 2 the medium was changed to RPMI(prf) + 2.5% HT-CSS with the addition of growth factor, drug or hormone to be examined. After 72Hrs incubation the medium was removed and blank serum free medium was added plus MTT solution and read as before.

VALIDATION OF THE MTT ASSAY

The MTT assay was validated against cell counts under three different growth conditions. Graphs of the growth curves from the four breast cancer cell lines and their average doubling times are shown on p72. The doubling time of each cell line under the three described growth conditions, closely agrees with the time taken for a two increase in optical density in the MTT assay. The cell growth curves can be directly compared with identical growth conditions using the MTT assay shown in Figures 3-2 and 4-11.

2.6.4 Preparation of Hormones, Drugs and Growth Factors

Table 2-2, lists all hormones, drugs, growth factors and antibodies used in the MTT assay throughout the course of this thesis. For the each factor there is a brief description of the factor as well as the supplier and the method of preparation and storage used.

TABLE 2-2 SUBSTANCES TESTED IN THE MTT ASSAY

Source and Preparation

Factor	Description	Preparation & Storage	Supplier
ß-Estradiol	Naturally occurring steroid hormone.	Stock solution dissolved in 2% ethanol in PBS, Stored at 4°C, further dilution in medium	Sigma
Insulin	Naturally occurring hormone,mimic IGF-I effect in culture	Lyophilisate diluted to 10mg/ml in sterile H_2O, aliquots stored at -20°C, further dilution in media	Boehringer Mannheim
EGF	Human recombinant epidermal growth factor	Lyophilisate diluted to 10 µg/ml in sterile H_2O and aliquots stored at -20°C. Further dilution in medium	BRL
Tamoxifen	Non-steroidal anti-oestrogen	Stock solution made up in ethanol at 10^{-3}M and stored at 4°C, further dilution in medium	ICI Pharmaceuticals

Factor	Description	Preparation & Storage	Supplier
IGF-I	recombinant human insulin-like growth factor I	Lyophilisate reconstituted in 0.1M acetic acid, further dilution in PBS to 10 µg/ml. Aliquots stored at -20°C, further dilution in medium	Boehringer Mannheim
IGF-II	Recombinant human insulin-like growth factor II	Lyophilisate reconstituted in 0.1M acetic acid, further dilution in PBS to 10 µg/ml. Aliquots stored at -20°C, further dilution in medium	Boehringer Mannheim
TGF-α	Recombinant human transforming growth factor-α	Stock solution stored in aliquots at -20°C at 200 µg/ml in 50mM sodium phosphate, further dilution in medium	BRL
bFGF	Recombinant human basic fibroblast growth factor	Lyophilisate reconstitutedin sterile PBS and stored in aliquots at 10 µg/ml	Boehringer Mannheim
IGF-Ir antibody IgG_1	Monoclonal antibody to insulin-like growth factor-1 receptor (Ab-1) Clone αIR3	Stock reconstituted in sterile H_2O to give a concentration of 100 µg/ml, stored at 4°C	Oncogene Science

2.7 CLONOGENIC CYTOTOXICITY ASSAY

Lethally irradiated Swiss 3T3 cells were plated onto 35mm^2 tissue culture plates (Nunclon) at a concentration of 10^5 cells/ml, 5mls per plate in RPMI containing 10% FCS. Cells were then incubated at 37°C for 24Hrs after which the medium was replaced RPMI(prf) + 2mM L-glutamine + 15mM Hepes buffer and the cells incubated for a further 24Hrs. On day 3 the medium was removed and 5mls of RPMI(prf) + 5%CSS containing either $2x10^4$ or 10^3 cells per ml of MCF-7WT cells was added to the Swiss 3T3 cell feeder layer. The medium was replaced on day 4 with RPMI(prf) + 5% CSS and test substance (hormone drug or growth factor). Cells remained in test medium for 72Hrs. On day 7 the medium was replaced with RPMI + 5%FCS to stimulate colony growth. Growth was continued until day 14 when the cultures were rinsed in PBS then fixed in methanol and stained with a 1% solution of crystal violet. Colonies greater than 50 cells were counted and colony survival in treated samples was compared to colonies in untreated controls.

2.8 INDIRECT IMMUNOFLUORESCENCE

Cell lines were generally plated in their normal growth medium on 8 well Lab-Tek glass slide tissue culture vessels (Gibco) at a concentration of 10^4 cells per well in 200 µl of medium. Following an overnight incubation at 37°C, semiconfluent cell cultures were briefly rinsed in PBS and immediately fixed and stained. The fixative and staining procedure varied with each antigen of interest therefore each is listed individually.

2.8.1 Vimentin Staining

Cells were fixed in methanol at -20°C for 5 minutes then air dried. At this stage preparations were able to be stored at -20°C. When ready to stain, 3% sheep serum in PBS was added at 200 µl/well and incubated at room temperature for 20 minutes. Wells were then washed with PBS and the primary antibody added which was a vimentin antibody (Ab-1) supplied by Oncogene Science at 100 µg/ml mouse IgG_1. It was diluted 1:50 in PBS containing 0.1% (w/v) BSA (PBS:BSA). The cells were incubated at room temperature in a humidified atmosphere for 60 minutes. Then the slides were washed five times in PBS:BSA. The second antibody, a goat anti-mouse IgG FITC conjugate (Sigma) was diluted 1:200 in PBS:BSA from a supplied concentration of 1mg/ml. This was added to the cells and incubated at room temperature for 45 minutes in the dark. Following incubation, the wells were washed five times in PBS:BSA and the slide mounted with the addition of anti-fade (2.5% 1,4-diazabicyclo-2.2.2.octane in

glycerol) and sealed. The slides were then examined for fluorescence intensity using a Polyvar fluorescence microscope (Leica UK Ltd). All other staining follows a similar procedure with small variations in the fixative and antibody dilutions.

2.8.2 Cytokeratin Staining

Cells were fixed and blocked as before, the first antibody was then applied to the cells. A monoclonal antibody to cytokeratin 1 to 19, called anti-cytokeratin pan IgG_1, clone Lu5 (Boehringer Mannheim). It was supplied at 40 µg/ml and was diluted 1:10 in PBS:BSA before application to the cells. The preparations were incubated in the presence of the primary antibody for 1 Hr then the procedure was continued as for the vimentin antibody.

2.8.3 EGF Receptor Staining

Cells were set up as before and rinsed briefly in PBS. The cells were then fixed by the addition of 200 µl acetone:methanol at -20°C per well for one minute and allowed to air dry. The cells were incubated with 3% sheep serum in PBS at 200 µl per well for 20 minutes at room temperature. After rinsing in PBS:BSA the EGF receptor antibody at a dilution of 1:500 in PBS:BSA was added to the cells in a volume of 200 µl per well. The EGF receptor antibody was rabbit polyclonal antibody BG48 kindly supplied by Dr Bill Gullick, ICRF. The wells were then incubated in a humidified atmosphere at room temperature for 2Hrs. Each well was then rinsed five times in PBS:BSA before addition of the secondary

antibody anti-rabbit IgG FITC conjugate (Sigma) diluted 1:100 in PBS:BSA, further incubation was continued in the dark at room temperature for 45 minutes. The cells were then washed five times in PBS:BSA and finally counterstained by the addition of 100ng/ml Hoechst 33258 stain (Sigma) for 5 minutes. The slides were then rinsed in PBS and mounted as before. Finally the cells were examined for immunofluorescence using a Polyvar fluorescence microscope.

OPTIMIZATION OF STAINING

In all cases antibody staining was checked for nonspecific binding by replacement of the primary antibody with an irrelevant antibody, on the first occasion. The antibody chosen, was on all occasions of the same class as the antibody being tested. Further expeiments with a previously tested antibody always contained a control well which received no primary antibody. Specific staining was taken to be the increase in fluorescence between the controls receiving no primary antibody and those receiving primary antibody.

RADIO-LIGAND ASSAY FOR EGFR

Multipoint Analysis was carried out with twelve points of increasing concentrations of 50 µl of[^{125}I]-EGF at the final concentration of 0.086, 0.208, 0.416, 0.616, 1.67, 5.0, 8.334, 13.34, 15.0, 16.7nM. Nonspecific binding was ascertained by incubating three aliquots containing in addition to the top three labelled concentrations a 100 fold excess of unlabelled EGF.

2.9 WESTERN BLOTTING

Table 2-6 ANTIBODIES USED FOR IMMUNODETECTION & IMMUNOPRECIPITATION

ANTIBODY	DESCRIPTION	DILUTION	SUPPLIER
EGFr	Rabbit polyclonal BG48	1:500	Bill Gullick ICRF
PHOSPHO-TYROSINE	Mouse monoclonal Clone PY54	1:1000	Affiniti
GAP	Mouse monoclonal (GAP 13)	1:500	Affiniti
ANNEXIN I	Mouse monoclonal Clone II-29	1:1000	Affiniti
ANNEXIN II	Mouse monoclonal Clone CPI-50-5-1	1:500	Affiniti
ANNEXIN IV	Mouse monoclonal Clone CPIII-16-5	1:1000	Affiniti
ANNEXIN VI	Mouse monoclonal Clone 73-5-4	1:500	Affiniti

2.9.1 Western Blot Solutions

Tris Buffered Saline (TBST)

Tris-HCl pH 7.4 10mM, NaCl 150mM and 0.05% v/v Tween 20

for phosphotyrosine work 1mM sodium orthovanadate added.

Stacking Buffer (Stacking Gel)

Tris-HCl pH 6.7 0.5M, SDS 14mM.

Gel Buffer (Resolving Gel)

Tris-HCl pH 8.9 1.5M, SDS 14mM.

Tank Buffer

Tris 50mM, Glycine 50mM, SDS 3.5mM

Transfer Buffer

Tris base 50mM, glycine 40mM, SDS 1.3mM.

SDS Lysis Buffer

SDS 70mM, EDTA 5mM, Glycerol 10%, ß mercaptoethanol 2.5%, Spacer buffer 25%, selection of protease inhibitors.

Solubilizing Buffer

NaCl 150mM, Tris-HCl pH 7.5 50mM, EGTA 5mM, Glycerol 10%, Triton X-100 1%, selection of protease inhibitors.

Sample Buffer

2x concentration lysis buffer plus bromophenol blue.

Cell lines were generally grown to sub-confluence in normal growth medium or in restricted growth conditions (RPMI(prf) + 2.5% HT-CSS) before

protein preparation.

2.9.2 Preparation of Cell Lysates

For detection of the EGF receptor cells were grown in 75cm^2 flasks then drained of medium and briefly washed in PBS, 2mls of SDS lysis buffer was added to each flask and the cells were scraped into the the buffer and the preparations were then sonicated to sheer the genomic DNA. For each sample total protein concentration was estimated using the Bio-rad protein assay. 50 μg of total protein were run per lane, with the addition of sample buffer containing bromophenol blue.

For the detection of phosphorylated tyrosine residues the cellular proteins were prepared differently. The cells were seeded into 25cm^2 or 75cm^2 flasks in normal growth medium and incubated for 24Hrs. After this time the medium was removed and RPMI(prf) + 2.5% HT-CSS medium was added and the cells were incubated for a further 48Hrs. The phosphatase inhibitor, sodium orthovanadate at a concentration of 0.05mM was added to the cells, with a growth factor or without for controls. Cultures were then incubated for 30 minutes at 37°C before the medium was removed and the cells rinsed in PBS. The cells were scraped into solubilizing buffer and centrifuged for 5 minutes in a microcentrifuge to remove all non-solubilized material from the preparation. Protein concentrations in the supernatant were measured and again 50 μg of protein in sample buffer was run per lane.

2.9.3 Gel Preparation

Gels were set up using a Bio-rad protein gel kit. The final dimensions of the slab gel were 16cm x 16cm x 1mm. 6% or 10% slab gels were used throughout the work and were prepared as follows:-

Constituent	**Resolving Gel (ml)**		**Stacking Gel (ml)**
	6%	**10%**	**4%**
Gel Buffer	8	8	3(stacking buffer)
Acrylamide(Severn)	6.4	10.7	1.6
Polyacryl.(1%soln)	3.2	3.2	1.2
H_2O	14.4	10.1	6.2
10% APS(Bio-Rad)	0.120	0.250	0.125
Temed(BRL)	0.015	0.020	0.015

The resolving gel was poured between the two glass plates and a layer of isopropanol was added to ensure an even surface on the gel whilst the gel set. The isopropanol was then poured off and the stacking gel was added plus the well

dividers. Soon after setting, the samples were added to the wells. On each gel one lane contained pre-stained protein molecular weight markers, as supplied by BRL.

Protein	Apparent MW
lysozyme	15 300
ß-lactoglobulin	17 950
carbonicanhydrase	28 500
ovalbumin	43 750
BSA	70 600
phosphorylase B	110 900
myosin (H-chain)	206 450

2.9.4 Electrophoresis

Gels were placed in tank buffer and an electric current was then applied. Gels were run during the day under a constant current of 40-60mAmps until the bromophenol tracker dye reached the bottom of the gel. Gels were occasionally left to run overnight. In this case the proteins were initially run into the slab gel at 60mAmps and then left to run overnight at 5-10mAmps.

2.9.5 Protein Transfer

After electrophoresis, proteins were transferred from the gel to an Immobilon-P transfer membrane (Millipore) using a Milliblot transfer unit (Millipore) this was carried out in transfer buffer at a constant current of

200mAmps for 45minutes. The voltage always remainded below 30 Volts. Protein transfer was checked by drying the blot and then soaking in transfer buffer + 20% methanol, bands were clearly visible . Proteins which remained on the gel were stained with coomassie blue solution (0.1% v/v coomassie blue, 50% v/v methanol and 10% v/v acetic acid) for at least 1Hr at room temperature then destained in a destain solution (10% v/v methanol, 10% v/v acetic acid).

2.9.6 Immunodetection

Transferred proteins held on Immobilon filters were washed and incubated in a blocking solution for 1Hr at room temperature or overnight at 4°C. For detection of the EGF receptor the blocking solution consisted of 5% w/v non fat dried milk powder (Marvel, Premier Brands) in TBST. For phosphotyrosine detection a block containing 3% w/v ovalbumin in TBST + phosphatase inhibitor (PI)(1mM sodium orthovanadate) was used. After blocking the filters were briefly washed in TBST and the primary antibody was added. The EGF receptor antibody was diluted 1:500 in blocking solution and the phosphotyrosine antibody was also diluted 1:500 in its blocking solution (sources of each are given in Table 2-3). In each case the primary antibody was added for 2Hrs at room temperature with gentle agitation. The filters were then washed in several changes of TBST or TBST + PI. Horseradish peroxidase linked second antibody or protein A (Amersham) were diluted 1:5000 in blocking solution and applied to the blots for 15mins at room temperature. The blots were then extensively washed in TBST +/- PI before addition of enhanced chemiluminescence (ECL) (Amersham)

reagents and finally exposure to autoradiography film (Kodak).

2.9.7 Immunoprecipitation

Cell lysates were prepared as for phosphotyrosine detection at a concentration of 1ml solubilizing buffer per 75cm^2 flask of subconfluent cells. A volume of 200µl of cellular protein solution was incubated overnight with 1µl of phosphotyrosine antibody at 4°C. This was followed by the addition of 15ml of a solution of Protein G sepharose beads (Sigma) resuspended in solubilizing buffer containing PI, for 40 minutes at 4°C. The complex was then spun in microcentrifuge and rinsed three times in solublizing buffer at 4°C before resuspension in 50µl of loading buffer. To separate the bead-antibody-antigen complex samples were boiled briefly then spun for 8 minutes. Supernatant was loaded onto the gel and proteins ran out as before, further transfer and immunodetection followed the methods already described. Table 2-3, lists all the antibodies used for immunodetection of phosphotyrosine immunoprecipitates. Blocking steps were all carried out in a 3% ovalbumin solution.

2.9.8 Stripping and Reprobing of Western Blots

Primary and secondary antibodies were removed from filters to allow reprobing using a different primary antibody. After initial immunodetection filters were submerged in stripping buffer containing 100mM 2-mercaptoethanol, 2% SDS, 62.5mM tris-HCl pH 6.7 and incubated at 50°C for 30 minutes with agitation. The filter was then washed for two 10 minute intervals in TBST and

blocked in normal blocking solution for 1Hr at room temperature of overnight at 4°C. A second immunodetection was then continued as normal.

CHAPTER 3

OESTROGEN & ANTIESTROGEN EFFECTS

CHAPTER 3

OESTROGEN AND ANTIESTROGEN GROWTH EFFECTS

3.1 INTRODUCTION

This chapter describes the responses to E_2 and tamoxifen of four different breast cancer cell lines using both an MTT assay and ^{3}H-thymidine incorporation into DNA.

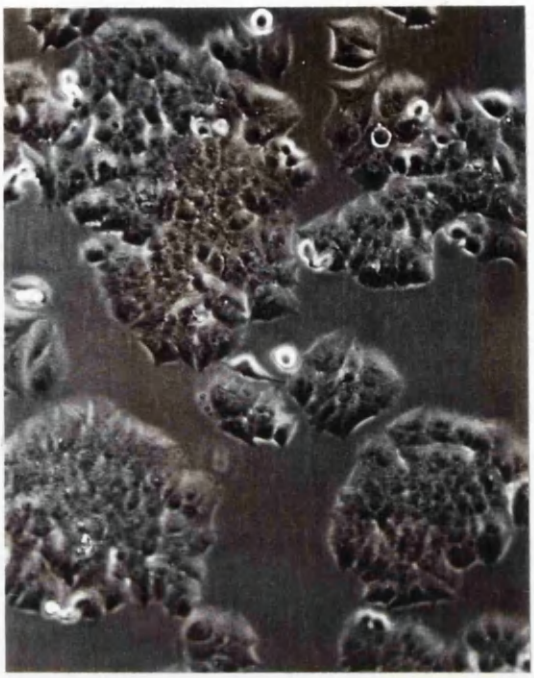
3.1.1 Selection of Cell Lines

Four cell lines were selected which have a range of phenotypic properties; MCF-7WT, MCF-7Adr, ZR-75-1 and MDA-MB-231 details of which are given in Table 2-1. Phase contrast photomicrographs of each breast cancer cell line are shown in Figure 3-1. Two of the cell lines are ER positive and two, ER negative. The MCF-7Adr line was a subline of MCF-7WT which has subsequently lost its receptors *in vitro* during longterm exposure to adriamycin, whilst the MDA-MB-231 cell line was derived from a pleural effusion as an ER negative line. Each cell line was checked for the presence of ER and PgR by competition analysis using a ten concentration assay for both the ER and PgR (Habib & Leake, 1987). The results of which are given below in Table 3-1.

Figure 3-1 Breast Cancer Cell Lines

ER Positive Lines

MCF-7WT



ZR-75-1

ER Negative Lines

MCF-7Adr

MDA-MB-231

Phase contrast photomicrographs of four breast cancer cell lines growing in monolayer culture in RPMI + 5% FCS. (x100)

Table 3-1 CELLULAR CONCENTRATIONS OF ER AND PgR

	ER fmoles/mg	PgR fmoles/mg
MCF-7 WT	81	41
MCF-7Adr	0	1.2
ZR-75-1	60	14
MDA-MB-231	0	6

Table 3-1
The ER and PgR concentrations in cell lines which have been grown to subconfluence in RPMI + 5%FCS shown as a fraction of the total cellular protein.

Selection of Media

The selection of an appropriate cell growth medium was critically important to experiments which were set up to identify an E_2 induced growth response. Cell doubling times were measured in a selection of media with varying levels of E_2, in order to identify the contribution of E_2 to cellular growth in each of the breast cancer cell lines.

Medium 1. Normal growth medium RPMI + 5% FCS

Medium 2. RPMI phenol red free (prf) + 5% CSS + 10^{-9}M E_2

Medium 3. RPMI (prf) + 5% CSS

Medium 1, should fully stimulate the growth of all the cell lines, it is a normal growth medium with a source of oestrogens present in the FCS. Medium 3, has had all sources of oestrogen removed by stripping the serum and removing phenol red from the medium. Phenol red has previously been shown to have weakly

oestrogenic properties (Welshons *et al.*, 1987), therefore growth in this medium should represent E_2 free growth. In medium 2, E_2 has been added back to the E_2 free medium, to identify the extent of oestrogen dependence in each of the selected cell lines. An E_2 concentration of 10^{-9}M was used to stimulate growth since this has previously been shown to be optimal for growth (Dabre *et al.*, 1983). The cell growth curves were carried out as stated in methods 2.2.6, and from these the cell doubling times were calculated.

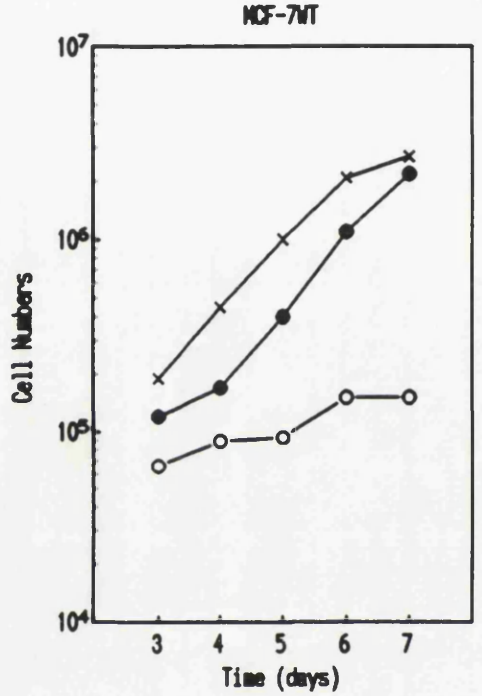
3.2 RESULTS

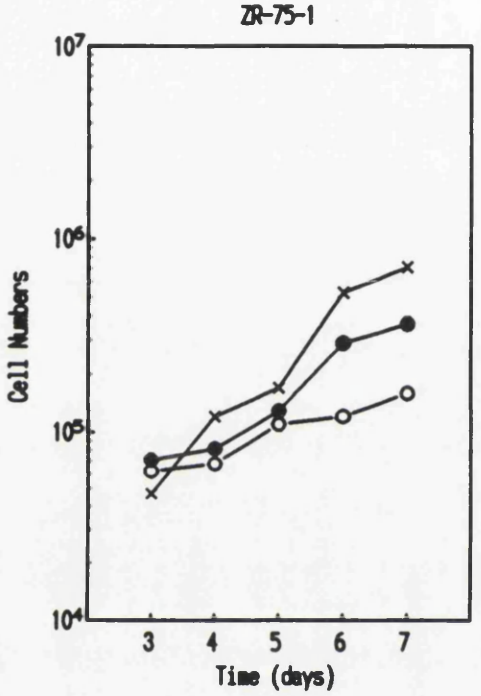
3.2.1 Cell Growth Curves

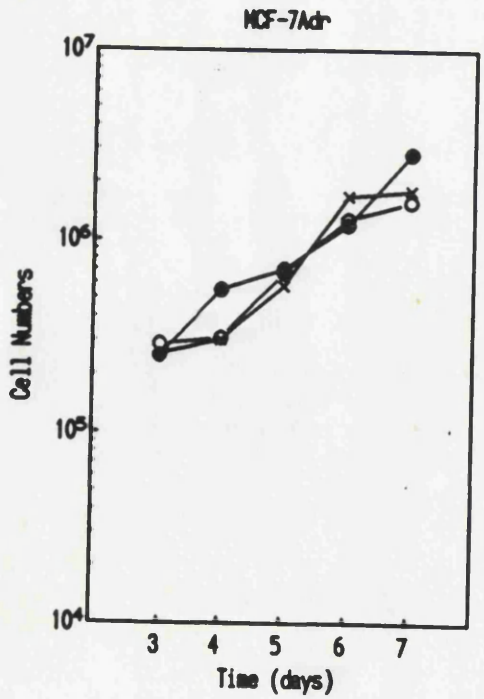
Table 3-2 gives the doubling times of each of the four cell lines grown in three different types of medium. The results clearly indicate that the growth of the ER positive cell lines, MCF-7WT and ZR-75-1, are dependent upon the presence of E_2. Both display very long doubling times in oestrogen free conditions which are barely measurable within the confines of this assay. On the other hand the two ER negative cell lines MCF-7Adr and MDA-MB-231, show little change in growth rate between the three medium types. The growth is delayed in the stripped medium whether in the presence or absence of E_2 (medium 2 & 3), compared to medium containing FCS therefore it would seem these cells respond to the presence of a serum factor other than E_2 which is removed from the FCS upon charcoal stripping.

CELL GROWTH CURVES

Graphs for Table 3-2, showing the growth curves used to derive cell doubling times. Each breast cancer cell line was grown in three different media according to the method outlined on page 47, (2.2.6). x medium 1 (RPMI + 5% FCS) ● medium 2 (RPMI(prf) + 5% CSS + 10^{-9}M E_2), ○ medium 3 (RPMI(prf) + 5% CSS). Each point represents the mean value of three replicate samples and in each case the coefficient of variation was within 10% of the mean. The doubling times of the cells have been checked on four independent occasions throughout the course of the work and show little variation.







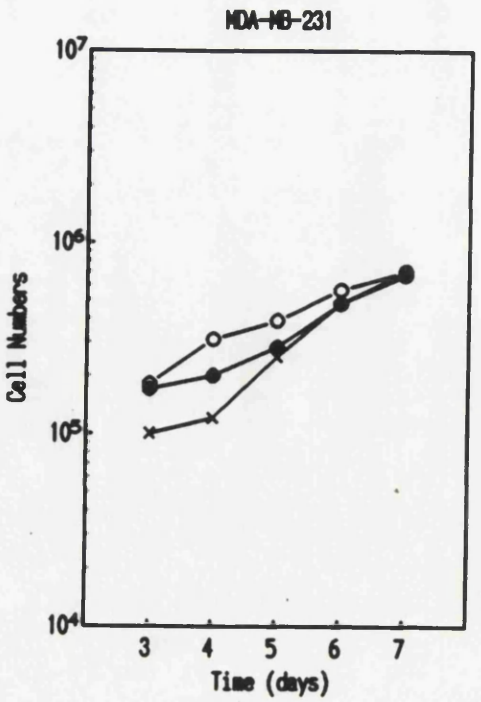


Table 3-2 MEDIUM DEPENDENT CELL DOUBLING TIMES

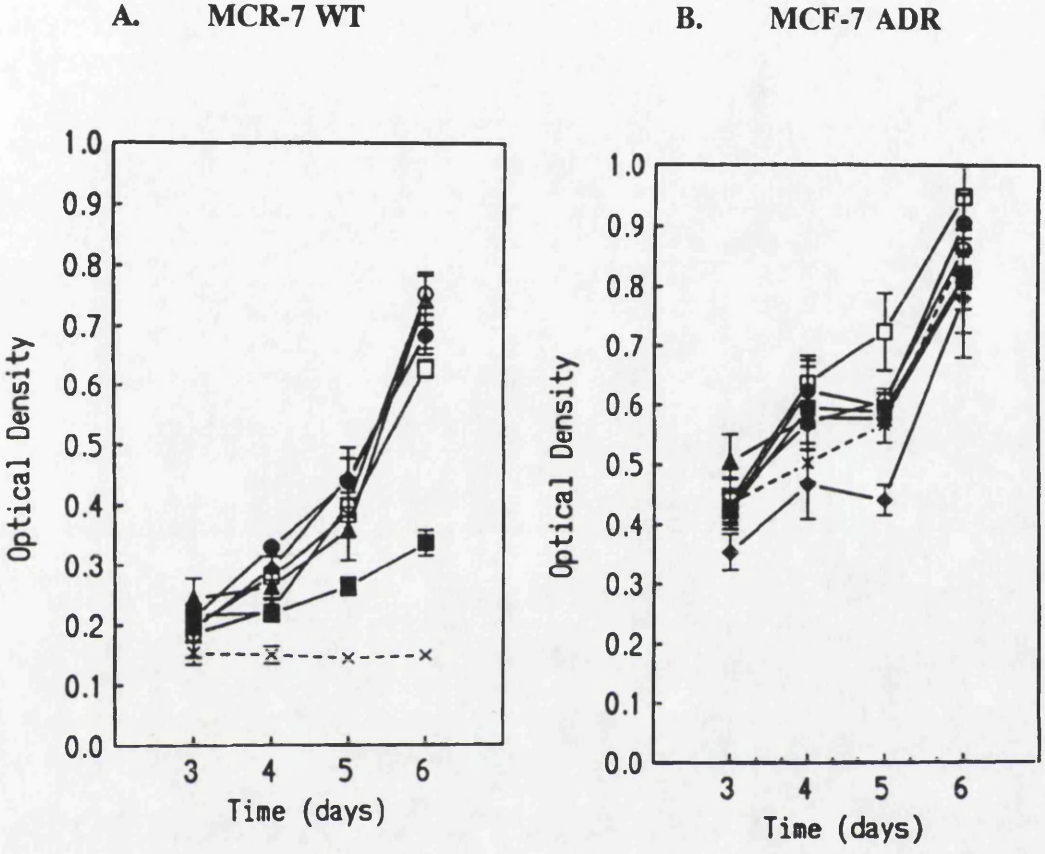
Cell Doubling Times (Hrs)

	Medium 1	Medium 2	Medium 3
MCF-7WT	20	24	75
MCF-7Adr	24	30	31
ZR-75-1	24	36	65
MDA-MB-231	29	43	48

3.2.2 Response to Oestradiol

The cell lines were characterised with respect to their growth responses to E_2. An MTT assay as described in methods 2.6.1, was set up with the cell lines MCF-7WT and MCF-7Adr. A range of E_2 concentrations were examined ranging from no E_2 to 10^{-6}M E_2. From days 3 to 6 of growth, triplicate plates from each cell type were analysed by measurement of optical density (O.D.) after MTT addition. Figures 3-2A and 3-2B show the growth curves of each cell type under a range of E_2 concentrations. The MCF-7WT cell line shows no measurable growth in E_2 free conditions, intermediate growth at a low concentration of E_2 (10^{-11}M) and highly stimulated growth at all other E_2 concentrations. On the other hand growth of the MCF-7Adr line under E_2 free conditions shows no difference from that in the presence of E_2. Its growth rate remains quite high under all conditions. It is clear these two cell lines are acting as expected with

Figure 3-2 Oestradiol Growth Response



Growth response in ER positive MCF-7 WT cells and ER negative MCF-7 Adr cells to a range of oestradiol (E_2) concentrations: x- - - - Control growth no E_2 present, ■ E_2 10^{-11} M, ▲ E_2 10^{-10} M, ◆ E_2 10^{-9}M, ● E_2 10^{-8} M, □ E_2 10^{-7} M, ○ E_2 10^{-6} M. Each point represents the mean of three plates (24 wells). Vertical bar represents one S.D.

Both cell lines were examined simultaneously. Data has been taken from a single experiment where both the control and optimal growth curves (10^{-9}M E_2) have been reproduced on more than three occasions.

MCF-7WT clearly dependent on the presence of E_2 and MCF-7Adr unresponsive to E_2.

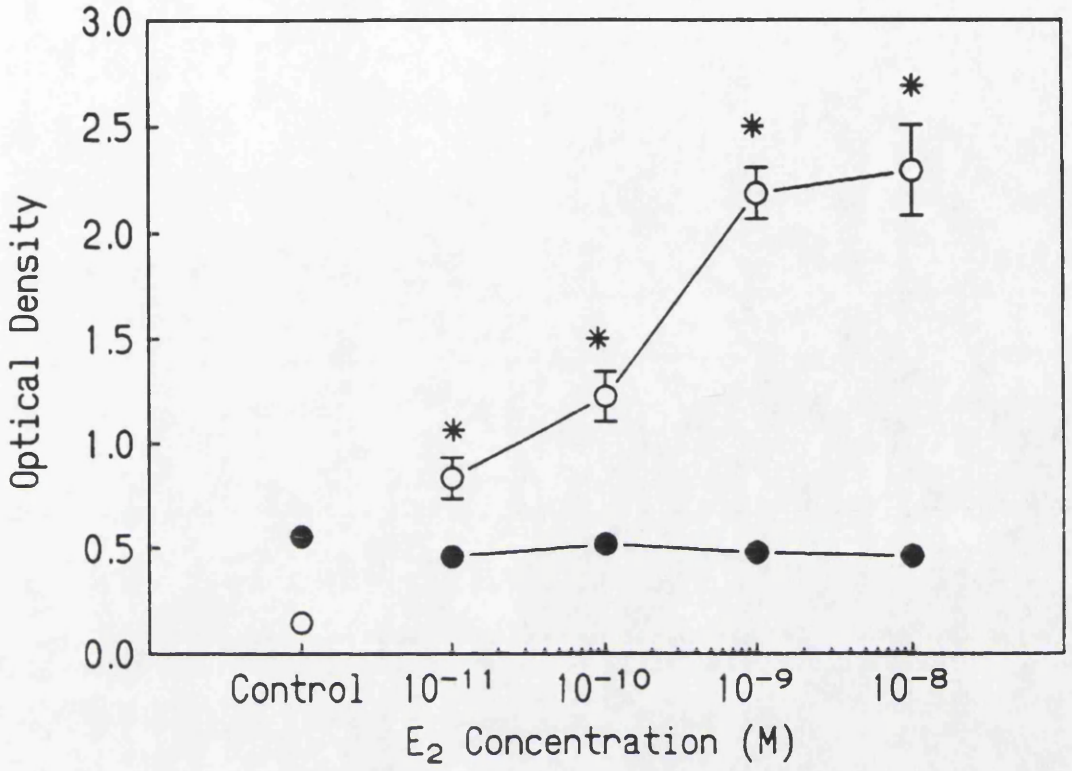
Figure 3-3, shows an E_2 dose response curve (DRC) at day 8 of growth, following MTT method 2.6.1, both the MCF-7 cell lines are displayed together. As in the previous experiment the MCF-7WT cells respond to E_2 with a significant difference of $P<0.01$ using a two sample t test when conditions containing 10^{-11}M E_2 and above are compared with E_2 free conditions. The MCF-7Adr cells remain unresponsive to E_2 showing no significant difference between E_2 free conditions and a range of E_2 containing conditions. From this work an E_2 concentration of 10^{-9}M was chosen as optimal for the growth of MCF-WT cells, in the MTT assay. This concentration agrees well with other published work (Bezwoda *et al.*, 1990, Cormier *et al.*, 1989 & Darbre *et al.*,1983).

3.2.3 Development of the MTT Assay

It was desirable to change the form of the MTT assay in order to reduce the number of medium changes required throughout the assay growth period and shorten the total length of this period. There were several reasons for this. Since one of the proposed mechanisms of growth by E_2 is through autocrine stimulation, the cells may be conditioning the medium which will have an overall effect on cell growth, this could be lost or diluted by regular medium changes. Numerous medium changes are more disruptive to the cells and over a long period of time will considerably increase the chance of contamination.

A short-term growth assay was set up at a number of seeding densities. On

Figure 3-3 Oestradiol Dose Response Curve



Oestradiol dose response curve after 8 days continuous exposure to oestradiol, ○ = MCR-7WT cell line, ● = MCR- 7Adr cell line, ✳ = Significant difference from control $p < 0.01$. Points represent mean of 3 or more plates. Vertical bars represent one S.D

Where vertical bars are not present they lie within the point. Results are representative of one experiment repeated on four independent occasions.

day 1 the cells were allowed to attach overnight in the RPMI(prf) + 5%CSS this was then changed to RPMI(prf) + 5%CSS +/- E_2 10^{-9}M and left on the cells for three days at which point MTT was added and the plates read. Figure 3-4 clearly shows a differential in O.D. between the presence and absence of E_2 at the highest plating density of $5x10^3$ cells per well or $2.5x10^4$ cells per ml. The difference between the two growth conditions was significant at $P<0.01$ using a two sample t test.

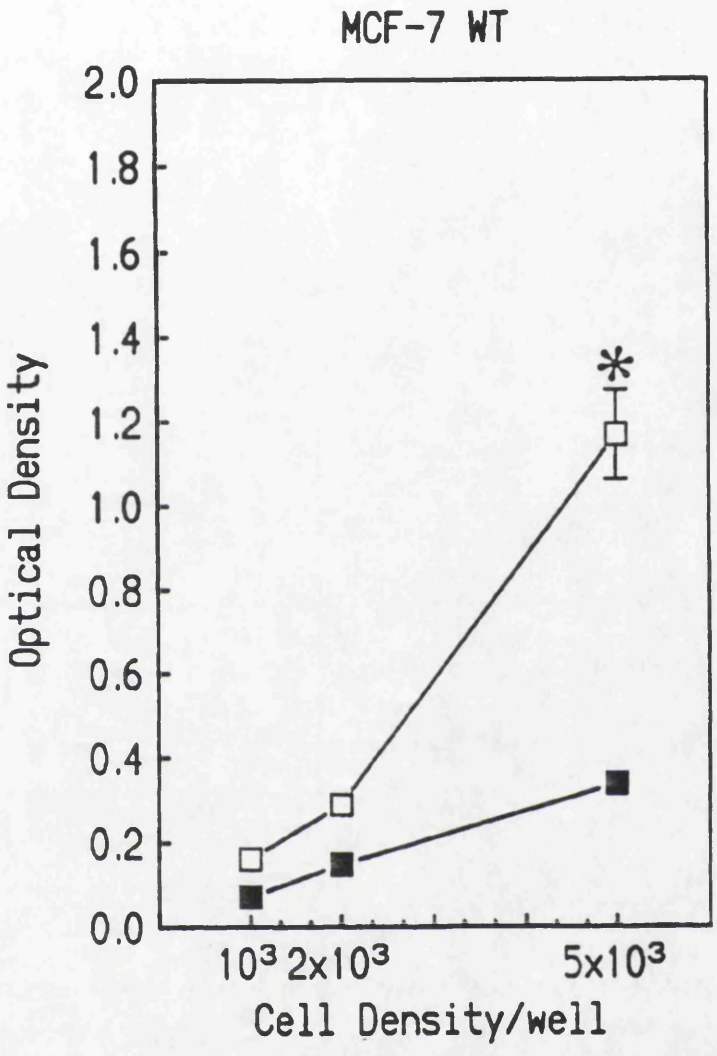
In order to optimise the differential between growth in control wells as against growth in test wells, a number of different media preparations were tested in optimal growth concentrations of E_2 and insulin 10 μg/ml ($1.7x10^{-6}$M). Figures 3-5(A-D) show clearly there are differences in cellular response to insulin and oestradiol which are dependent upon the controlling growth medium. This is only evident in the two ER positive cell lines MCF-7WT and ZR-75-1. The more restricted the growth ability of the medium the greater the measurable stimulus by mitogens such as oestradiol and insulin.

Concentrating on the most responsive cell line MCF-7WT, the same data has been displayed as a percentage of control (Fig. 3-6) where the O.D. measured for the sample in the presence of insulin and E_2 is displayed as a percentage of its own medium control. This graph only displays the increase in growth response to insulin and E_2.

3.2.4. Oestradiol and Tamoxifen Responses

As already discussed (1.3) tamoxifen is a non-steroidal ER antagonist. Its

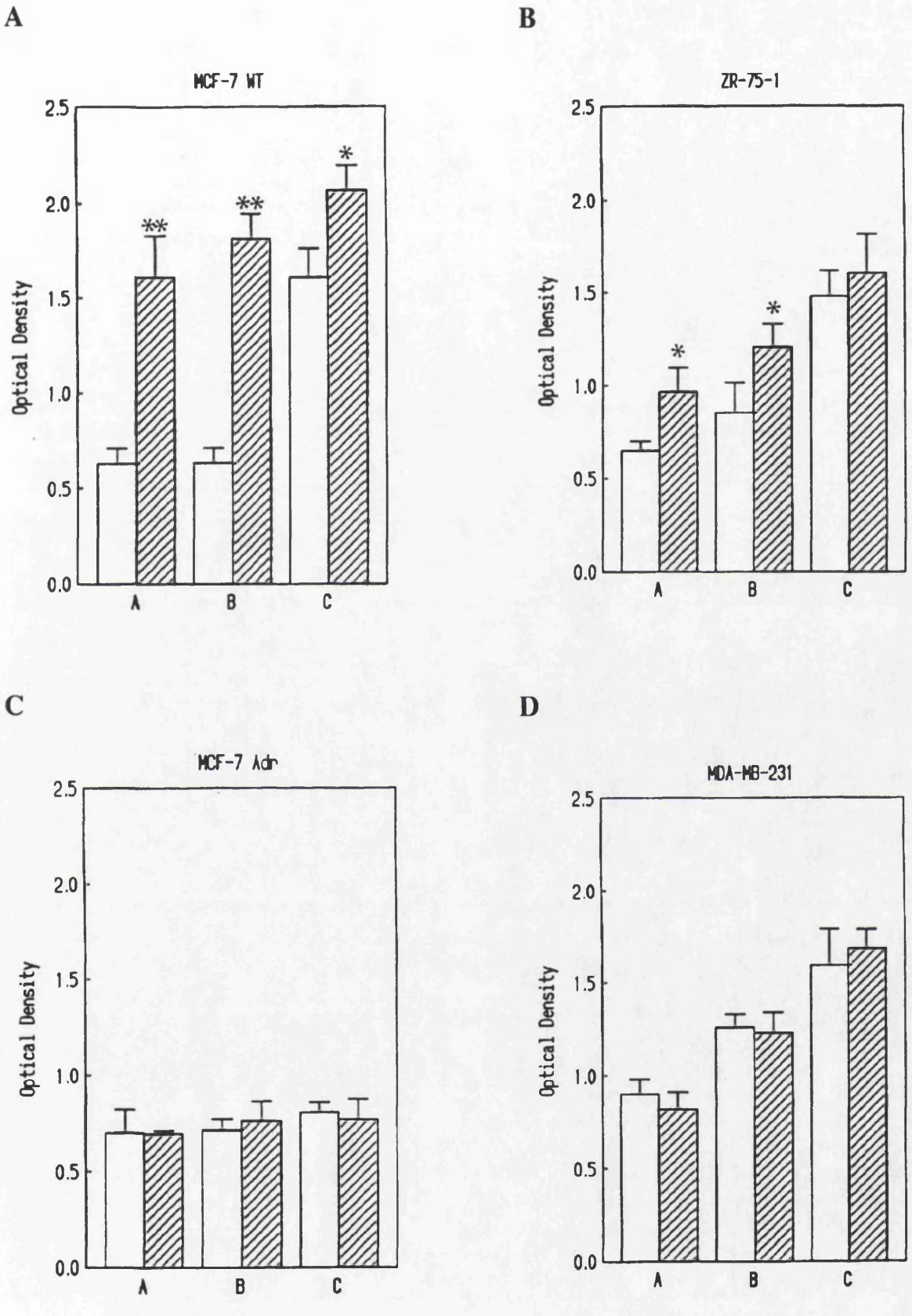
Figure 3-4 Plating Density Experiments



MCF-7 WT cells were grown in the presence (□) or absence (■) of 10^{-9}M oestradiol over 3 days at a number of cell densities: 10^3 cells/well ($5x10^3$ cells/ml), $2x10^3$ cell/well (10^4 cells/ml), and $5x10^3$ cells/well ($2.5x10^4$ cells/ml). * represents significant difference at $P<0.01$ in optical density between oestradiol presence or absence. Bars represent one S.D.

Where vertical bars are not present they lie within the point. Significant differences were calculated using a two sample t-test. Results are representative of one single experiment from a series of three.

Figure 3-5 Medium Dependent Growth Response (1)



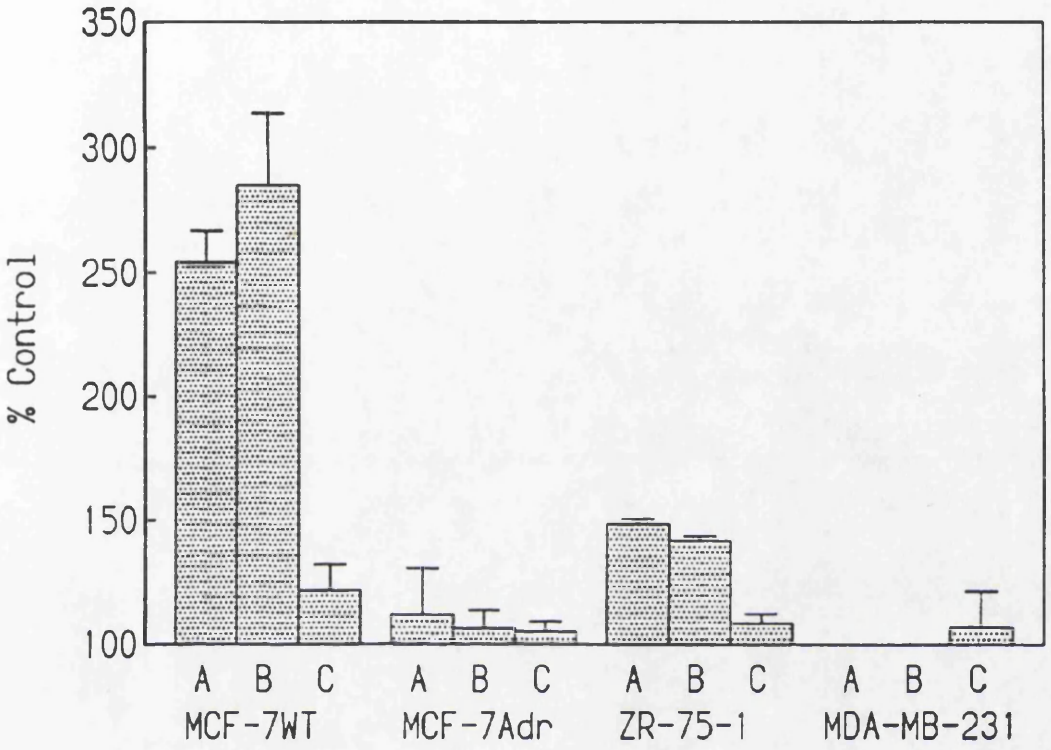
Growth response in blank control medium (□), or medium containing oestradiol (10^{-9}M) +insulin 10μg/ml (1.7×10^{-6}M) (▨).

A:— RPMI(prf)+2.5% HT-CSS, **B**:— RPMI(prf)+5% CSS, **C**:— RPMI+5% FCS.

** represents significant difference between blank and treated media at $P<0.01$. * represents significant difference at $P<0.05$. Vertical lines represent one S.D. Each bar represents mean value of triplicate plates. Single representative experiment repeated twice.

Figure 3-6 Medium Dependent Growth Response (2)

(shown as a percentage of control values)



Growth response to oestradiol (10^{-9}M) and insulin 10μg/ml (1.7 x 10^{-6} M) shown as a percentage of blank control values. A-RPMI (prf) + 2.5% HT-CSS , B-RPMI (prf) +5% CSS, C- RPMI (prf) + 5% CSS, C-RPMI + 5% FCS. Vertical lines represent one S.D. Each bar represents the mean value of triplicate plates. Single representative experiment repeated twice.

mechanism of action is not entirely clear but it is known to display partial agonist properties at lower concentrations. In oestrogen free conditions, tamoxifen has been shown to stimulate the growth of MCF-7 cells (Wakeling, 1989 & Cormier & Jordan, 1989).

Growth responses to tamoxifen were investigated in the MCF-7WT cell line, using both the MTT assay and ^{3}H-thymidine incorporation, where it was expected that tamoxifen would inhibit the E_2 stimulated growth response. Figure 3-7 shows the responses of cells exposed to tamoxifen, alone and in the presence of E_2 10^{-9}M, over 7 days (MTT Method 1). From the E_2 control, tamoxifen does bring about a concentration dependent inhibition of growth although it is not until a tamoxifen concentration of 10^{-6}M that we are seeing anything like a full inhibition of the E_2 responsive growth. Tamoxifen alone has an independent effect upon cellular growth, with a small growth response to tamoxifen at a concentration of 10^{-6}M and below.

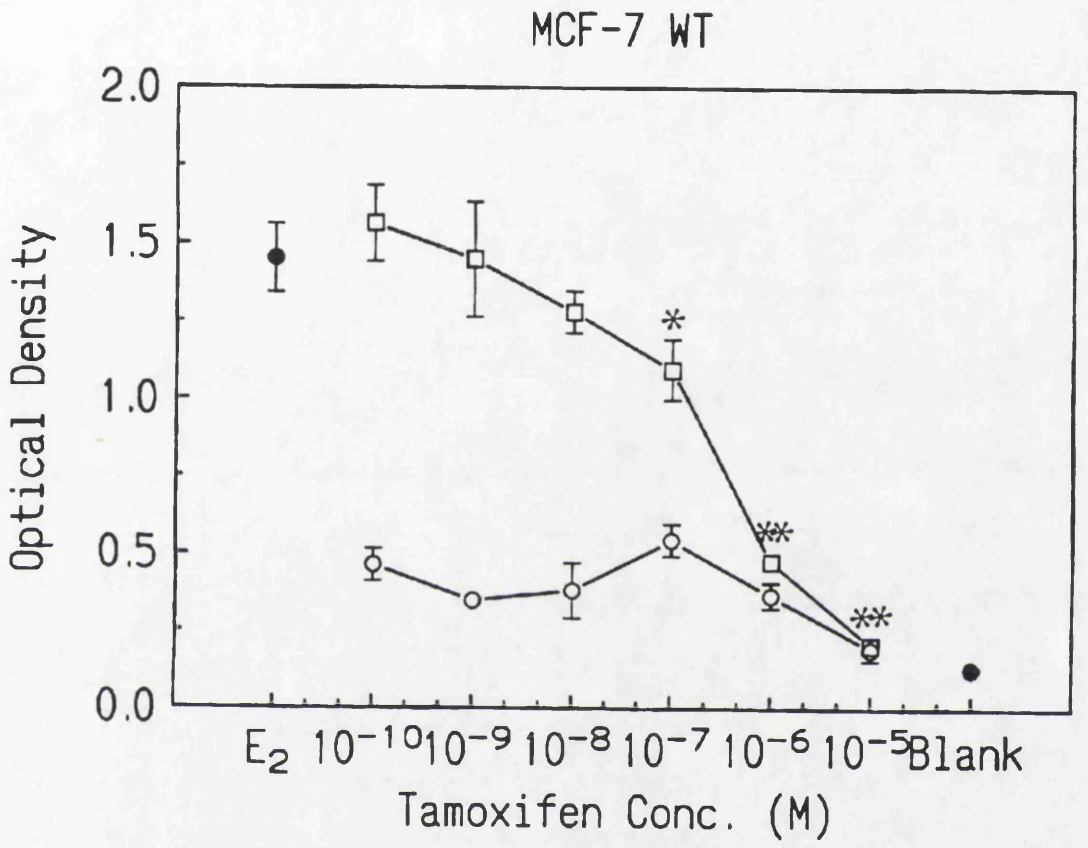
A similar experiment, using MTT method 2 is summarized in Figure 3-8 and allows comparison of methods. The first half of the figure displays a dose response to E_2, which clearly shows optimal stimulation at a concentration of 10^{-9}M with no further stimulation at higher concentrations. This optimal dose of E_2 (10^{-9}M) was then used as a steady stimulus to examine the cells dose response characteristics to tamoxifen. Using the shorter term assay; three days in specific growth medium, it is clear tamoxifen is only able to inhibit the E_2 regulated growth response at concentrations greater than 10^{-7}M. At 10^{-6}M tamoxifen there is a significant decrease in the growth response ($p<0.01$) which is reduced still

further at a 10^{-5}M concentration of tamoxifen. At concentrations of 10^{-5}M tamoxifen and above there is evidence to suggest the effect on cell growth is mainly cytotoxic. To assess tamoxifen cytotoxicity a clonogenic assay was set up with the MCF-7WT cells (methods 2.7). A range of tamoxifen concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M) were examined using this assay. At a concentration of 10^{-5}M tamoxifen, no well formed colonies were to be found. So it is clear that at 10^{-5}M tamoxifen, the effect on cell growth is mainly cytotoxic.

In Figures 3-9 (A-D) all the cell lines have been examined in the MTT assay (method 2) for their response to tamoxifen both in the presence and in the absence of E_2. In this case the results are all displayed as a percentage of control, where the controls at 100% are equal to the O.D. of cells grown in basal medium (RPMI(prf) + 5%CSS). For the MCF-7WT cell line, both tamoxifen and tamoxifen + E_2 bring about stimulation of cellular growth. The pattern follows previous experiments with inhibition of E_2 regulated growth not seen until tamoxifen concentrations are greater than 10^{-7}M, with full competitive inhibition seen at $5x10^{-5}$M tamoxifen. The agonist effect of tamoxifen is most pronounced at lower tamoxifen concentrations appearing to be optimal at 10^{-8}M and is reduced as the tamoxifen concentration increases. Again at 10^{-5}M tamoxifen there is almost certainly a cytotoxic effect. Interestingly tamoxifen is more cytotoxic at high concentrations in the presence of E_2 than in its absence. This appears to be true of all the cell lines, including the ER negative cell lines suggesting the effect is independent of the ER.

In the other ER positive cell line ZR-75-1, there is a small E_2 growth

Figure 3-7 Tamoxifen Dose Response Curves

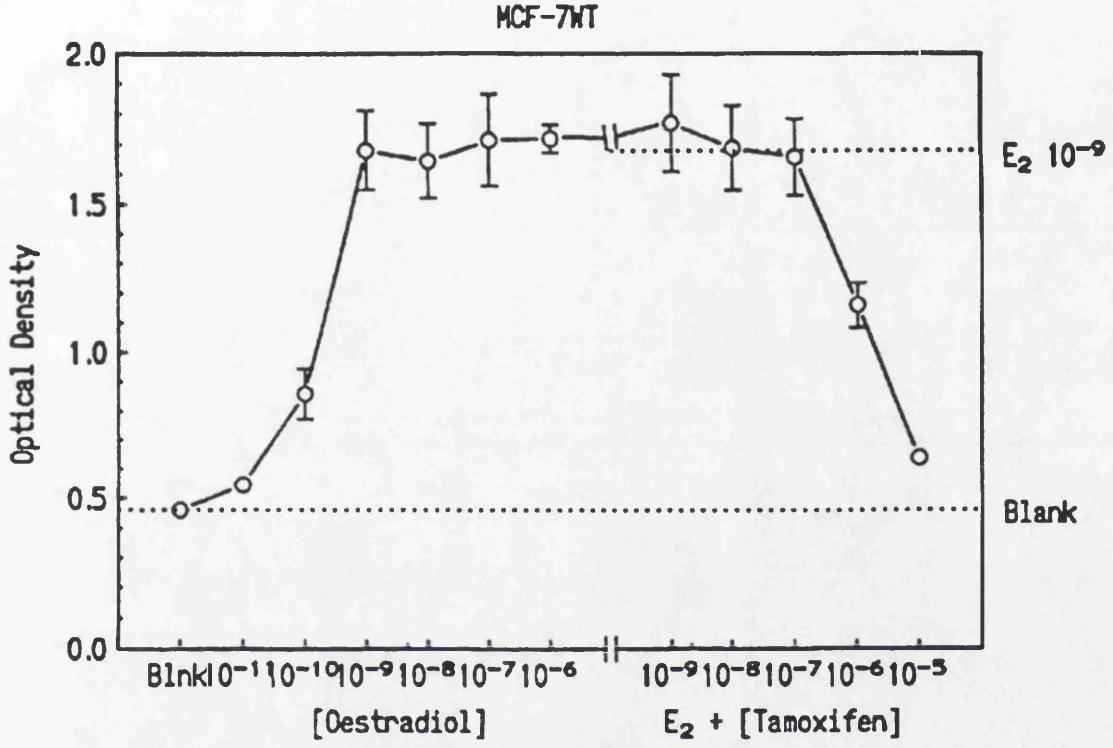


MCF-7WT growth response to increasing concentrations of tamoxifen alone and in the presence of E_2.

Dose response curve for tamoxifen alone(○), tamoxifen + oestradiol(10^{-9}M)(□) Control growth, oestradiol 10^{-9}M and blank control (●). ** represents significant difference from oestradiol control $P < 0.01$, * $P < 0.05$. Vertical bars represent one S.D.

Blank, refers to growth in the absence of added E_2 or tamoxifen. Single representative experiment from a series of four.

Figure 3-8 Oestradiol and Tamoxifen Dose response Curves

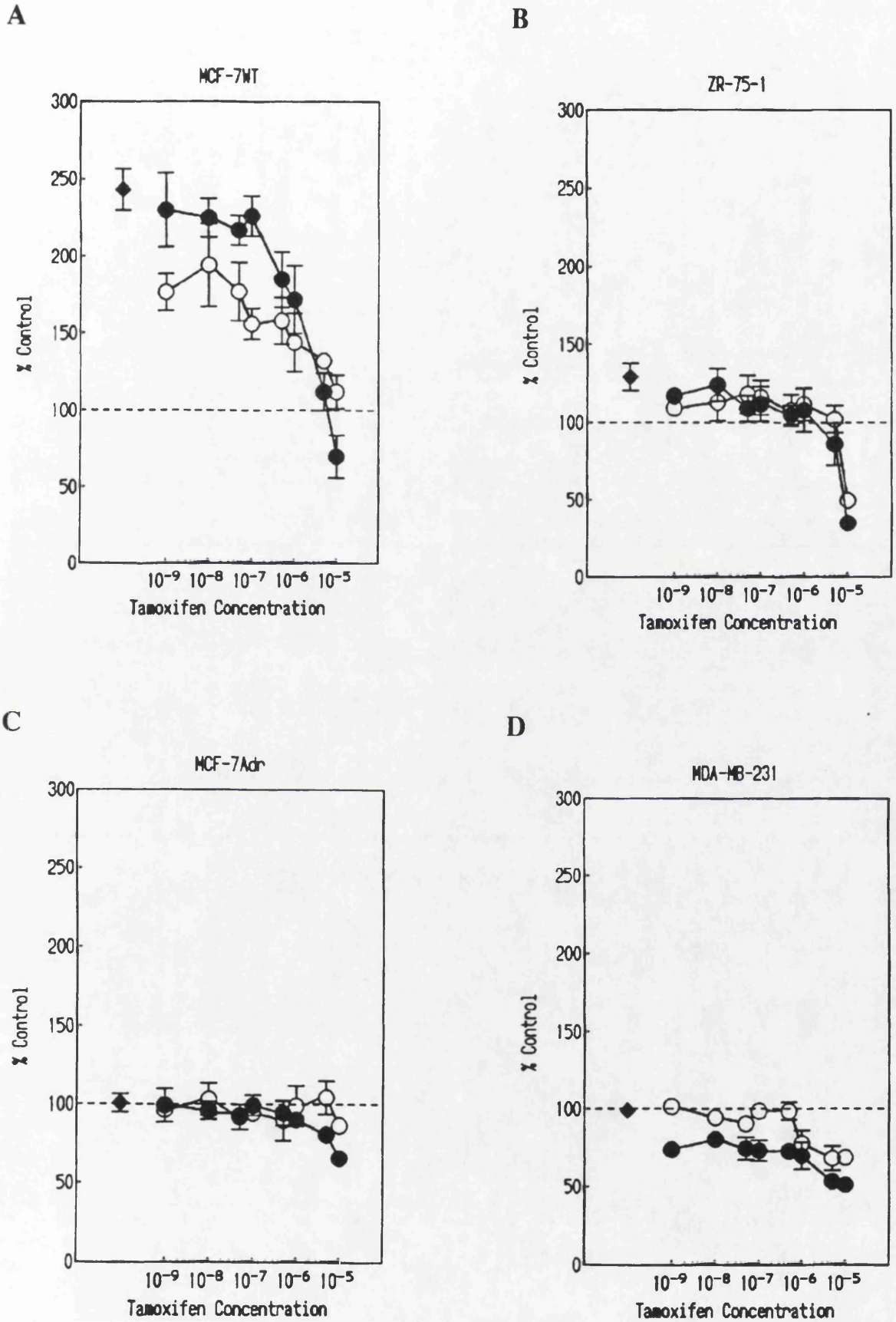


MTT assay showing growth response to E_2 and $E_2[10^{-9}M]$ + tamoxifen.

First half of graph depicts MCF- 7WT dose response curve for oestradiol over 3 day MTT assay. Second half depicts MCF 7 WT do se response curve for tamoxifen in the presence of oestradiol (10^{-9}). Dotted line represents mean growth response in blank medium and medium + oestradiol (10^{-9}M). Vertical bars represent one S.D.

Representative experiment repeated on three occasions.

Figure 3-9 Tamoxifen Response in Four Cell Lines



Growth response in four breast cancer cell lines to tamoxifen in the presence and absence of $E_2(10^{-9}M)$. Growth is represented as a percentage of the control growth in E_2 and tamoxifen free medium and taken to be 100%. Growth in response to: ♦ E_2 ($10^{-9}M$), ● tamoxifen + $E_2(10^{-9}M)$, ○ tamoxifen alone. Growth measured using the MTT assay (Method 2). Each data point represents the mean of three observations +/- S.D. of one representative experiment from a series of three.

response which appears to be inhibited by tamoxifen. Tamoxifen itself has a small but insignificant growth effect upon these cells. For both the ER negative lines no stimulation above control levels is seen by either tamoxifen or E_2. It is interesting that the MDA-MB-231 cell line shows greater growth inhibition to the combination of E_2 and tamoxifen than tamoxifen alone at all the tamoxifen concentrations tested. Since cytotoxic effects of tamoxifen would not be expected at concentrations of 10^{-6}M and below it is likely that tamoxifen and E_2 are together causing inhibition of cell growth through an unknown mechanism.

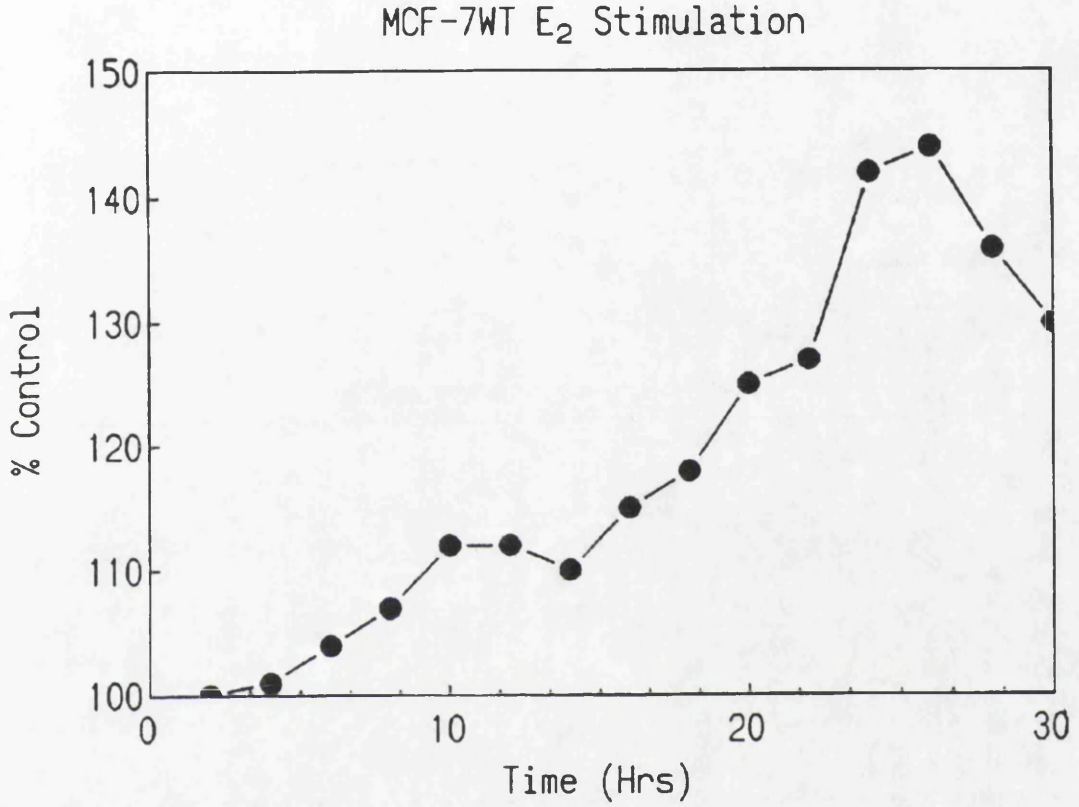
3.2.5 ^{3}H-Thymidine Response to E_2 and Tamoxifen

As a further check on the E_2 and tamoxifen growth effects, an assay to determine E_2 regulation of DNA synthesis was set up. This allows examination of the E_2 stimulus in a serum free environment which should give cleaner results than the growth assays.

In the first instance the optimal time of E_2 regulated uptake of H^3-thymidine was deduced from a time course study where one hour ^{3}H-thymidine exposures were made every 2Hrs, for a total of 30Hrs. Maximal stimulation was found to occur at 26Hrs (Fig.3-10). Further experiments looking at E_2 and tamoxifen effects were carried out at 26Hrs.

Figures 3-11(A&B) show the dose response curves to E_2 in both the MCF-7WT and the MCF-7Adr cell lines. For the MCF-7WT line there was a dose dependent growth response to E_2. The overall increase in DNA synthesis did not relate on a percentage basis to the growth assays, which was probably due

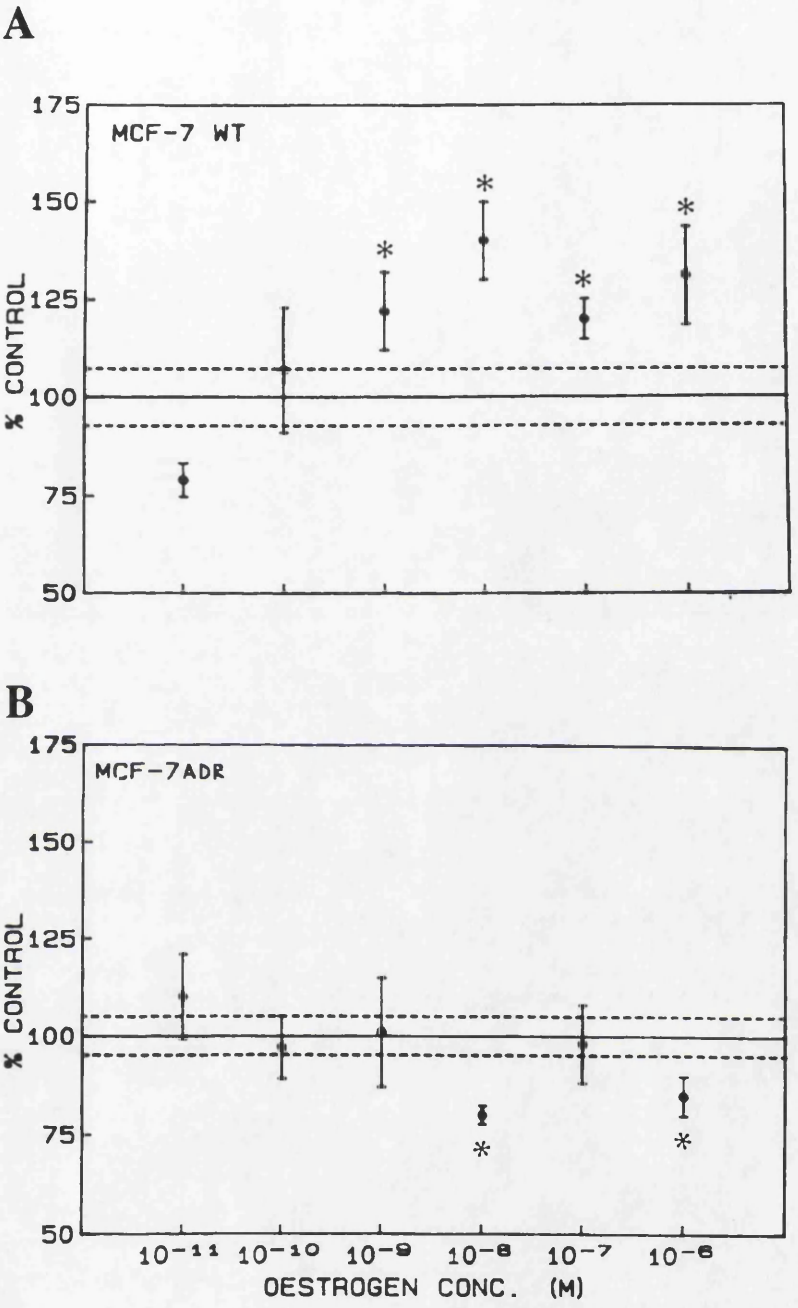
Figure 3-10 Oestradiol Stimulation of ^{3}H- Thymidine Incorporation



Oestradiol stimulation measured as ^{3}H- thymidine incorporation after 1 hour exposure to ^{3}H- thymidine at a number of intervals after addition of oestradiol. Points represent percentage increase in ^{3}H- thymidine uptake between oestradiol treated samples and time matched controls. (All points have been calculated from the mean of 3 individual samples).

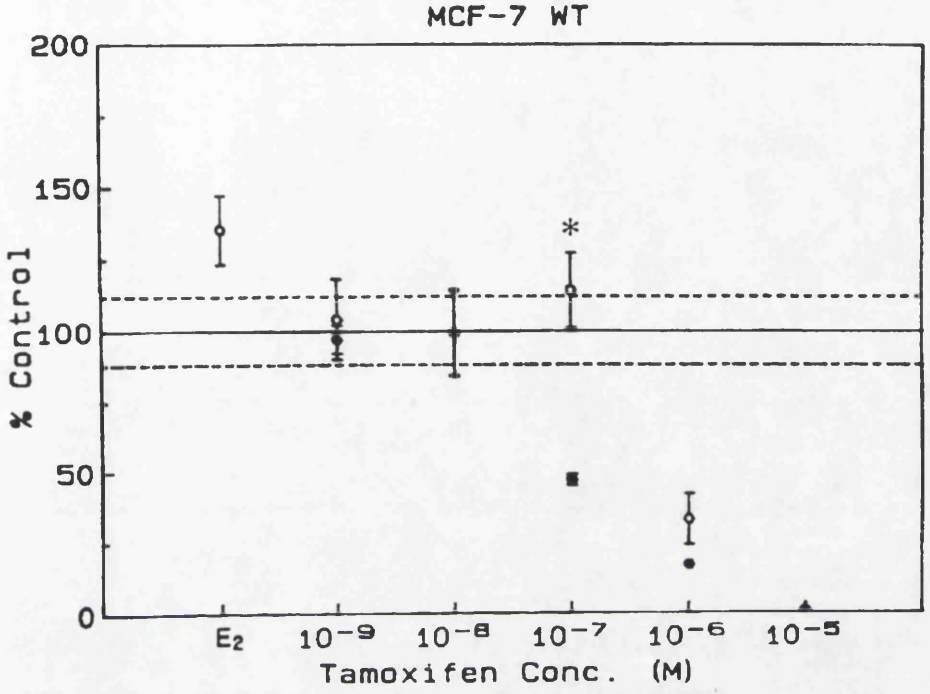
S.D. within 10% of mean values. This data is taken from one single experiment.

Figure 3-11 Oestradiol Dose Response Curve (^{3}H- Thymidine Uptake)



Effect of oestradiol concentration on ^{3}H- thymidine incorporation into cells. Results expressed as a percentage of control counts from untreated cells. MCF-7WT 100% = 253644 DPM (——) $^+/_-$ S.D = 41442 DPM (- - - -). MCF-7Adr 100% = 212508 DPM (——) $^+/_-$ S.D = 23666 DPM (- - - -). Each of the control lines is taken as a mean of 6 plates. Individual points are the mean of 3 plates with vertical bar denoting one S.D. * represents significant difference from control $p < 0.01$. Graphs show one representative experiment repeated three times.

Figure 3-12 Tamoxifen Dose Response Curve (^{3}H- Thymidine Uptake)



MCF-7 WT cellular dose response to tamoxifen $^{+}/_{-}$ oestradiol (10^{-9}M) measured as ^{3}H- thymidine incorporation. Results expressed as percentage of control in drug free medium. 100% (mean of 6 plates)= 131944 DPM (——) $^{+}/_{-}$ S.D = 14960 DPM (- - - -), Oestradiol (10^{-9}M) + tamoxifen [o], tamoxifen alone [●]. Individual points are mean of 3 plates, vertical bar denoting one S.D.

* represents a significant difference between tamoxifen + oestradiol and tamoxifen alone. One representative experiment repeated three times.

to the limited time period of the assay i.e. 26Hrs where only one single cell cycle is examined as opposed to 3 or 4 cell cycles in the MTT assay. Also, since this assay was carried out in serum free culture conditions therefore lacking the presence of any growth factors the growth response to E_2 may alter, since it has been suggested the growth response to E_2 is dependent on the presence of co-factors (van der Burg *et al.*, 1988). In the MCF-7Adr line, there is no increased response to E_2 as would be expected. When E_2 and tamoxifen are examined together (Fig.3-12), tamoxifen shows clear inhibition of any E_2 induced response at comparatively low levels 10^{-9}M and above. Interestingly at 10^{-7}M tamoxifen the E_2 stimulus is inhibited, whereas tamoxifen alone at 10^{-7}M reduces the level of DNA synthesis below the basal control values. At 10^{-6}M tamoxifen, a depressed level of DNA synthesis is seen in both the tamoxifen alone and the E_2 and tamoxifen experiments. At 10^{-5}M it is certain tamoxifen is acting as a cytotoxin in serum free conditions.

3.3 DISCUSSION

When examining the effect of oestrogens on cellular growth, the selection of an appropriate medium was very important. A number of possibities were then explored. A serum free system would have been ideal for this work and the growth factor responses which are examined in Chapter 4. Such a system was tried based on the medium suggested by Barnes and Sato,(1979), unfortunately, cell growth was very low and the cell morphology changed considerably. The cells

grew in small clusters, rather than the large cuboidal cells seen in the presence of serum. In order to study the effects of steroids on cellular growth a compromise was reached by substituting charcoal stripped serum. This allowed the cells to maintain their morphology and steroid sensitive cell growth to be stimulated by E_2 to the same levels which could be achieved with optimal FCS. In this steroid reduced medium, the MCF-7WT cell line showed almost no growth without the addition of E_2. The essential property required of the medium, was to maximize the E_2 responsive growth in an oestrogen responsive cell line. This was achieved at a 5% level of CSS, which still enabled the cells to attach and form monolayers after trypsinisation. On developing the MTT assay, a further reduction in serum factors was tested by heat treating the stripped serum and reducing its level to only 2.5% of total medium volume. This medium has lost a number of growth factor properties and therefore was useful for the examination of growth factor effects. This medium had lost the active factors required to enable the cells to form monolayer cultures and therefore was not used for examining steroid induced growth.

The MTT assay was first described by Mosmann in 1983, and has since been used extensively for chemosensitivity testing in a large variety of cell lines. In this work the assay has been developed for the study of cell growth, rather than cell kill. This was done by growing the cells in a basal growth medium and adding growth stimuli to the cells. The stimulatory effect could then be measured as the difference in final cell density between cells grown in basal medium and those supplemented with stimulatory factors. This worked out well for measuring

the growth effects of E_2, and compares favourably with other methods used to measure this stimulus (Dabre *et al.*, 1989 & Cormier *et al.*, 1989).

The ^{3}H-thymidine assay allowed for a restricted period of growth in serum free culture, whilst still allowing the cells to be stimulated by E_2. This assay was used in parallel with the MTT assay and both showed similar stimulatory and inhibitory trends with oestradiol and tamoxifen. Careful interpretation of thymidine incorporation in the MCF-7 cell line are required, since a number of problems have been highlighted (Lippman & Aitken, 1981). These include; destabilization of the experimental system by the addition of excess thymidine, compartmentilization of DNA pools and incorporation of label into non DNA material. Since both types of assay gave similar results, these problems do not appear to have affected the overall purpose of the experiment in this situation.

The cell lines examined here show varying responses to E_2. The MCF-7WT line shows a large growth response to E_2, while the ZR-75-1 cell line shows only a small growth response to E_2 and is not dependent on E_2 for growth, both are ER positive. This was in marked contrast to the results of Darbre & Daly, 1989 who found the ZR-75-1 cell line to be E_2 dependent and highly growth responsive to the addition of E_2. Neither of the two ER negative cell lines show any growth effects upon E_2 addition.

Tamoxifen effects on the inhibition of E_2 stimulation were only clearly seen in the MCF-7WT line where the highest rates of E_2 stimulation could be achieved. Focussing on this cell line, it is clear that tamoxifen antagonism of E_2 stimulation can only be achieved in cell culture at a high concentration of drug ($5x10^{-7}$M tamoxifen), approximately a thousand fold greater molar concentration than E_2. In human patients concentrations of tamoxifen approximating to $7x10^{-6}$M

have been found in tumour homogenates, a level at which tamoxifen should be able to inhibit all the E_2 regulated growth (reviewed in Wakeling, 1988). The major metabolite 4-hydroxytamoxifen, has also been widely studied and found to have a hundred fold greater affinity for the ER than the parent compound, but its importance *in vivo* is uncertain since it is rapidly lost from the circulation (Rochefort *et al.*, 1983). MCF-7 cells have been found incapable of forming this metabolite of tamoxifen in cell culture (Coezy *et al.*, 1982) therefore it does not play a role in the inhibitory actions of tamoxifen in these experiments.

It is very interesting to note the effects of tamoxifen in the ^{3}H-thymidine assay. Clearly tamoxifen is having a strong inhibitory effect on the basal rate of DNA synthesis, at concentrations of 10^{-7}M and above. A level where no cytotoxicity will be present. This inhibition is rescued in the presence of E_2 10^{-9}M, but at a tamoxifen concentration of 10^{-6}M, E_2 is no longer able to compete out the effect. It would appear that tamoxifen is having an inhibitory effect not attributable to antagonism of the ER. A number growth inhibitory biochemical mechanisms have been identified which work indepently of the ER. The drug is known to bind to protein kinase C (PKC) and thereby inhibit its actvity (O'Brian *et al.*, 1988) it may also inhibit cellular uptake of Ca^{2+} (Ferno *et al.*, 1985). Both of these, will have profound effects upon cell growth and both explanations would fit the data, but further work is needed to identify which effects are actually occurring. As the tamoxifen concentration increases to 10^{-5}M we are seeing non specific cytotoxicity.

When comparing the two assays; MTT assay with the ^{3}H-thymidine

incorporation assay, the tamoxifen response is different. In the ^{3}H-thymidine assay tamoxifen appears to act as a more potent inhibitor of E_2 growth regulation and also shows ER independent growth inhibition at a lower concentration than the MTT assay. The major difference between these two assays is the presence of serum in the MTT assay which may be responsible for altering the potency of tamoxifen by binding the drug.

3.4 SUMMARY AND CONCLUSIONS

The MTT assay is a simple relatively quick method for examining mitogenic responses in monolayer cell lines. It has been used successfully for the characterisation of oestrogen responses in a panel of four breast cancer cell lines. In each case, these responses have agreed with ER receptor status of the cell, with no oestrogen growth regulation seen in the two ER negative cell lines examined. Under optimal E_2 stimulation it was possible to examine tamoxifen inhibition of cell growth, which was apparent only in the ER positive cell lines. Further examination of tamoxifen responses revealed the possibility of ER independent effects.

CHAPTER 4

GROWTH FACTOR REGULATION OF CELL GROWTH

CHAPTER 4

GROWTH FACTOR REGULATION OF CELL GROWTH

4.1 INTRODUCTION

The influence of growth factors in autocrine and paracrine growth control is an important aspect of breast tumour growth. A number of growth factors thought to play a role in growth regulation have been examined individually and in combination.

4.1.1 The Role of Growth Factors in Growth Regulation of Breast Tumour Cell Lines

Oestrogen regulation of growth is thought to be partly regulated by a number of autocrine growth factor loops. The growth factor TGF-α has been a prime candidate for such control (section 1.4.1), although other growth factors may also be involved. Tamoxifen has been thought to induce increased synthesis of TGF-ß, an inhibitor of epithelial cell growth (see section 1.3.1). In both the assays used here; the MTT assay and ^{3}H-thymidine incorporation, TGF-ß was not found inhibitory to the growth of MCF-7WT cells. Growth factors synthesised by neighbouring stromal cells are also strongly suspected to play an important role in the growth of breast tumour cells (section 1.6.2); IGF-I, IGF-II and bFGF have been examined as candidate peptides involved in this type of growth regulation.

4.2. RESULTS

4.2.1 EGF and the EGF Receptor

Expression of the EGF receptor was found in each of the four breast cancer cell lines at varying levels. Scatchard analysis was used to determine the concentration of EGF receptor in the MCF-7WT and MCF-Adr cell lines using a 14 point concentration assay (Owens *et al.*,1991). For the cell lines ZR-75-1 and MDA-MB-231 full scatchard analysis was not performed but an estimate was made of the EGF receptor concentration from single point analysis. The results of the analysis are given below in Table 4-1, as the amount of EGF receptor protein per mg of cellular protein. This assay measures levels of unoccupied receptor since no stripping procedure was adopted to remove any endogenous ligand from the receptors, although some occupied receptor will be competed out by the radioactive ligand. The MCF-7Adr line appears to have approximately twice as many receptors as the parent MCF-7WT line when measured using scatchard analysis. For both the MCF-7 lines only single affinity binding sites were identified from the scatchard plots.

Table 4-1 CELLULAR CONCENTRATIONS OF EGF RECEPTOR

	EGFr fmoles/mg
MCF-7WT	9.2
MCF-7Adr	19.2
ZR-75-1	(+) <12
MDA-MB-231	(++) >15

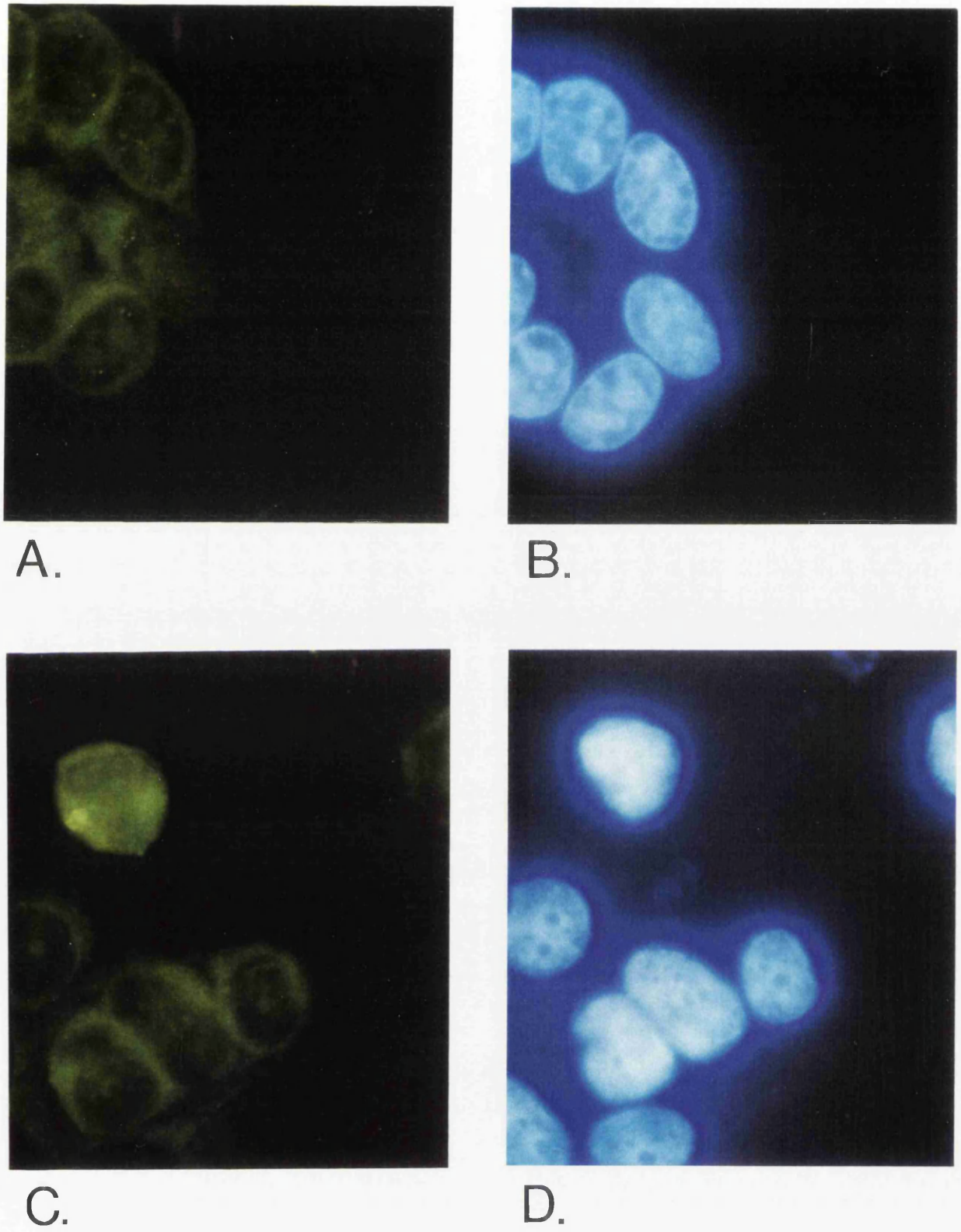
EGFr concentrations measured using Scatchard analysis. Results shown as fmoles of EGFr per mg cellular protein. Results represent single assay where all cell lines grown in RPMI+5% FCS. Full Scatchard analysis was not available for ZR-75-1 and MDA-MB-231 cells. Values estimated from single points (+)<12 fmoles/mg, (++)>15 fmoles/mg.

To further verify this work, the rabbit polyclonal antibody BG48 raised against the EGF receptor has been used successfully in each of the breast cancer cell lines to identify the EGF receptor by immunohistochemistry and western analysis. Figures 4-1(A-D), clearly show the presence of the EGF receptor in the cytoplasm of each of the two MCF-7 cell lines using indirect immunofluorescence (method 2.8.4). This technique gives little quantitative information so the same antibody was also employed as a probe in western blots, a method (section 2.9) which gives a much more quantitative examination of the EGF receptor (Fig. 4-2). Equal quantities of cellular proteins from whole cell lysates were ran out on a gel, transferred to nitrocellulose and immunoblotted with the same polyclonal antibody. The A431 cell line was also ran out on the same gel as a positive control. Clearly high EGF receptor expression levels exist in the two ER negative cell lines; MCF-7Adr and MDA-MB-231, whilst the two ER positive cell lines contain levels of EGF receptor below the level of detection of this technique. Subsequent gels have shown faint evidence of the EGF receptor in both these lines.

Since the two quantitative methods; scatchard analysis and western blotting are actually measuring two different end points i.e. unoccupied receptor and total receptor respectively, the discrepancy between the two results is quite understandable.

EGF was added to the MCF-7WT cell line to examine the growth response in an MTT assay (method 2.6.2). Figure 4-3 shows a dose response curve (DRC) for EGF, with only a modest, but significant ($p < 0.05$) increase in growth to 140% of control levels at an optimal EGF concentration (10ng/ml, 1.7×10^{-9}M). The

Figure 4-1 Indirect Immunofluorescence of the EGF Receptor



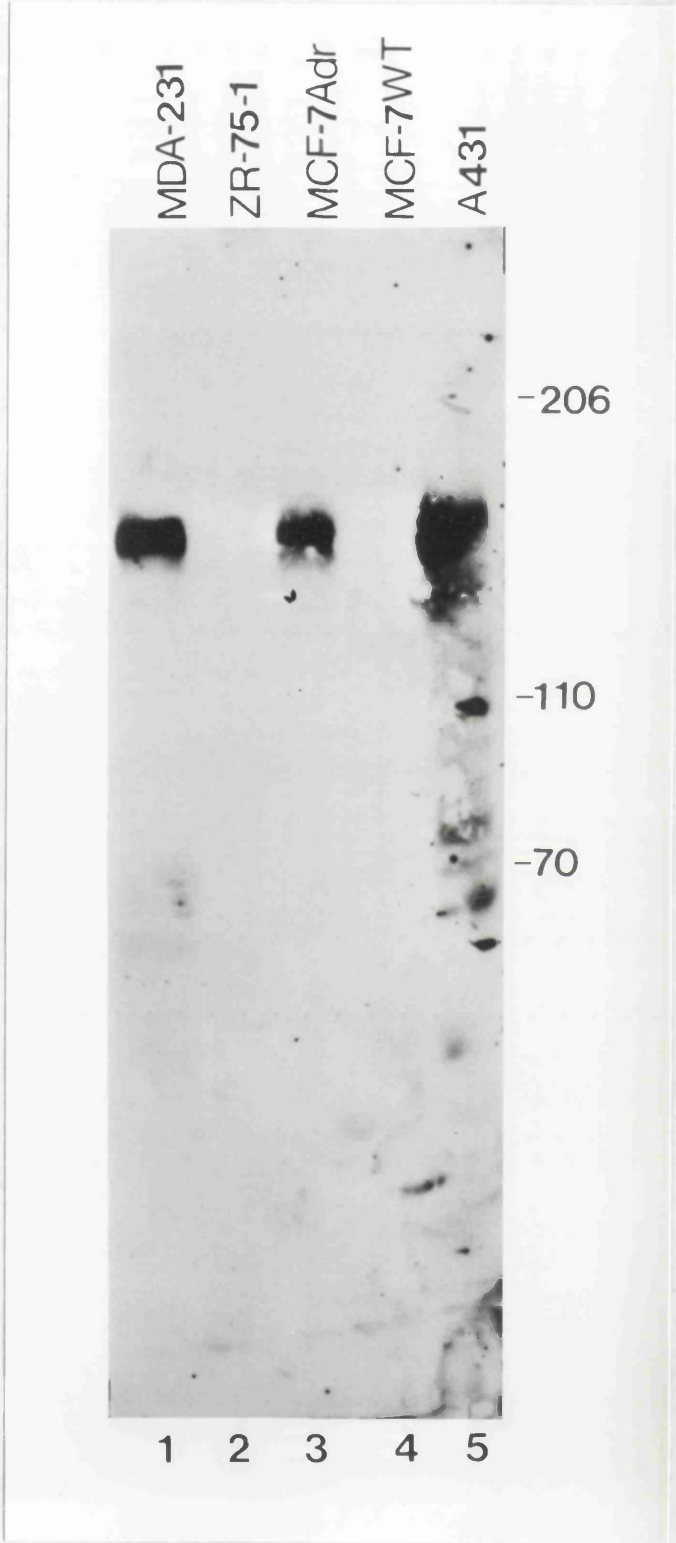
Indirect immunofluorescent staining of the EGF receptor

A. MCF-7WT EGF receptor staining **B.** MCF-7WT H33258 counterstaining

C. MCF-7Adr EGF receptor staining **D.** MCF-7Adr H33258 counterstaining

EGFr immunofluorescence staining carried out as stated in Methods section, (x1000).

Figure 4-2 Western Analysis of the EGF Receptor



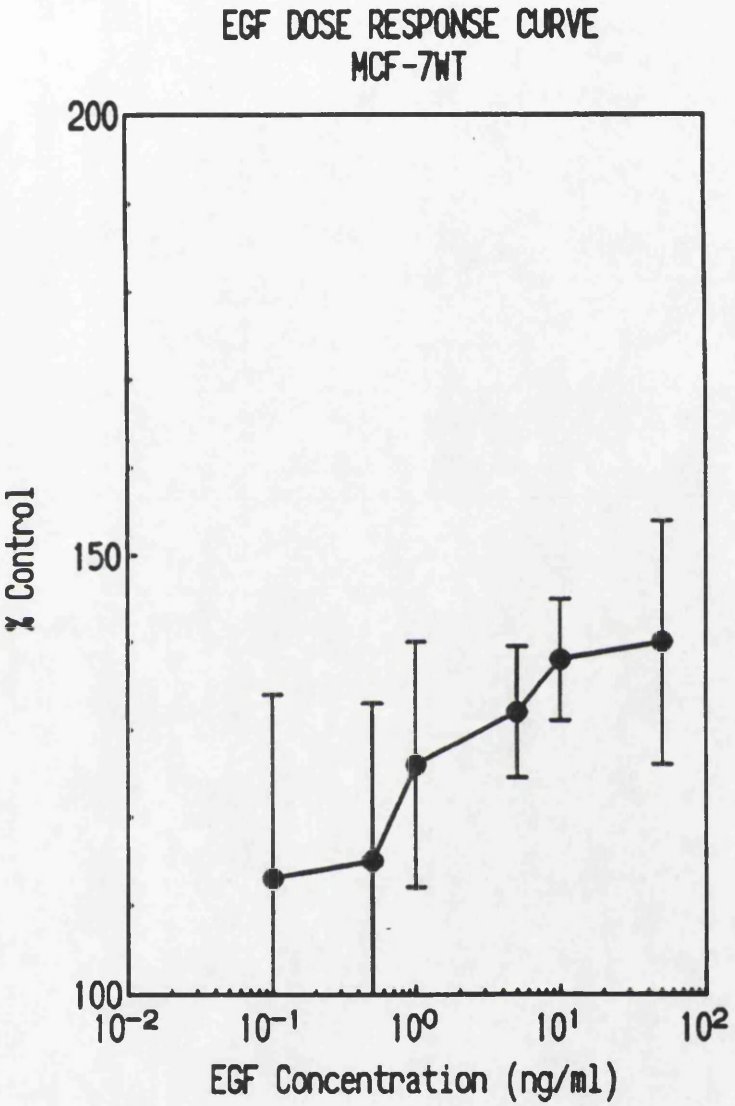
Western blot of EGFr in 5 cell lines. Total cellular protein extracted from each cell line grown in RPMI+5%FCS at subconfluence. 50 µg of protein were ran on a 6% SDS-Page gel and transferred proteins immunoblotted with the EGFr antibody. EGFr expression was detected in MDA-MB-231 and MCF-7Adr breast cancer cell lines. The A431 cell line was present as a positive control. The EGFr was undetectable in the MCF-7WT and ZR-75-1 cells using this technique. Numbers on the RHS represent MW markers.

same experiment repeated in the MCF-7Adr cell line showed no significant growth response to EGF (data not shown). The response to EGF was also tested in a ^{3}H-thymidine assay. After ascertaining the optimal time for ^{3}H-thymidine uptake to be 22Hrs (Figure 4-4), when MCF-7WT cells were treated with 10ng/ml EGF over a period of 30 Hrs. The response to a range of EGF concentrations was measured at this time point and is shown in Figure 4-5A for MCF-7WT cells and Figure 4-5B for MCF-7Adr cells. For the MCF-7WT cell line increasing concentrations of EGF produced a bell-shaped DRC with optimal stimulation of DNA synthesis at 10ng/ml EGF, dropping back to control levels at an EGF concentration of 1 μg/ml. This concentration of EGF was tested for any possible cytotoxic effects by clonogenic assay but none were found. The MCF-7Adr cell line which expresses much higher levels of the EGF receptor shows no growth response to EGF at any concentration and if anything may be growth inhibited by the addition of high concentrations of EGF(1 μg/ml). Since all the ^{3}H-thymidine experiments are carried out in serum free conditions, these responses should be free from interference from background levels of growth factors.

4.2.2 Insulin, IGF-I and IGF-II, Growth Effects on MCF-7WT Cells

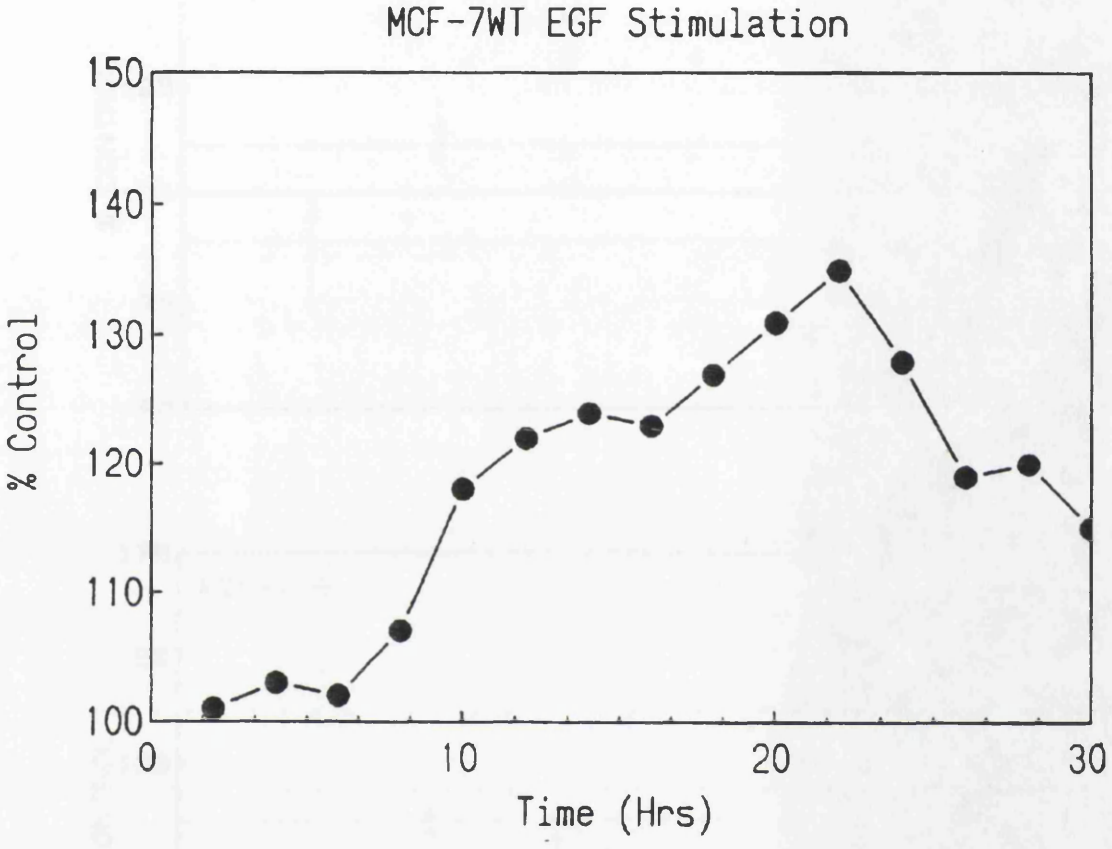
The capacity of insulin, IGF-I and IGF-II to stimulate the growth of the MCF-7WT cell line was examined using the MTT assay. Figure 4-6, shows the DRC for insulin, which rises to a level of 275% of control values at the optimal concentration of 10 μg/ml (1.7×10^{-6}M). All concentrations of insulin greater than 5ng/ml showed a growth response significantly greater than control, ($P < 0.01$), using the MTT assay. The insulin concentration of 10 μg/ml, has been used

Figure 4-3 **EGF Induced Dose Response Curve MCF-7WT cells)**



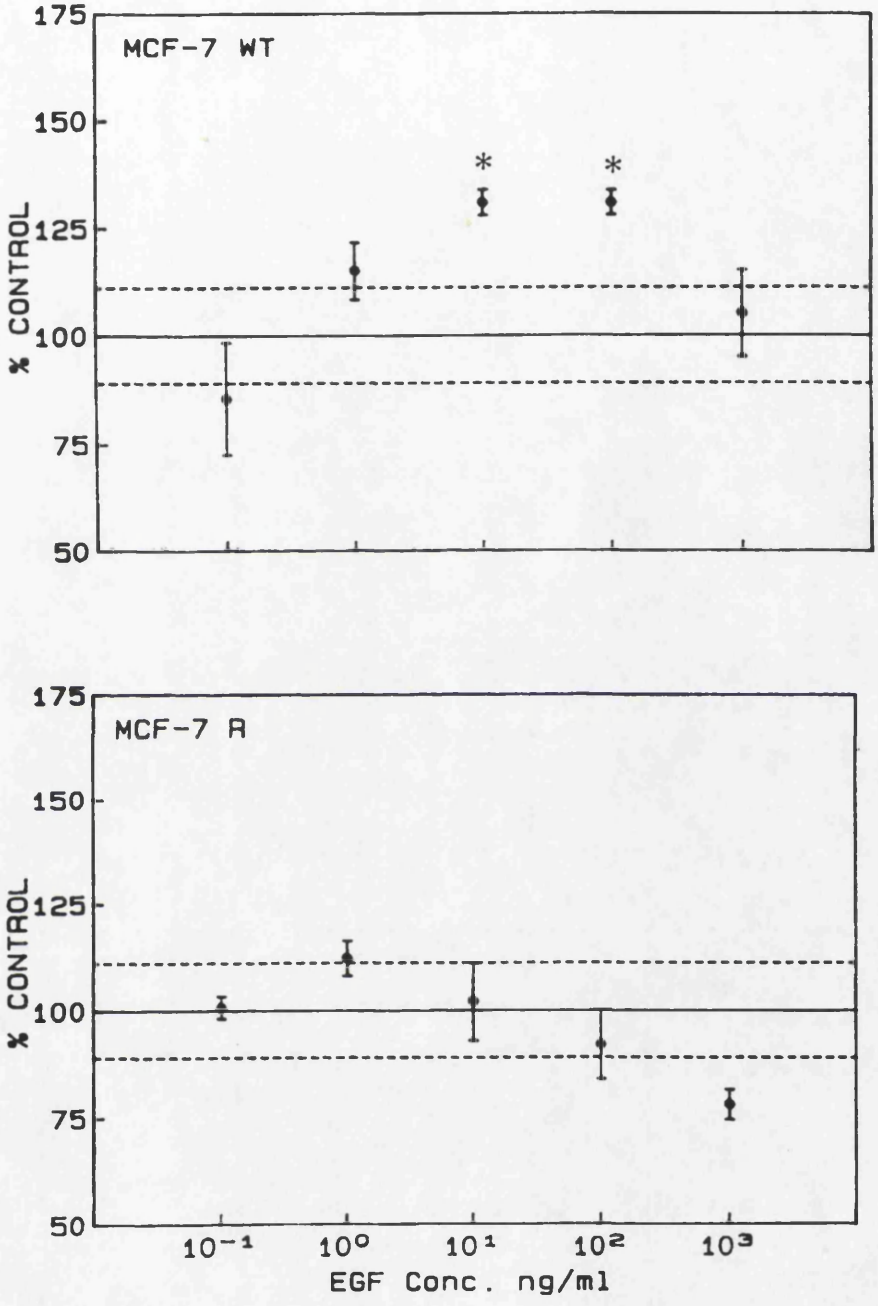
Growth response to EGF measured using the MTT assay. Each point is the mean of three samples and is expressed as a percentage of control values. Vertical bars represent one S.D. One representative experiment from a series of four.

Figure 4-4 EGF Stimulation of ^{3}H- Thymidine Incorporation



EGF stimulation measured as ^{3}H- thymidine incorporation after 1 hour exposure to ^{3}H- thymidine at a number of intervals after addition of EGF (10 μ/ml). Points represent percentage increase in ^{3}H- thymidine uptake between EGF treated samples and time matched controls. (All points have been calculated from the mean of 3 individual samples). S.D. within 10% of mean values. This data is taken from one single experiment.

Figure 4-5 EGF Dose Response Curve (^{3}H- Thymidine Uptake)

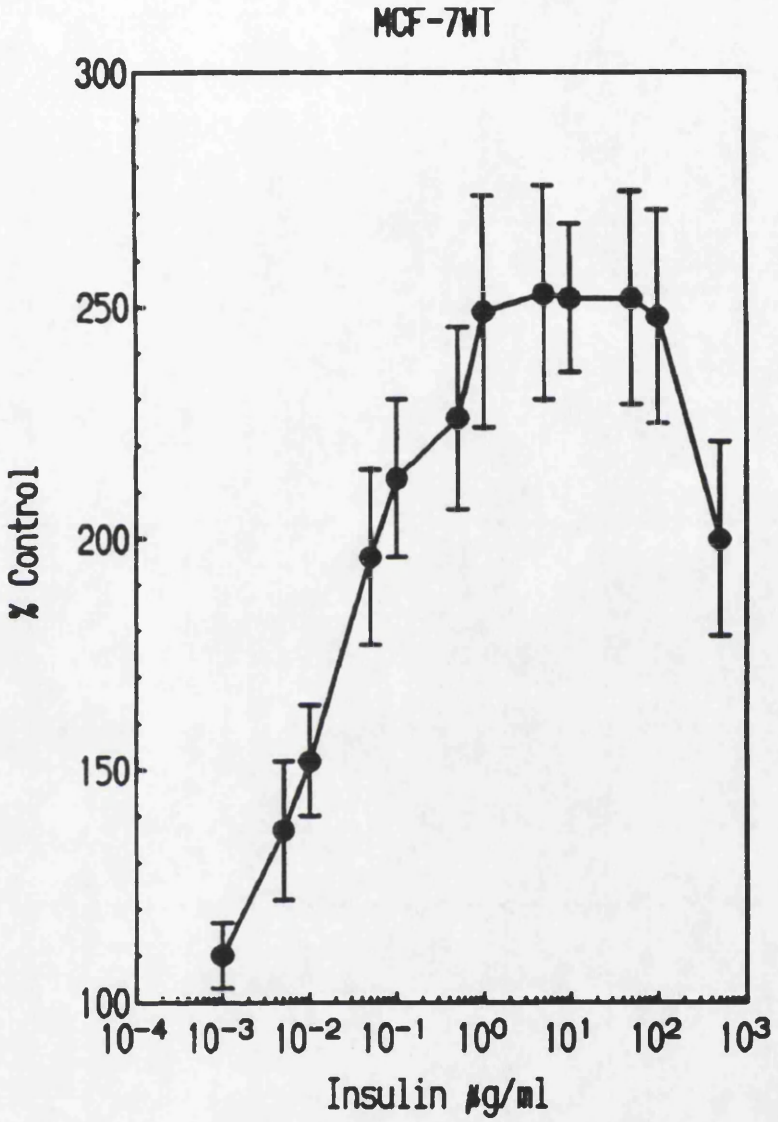


Effect of EGF concentration on ^{3}H- thymidine incorporation into cells. Results expressed as a percentage of control counts from untreated cells.
MCF-7WT 100%= 150724 DPM (——) $^{+}/_{-}$ S.D= 16929 (- - - - -).
MCF-7Adr 100%= 124477 (——), S.D= 14174 (- - - - -).
Each of the control lines is taken as a mean of 6 plates. Individual points are the mean of 3 plates with vertical bar denoting one S.D. * represents a significant difference from control.

repeatedly throughout the work with the addition of 10^{-9}M E_2 as a positive growth control in the MTT assay. The insulin DRC was repeated in medium conditions containing 2.5% HT-CSS in case serum insulin or insulin-like growth factors were present in the CSS and altering the response to additional insulin. In actual fact the response was reduced to 220% of control values at the peak response when the cells were growing in RPMI(prf) medium with the addition of 2.5% HT-CSS. This was statistically different ($P<0.01$) from the 275% increase in growth found with CSS containing medium. This is due to the increased growth potential of the CSS as oppose to the HT-CSS. The DRC for IGF-I (Fig. 4-7) shows a similar profile to insulin but achieves maximum proliferation at a concentration of 50ng/ml ($6.6x10^{-9}$M) approximately 100 fold less than insulin. IGF-I achieves a statistically significant ($p<0.01$) increase in the growth of MCF-7WT cells at a concentration of 1ng/ml and above. IGF-II stimulates MCF-7WT proliferation to a similar extent (Fig. 4-8), with an optimal response at 100ng/ml ($1.3x10^{-8}$M) concentration showing slightly less potency than IGF-I, again all concentrations of IGF-II of 0.05ng/ml and above cause a statistically significant increase in cell growth.

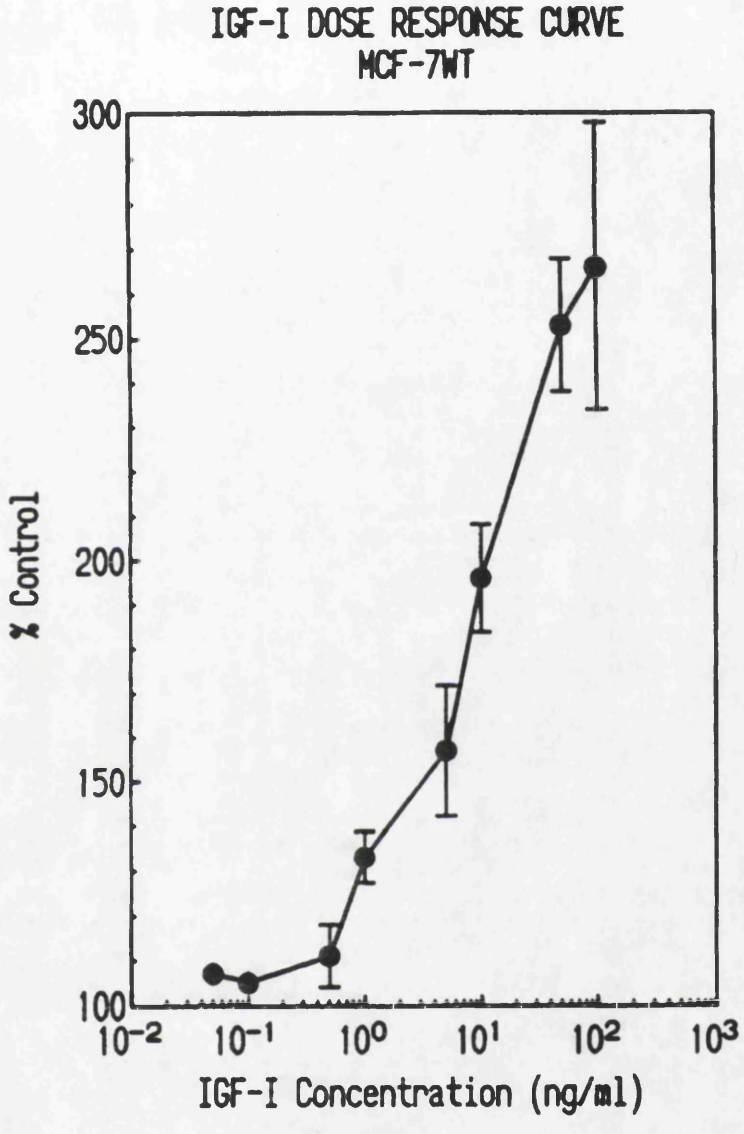
The αIR-3 monoclonal antibody to the IGF-I receptor is known to have a neutralizing function on the IGF-I receptor (Rohlik *et al.*,1987) and was used to determine whether the activation of IGF-I receptor was involved in the insulin and IGF-I growth responses seen in MCF-7WT cells, rather than the insulin receptor. Figure 4-9, shows the effect of increasing concentrations of this antibody on cells in the presence and absence of an optimal concentration of IGF-I (50ng/ml). The growth response is reduced to control levels at an antibody

Figure 4-6 Insulin Induced Dose Response Curve (MCF-7WT Cells)



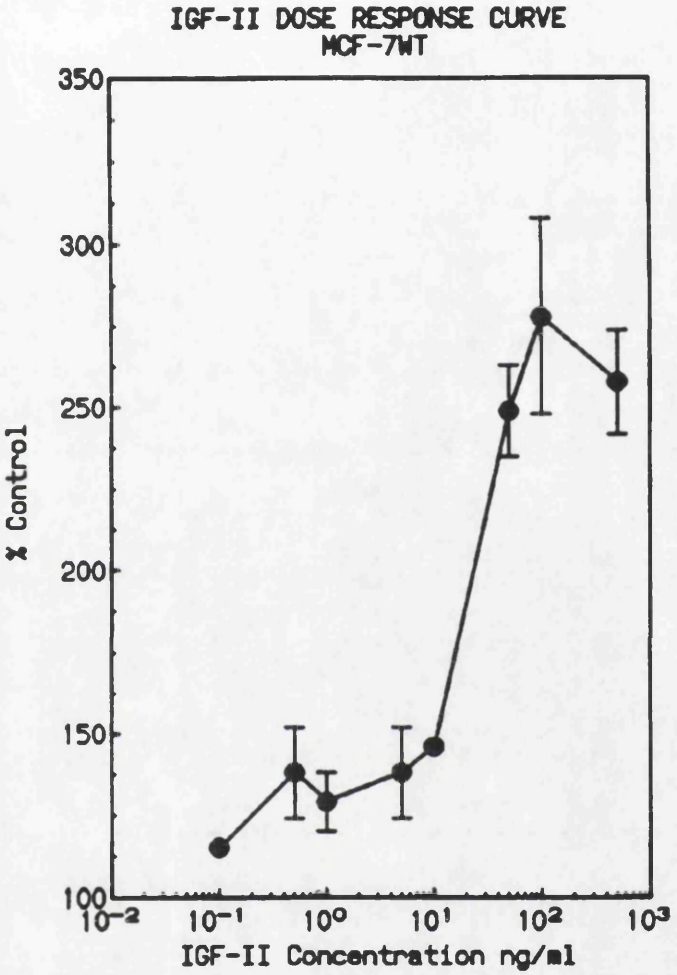
Growth response to insulin measured using the MTT assay. Each point is the mean of three samples and is expressed as a percentage of control values. Vertical bars represent one S.D One representative experiment from a series of three.

Figure 4-7 IGF- I Induced Dose Response Curve (MCF-7WT cells)



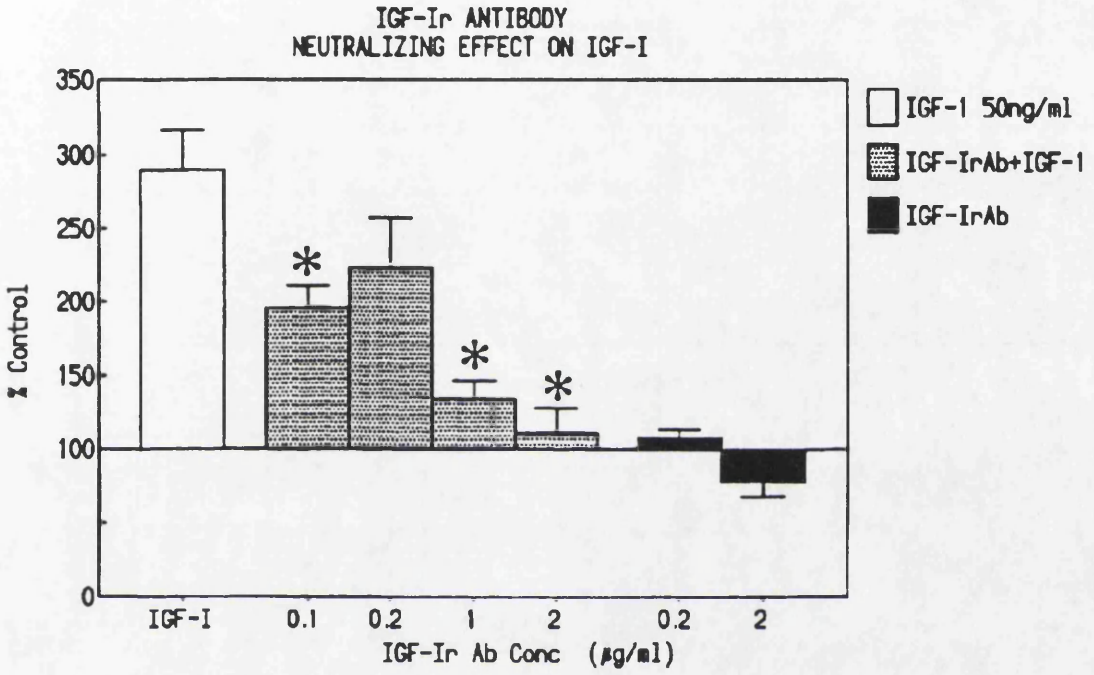
Growth response to IGF- I, measured using the MTT assay. Each point is the mean of three samples and is expressed as a percentage of control values. Vertical bars represent one S.D. One representative experiment from a series of three.

Figure 4-8 IGF- II Induced Response Curve (MCF-7WT cells)



Growth response to IGF-II measured using the MTT assay. Each point is the mean of three samples and is expressed as a percentage of control values. Vertical bars represent one S.D. One representative experiment from a series of three.

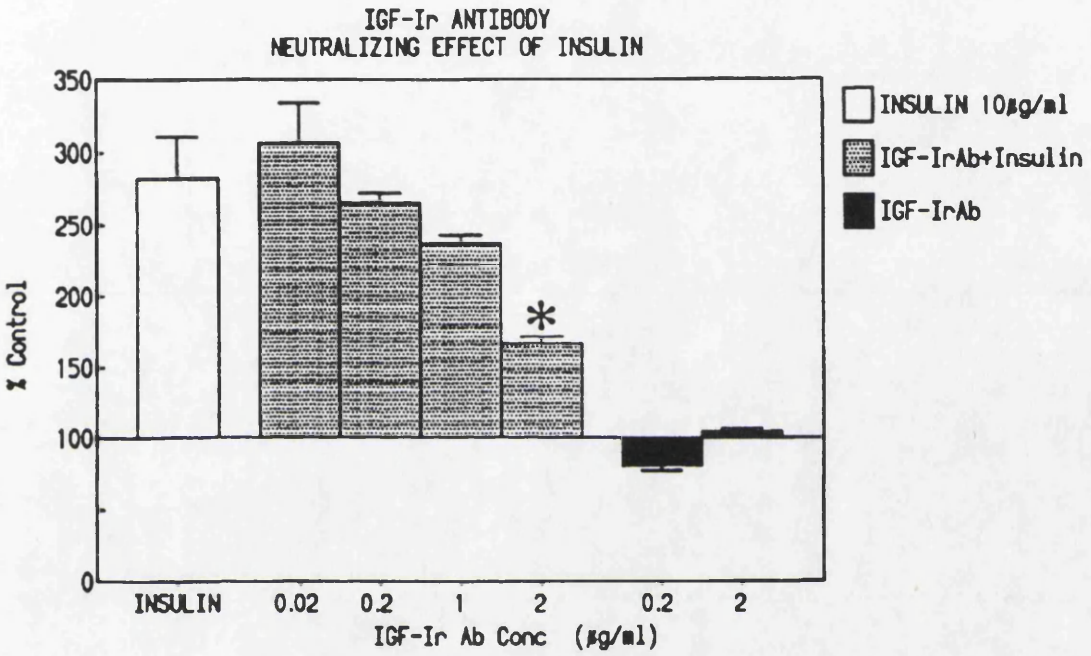
Figure 4-9 IGF-I Growth Response through IGF-I Receptor



Growth responses to IGF-I were measured in the MCF-7WT cell line using the MTT assay. Cells were treated with increasing concentrations of an IGF-I receptor neutralizing antibody. Growth effects of antibody alone have been examined at two concentrations with no significant difference from control values. * represents significant difference from IGF-I positive control ($P < 0.01$). Vertical lines represent one S.D.

One representative experiment from a series of three. As a control bFGF IgG_1 antibody caused no inhibition of growth in the presence of IGF-I.

Figure 4-10 Insulin Growth Response through IGF- I Receptor



Growth responses to insulin were measured in the MCF-7WT cell line using the MTT assay. Cells were treated with of increasing concentrations of an IGF-I receptor neutralizing antibody. Growth effects of antibody alone have been examined at two concentrations with no significant difference from control values.*represents significant difference from insulin positive control ($p<0.01$). Vertical lines represent one S.D.

One representative experiment from a series of four. As a control bFGF IgG_1 antibody caused no inhibition of growth in the presence of insulin (10 μg/ml).

concentration of 2 μg/ml, suggesting the IGF-I receptor is responsible for the IGF-I induced growth response. When the same experiment was carried out in the presence of insulin rather than IGF-I (Fig. 4-10) an antibody concentration dependent decrease in the rate of growth was apparent but levels were not fully reduced to the control rate at 2 μg/ml antibody.

4.2.3 Breast Cancer Cell Line Responses to Combinations of E_2 and Growth Factors

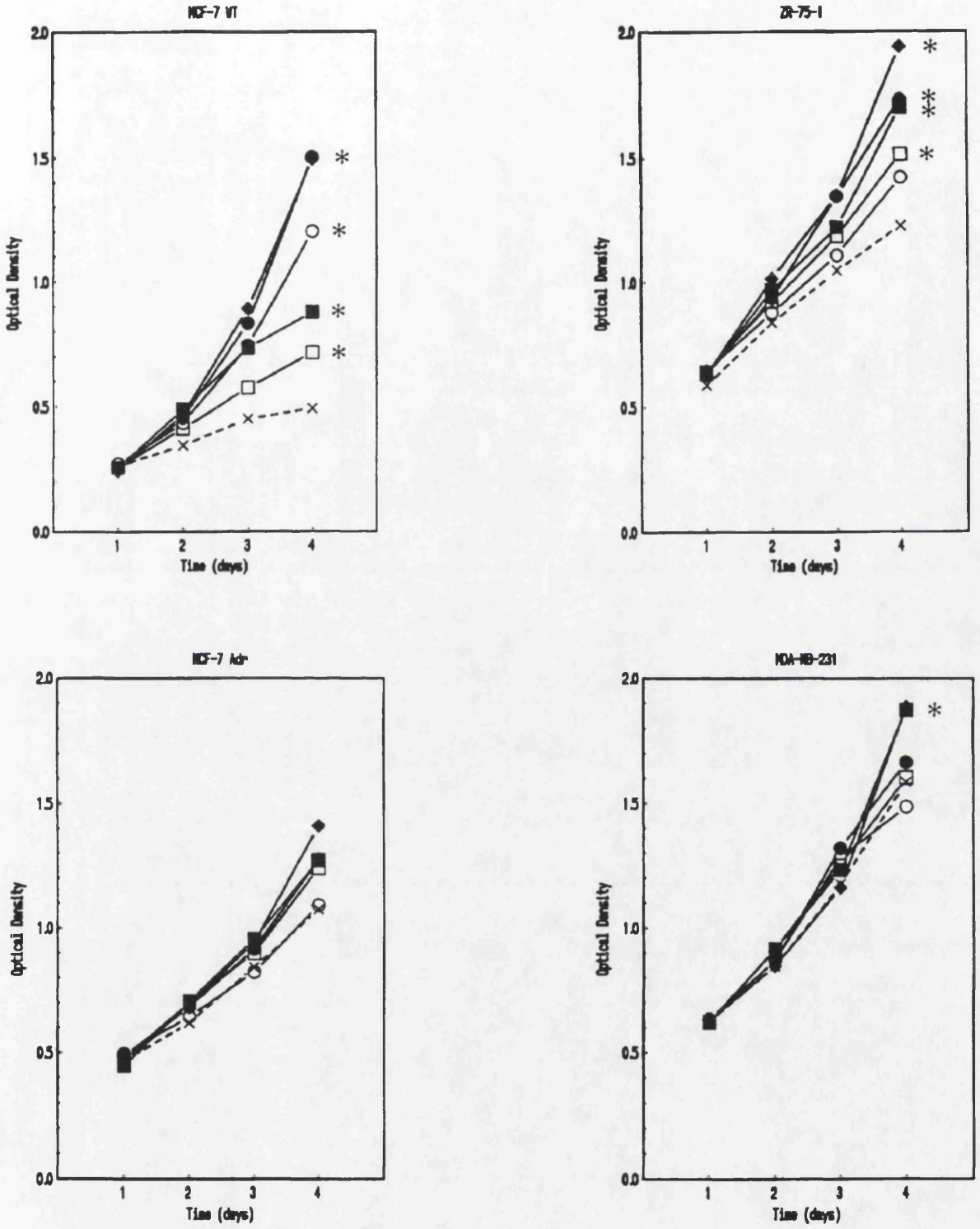
In Figures 11 and 12 cellular growth responses to optimal concentrations of E_2, insulin and EGF and combinations of each of these are dealt with. Growth curves in each of the four breast cancer cell lines are illustrated in Figures 4-11(A-D) over the 3 day stimulated growth period of the MTT assay. Only in the two ER positive cell lines MCF-7WT and ZR-75-1 is a stimulatory response seen. The MCF-7WT line (Fig. 4-11A) shows a small increase in growth rate after EGF treatment, which is increased further with the addition of insulin. Maximal growth is achieved in this cell line with the addition of insulin and E_2, EGF causes no further increase in growth. In the ZR-75-1 cell line, the basal growth rate is much higher in control medium than MCF-7WT cells and the response to mitogens is far less pronounced. EGF appears to be a stronger mitogen for this cell line, whilst E_2 has a much smaller mitogenic effect. On this occasion EGF is able to cause a further increase in the growth response brought about by insulin and E_2. The two ER negative cell lines both have very high basal levels of growth in the depleted medium and neither show large growth responses to the addition of mitogens. In both cases E_2 brought about no growth response and only the

addition of EGF and insulin together were able to increase the growth rate above control levels, reaching significance for the MDA-MB-231 cell line. In Figures 4-12(A-D) the same combinations of mitogens have again been tested, with the results expressed as a percentage of control. These figures show more clearly the growth increase brought about by each mitogen and combination of mitogens and allows easy comparison of responses from each cell line. MCF-7WT cells respond to each of the mitogens tested: E_2, insulin and EGF. Combinations of each mitogen produced a further increase in the growth. This was also true of the ZR-75-1 cells although significance was only reached after combined treatment. Again the two ER negative cells showed little growth response to any of the mitogens.

Low doses of insulin and IGF-I in combination with E_2 were examined for possible synergy between the two factors (Fig. 4-13). The graph shows growth responses to E_2, insulin and IGF-I alone and in combination but in each case the combined factors resulted in a growth response equal to or less than its two constituent parts, suggesting it is highly unlikely either insulin or IGF-I are able to synergise the effect of E_2 in these assay conditions.

bFGF also causes a growth response in the MCF-7WT cell line. A DRC for this growth factor is illustrated in Figure 4-14, and it clearly shows the pronounced effects on growth this family of growth factors can achieve. All concentrations of bFGF tested from 0.001ng/ml to 500ng/ml caused a significant increase ($p<0.01$) in cell growth when compared to controls.

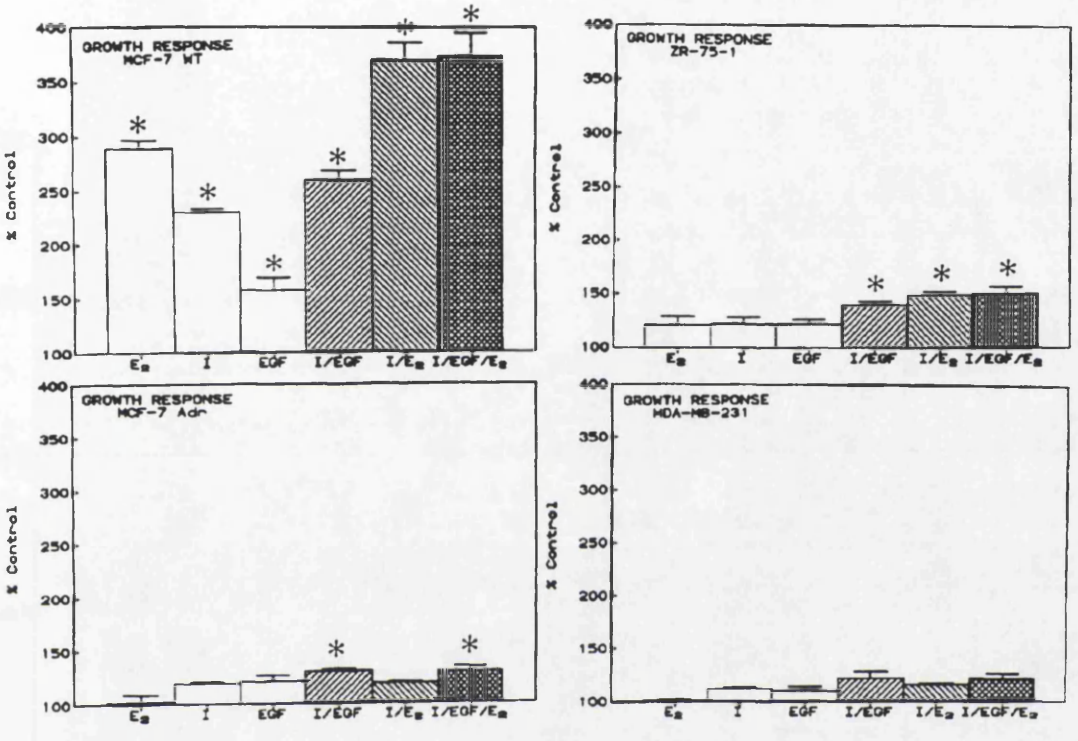
Figure 4-11 Growth Curves in Response to Mitogens



Growth curves under different mitogen conditions. x- - - represents control growth in RPMI(prf)+5% CSS, (□)EGF 10ng/ml, (■)EGF+Insulin 10μg/ml, (○)E_2 10^{-9}M, (●)E_2+Insulin, (◆)E_2+Insulin+EGF.
Each point represents the mean of 3 samples. * represents signiificant difference from control at day 4 of growth. ($p < 0.01$)

MCF-7WT and MCF-7Adr graphs are representative experiments carried out on three independent occasions. ZR-75-1 and MDA-MB-231 experiments carried out twice.

Figure 4-12 Growth Response to a Range of Mitogens



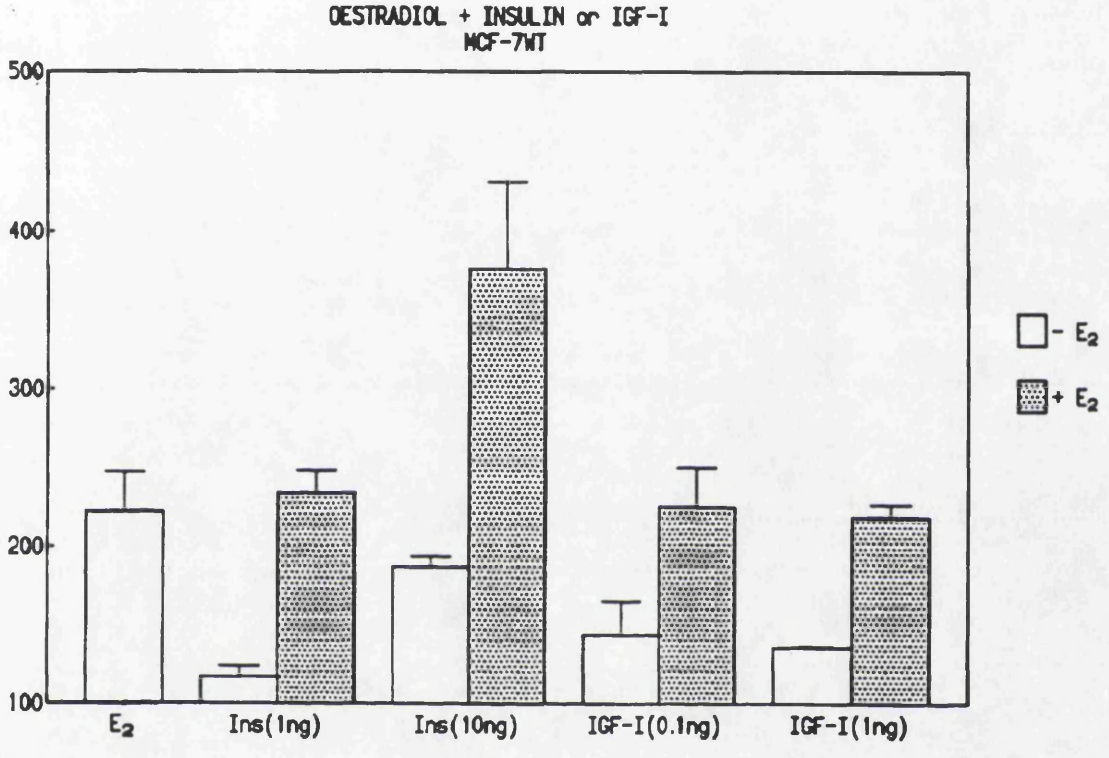
Stimulation of cell growth in MCF-7 WT, ZR-75-1, MCF-7 Adr and MDA-MB-231, by E_2, insulin (I) and EGF over 3 days of growth in an MTT assay. Response is measured as a percentage of growth in control wells.

Individual growth response to E_2 (10^{-9}M) , I 10 μg/ml (1.7 μM), EGF 10μg/ml (1.7nM), I+EGF, I+E_2, I+EGF+E_2. Each bar represents the mean of 3 plates (24 wells) with vertical lines denoting one S.D.

$*$ represents significant difference from control growth ($p<0.01$).

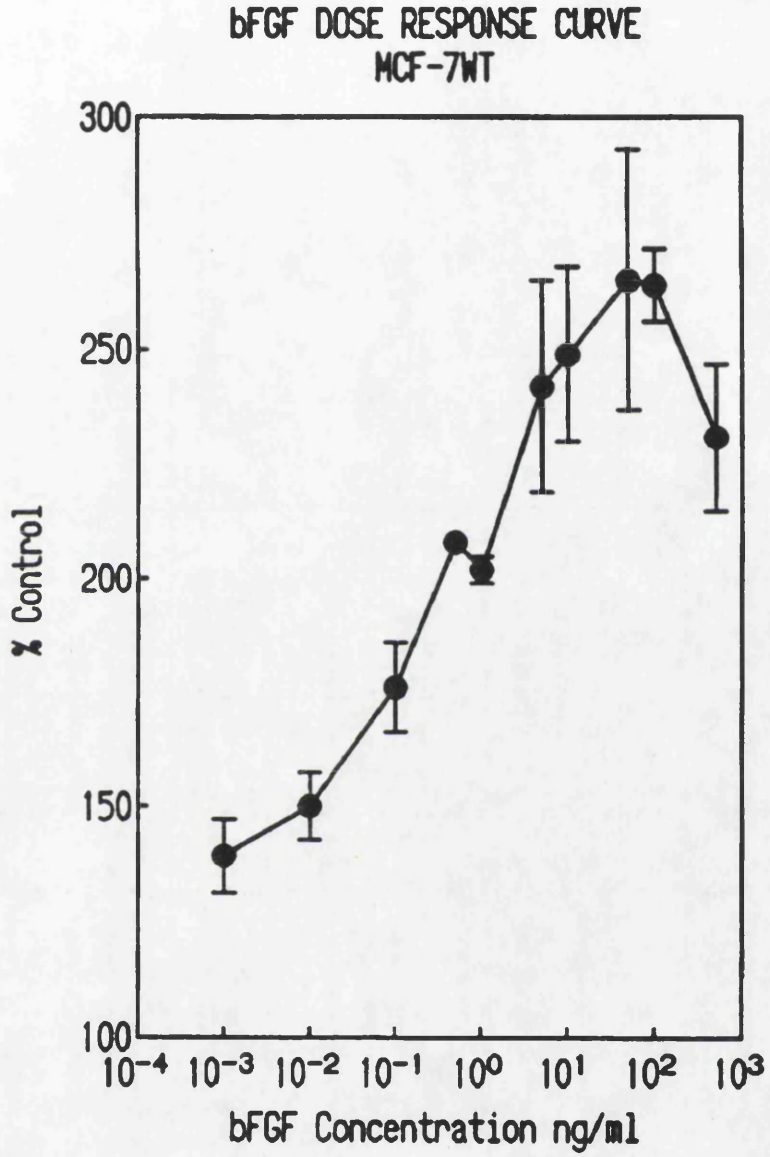
One representative series of experiments which were carried out on two occasions.

Figure 4-13 Growth Response to E_2 + Insulin and E_2 + IGF- I.



Growth response in MCF- 7WT cells to E_2 10^{-9}M and suboptimal concentrations of insulin (Ins) and IGF-I. Each bar represents the mean of 3 samples as measured by a 3 day MTT assay and represented as a percentage of control values. Vertical bars represent one S.D

Figure 4-14 bFGF Induced Dose Response Curve (MCF 7WT cells)



Growth response to bFGF measured using the MTT assay. Each point is the mean of three samples and is expressed as a percentage of control values. Vertical bars represent one S.D.

One representative experiment from a series of two.

4.3 DISCUSSION

The main theme of this chapter has been the growth effect of a range of growth factors on each of the four breast cancer cell lines. In general, the two ER positive cell lines, MCF-7WT and ZR-75-1 are growth stimulated by each of the growth factors tested, whilst the two ER negative cell lines, MCF-7Adr and MDA-MB-231 show only marginal growth effects. This would tend to suggest major changes in cellular growth regulation have occurred during the process of loosing a functional ER.

The relationship between ER and EGF receptor expression in breast cancer has been examined in a number of cell lines and it has been repeatedly found that expression of the EGF receptor increases as the ER expression decreases (Davidson *et al.*,1987). The increased levels of EGF receptor in the ER negative cell lines were generally found to be due to increased levels of gene transcription and not amplification of the gene itself.

During development of the MCF-7Adr cell phenotype there was loss of the ER and an increase in the level of EGF receptor expression, associated with the development of multidrug-resistance (Vickers *et al.*,1988). A study carried out in variant phenotypes of the ZR-75-1 cell line showed a pattern of EGF receptor expression in close agreement with that seen in the MCF-7WT and MCF-7Adr cell lines. Long *et al,* (1992), found that a tamoxifen resistant variant of the ZR-75-1 cell line showing loss of ER also showed an increase in the level of EGF receptor, when the resistance was reversed there was a fall in EGF receptor levels.

The inability of breast cancer cells to respond to EGF stimulation *in vitro*

has repeatedly been found to be a characteristic of cell lines known to express high levels of the EGF receptor (Davidson *et al.*,1987) and is also dependent on the culture conditions of the cells (*Nelson *et al.*, 1990). The MCF-7WT cell line response to EGF has been found to be stimulatory (* Osborne *et al.*, 1980), and inhibitory (*Imai *et al.*, 1982), suggesting the response to EGF can vary with cell strain and method of treatment. Dong *et al,* (1991), suggest cells expressing high levels of EGF receptor have dual affinity binding sites (high and low affinity) and upon occupation of the low affinity sites the response to EGF is switched off. In the A431 cell line the level of activated receptor was found to associate with a particular response; growth stimulation associated with intermediate levels of receptor activation and growth inhibition with high levels of receptor activation (Kawamoto *et al.*,1984). These observations tend to suggest that in the MCF-7Adr line and the MDA-MB-231 line, addition of exogenous EGF is reaching a level of EGF receptor occupation associated with loss of response. Involvement of autocrine TGF-α pathways could also play a significant role in this process, with the majority of high affinity receptors already activated by endogenously produced TGF-α. Levels of TGF-α mRNA expression were found to be high in the MDA-MB-231 breast cell line, higher than levels found in E_2 induced MCF-7 ER positive cells (Bates *et al.*,1988). A separate ER negative breast cancer cell line MDA-468 was found to be growth inhibited by anti-EGF receptor antibody, suggesting the cells growth is under autocrine control through production of TGF-α (Ennis *et al.*,1989).

Whether a similar process can account for the reduced growth response seen in the two ER negative cell lines to insulin and the IGFs is unknown, no such studies have been carried out with regard to the insulin or IGF-I receptors.

(* references to be found at back of reference section)

In the ER positive lines, particularly MCF-7WT, insulin, IGF-I and IGF-II are all able to substantially increase the rate of proliferation. They each show different affinities for the receptor involved, with IGF-I showing the greatest affinity followed by IGF-II and insulin with the relative affinities of 1:2:100 respectively, this is in close agreement with other published work looking at the relative affinities of each of these factors for the IGF-I receptor (Rechlar *et al.*,1980). From these results it appears the growth response to IGF-I, IGF-II and insulin is due to activation of the IGF-I receptor and the capability of the αIR-3 antibody to abrogate this response would tend to support this theory. Since the antibody is unable to completely inhibit insulin regulated growth, it cannot be ruled out that some of the growth response to insulin is due to the insulin receptor.

A tumour cell growing *in situ,* is likely to receive a complex mixture of signals, reaching the cell through autocrine, paracrine and endocrine routes. It is unlikely each of the hormones and growth factors are working in isolation on the cell and there is a strong possibility of synergy between factors or modulation of one response by another. A synergistic growth effect between insulin and E_2 on the proliferation of MCF-7 cells in a growth factor defined environment was proposed by van der Burg *et al,* (1988). Using the MTT assay, no such synergy was seen between E_2 and suboptimal concentrations of IGF-I or insulin, although in one case an additive response was apparent. The different responses may have been due to a different strain of MCF-7WT cell line or a more likely explanation would be the presence of enough growth factor in the CSS to cause the cells to already show maximum E_2 response i.e. they are already synergised with a low level of IGF present in the stripped serum. Work by van der Burg was carried

out in a CSS further chemically treated to inactivate all growth factors. Both insulin (IGF-I, IGF-II) and E_2 are required by the ER positive cell lines to maximise their rate of proliferation, whilst EGF is only able to further increase the growth rate in the ZR-75-1 cell line. Insulin has a small effect on the growth of the ER negative cell lines.

4.4 SUMMARY AND CONCLUSIONS

ER negative cells *in vitro* show higher rates of growth in steroid reduced culture conditions, higher levels of EGF receptor expression and none or very low levels of growth response to exogenously applied growth factors. The ER positive cell line MCF-7WT shows very high levels of growth response to a range of growth factors including; EGF, insulin, IGF-I, IGF-II and bFGF. When applied together, insulin and EGF or insulin and E_2 produce only additive effects in the growth of the MCF-7WT cell line.

CHAPTER 5

TYROSINE PHOSPHORYLATION IN BREAST CANCER CELL LINES

CHAPTER 5

TYROSINE PHOSPHORYLATION IN BREAST CANCER CELL LINES

5.1 INTRODUCTION

Breast cancer cell lines were compared for levels of tyrosine phosphorylation. Interestingly, quantitative and qualitative differences between the two MCF-7 cell lines were observed which led to a more detailed study. Both the MCF-7 lines were examined for tyrosine phosphorylation after stimulation by a range of growth factors. Differences in phosphotyrosine patterns were in response to growth factor stimulation were apparent between the cell lines but there was also a number of shared responses. Many of the phosphorylated substrate proteins were common to a range of different growth factors and some evidence toward their identification has also been presented.

5.1.1 Background

Proliferation of MCF-7WT cells in response to growth factors is in part regulated by stimulation of specific tyrosine kinase linked receptors (reviewed in 1.4). Ligand binding to the receptor results in an increase of receptor tyrosine kinase activity causing receptor phosphorylation and phosphorylation of a number of substrate proteins. These patterns of tyrosine phosphorylation represent an early part of the signal transduction process which eventually leads to cellular response.

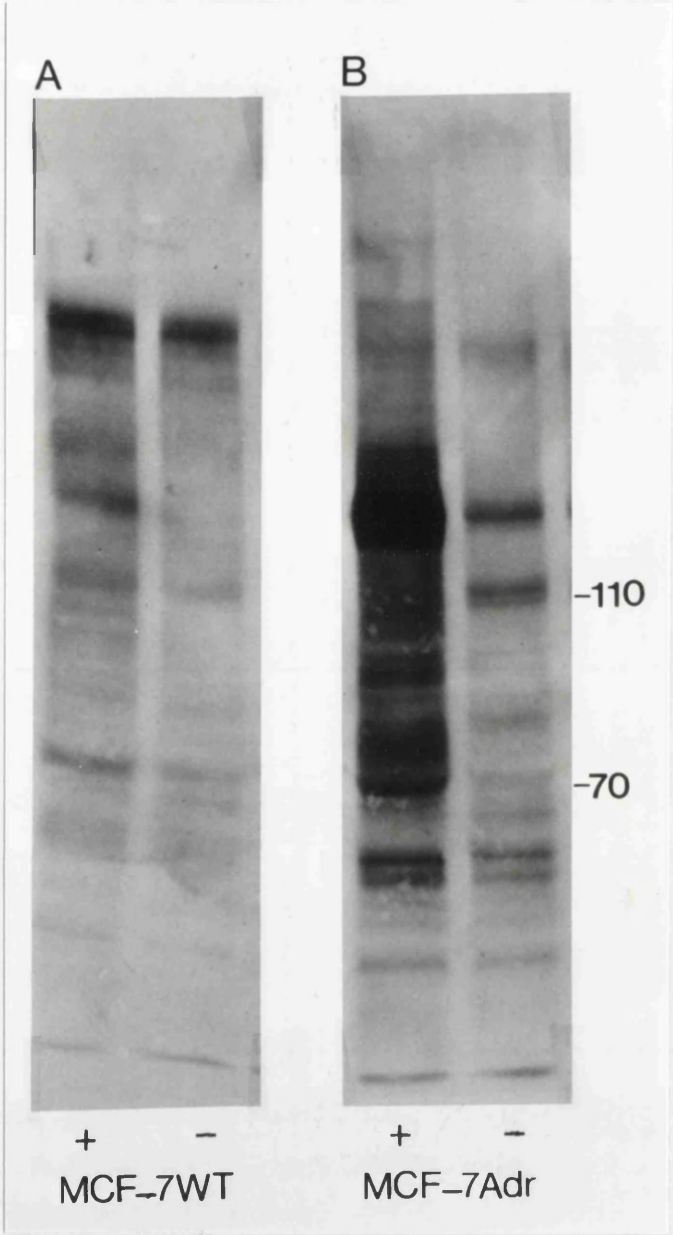
Tyrosine phosphorylation has been examined in a number of unstimulated breast cancer cell lines and in the two MCF-7 cell lines after growth factor

stimulation. Western analysis was employed to examine tyrosine phosphorylation in the four breast cancer cell lines. Cell lysates were prepared and run out on SDS Page gels then transferred and immunoblotted with an anti-phosphotyrosine antibody (methods 2.9). To examine the response to growth factors of MCF-7WT cells in greater detail, patterns of phosphotyrosine activity upon growth factor activation were examined. These were compared to MCF-7Adr cells treated under the same conditions, since it is a drug resistant clone of MCF-7WT which shows very different growth responses to exogenously applied growth factors (see chapter 4). Cells were treated under similar experimental conditions in the phosphotyrosine assay as the MTT assay so that a direct comparison could be made between phosphotyrosine activity and cell growth. Cells were plated into flasks at a low density and held in basal growth medium RPMI(prf) + 2.5% HT-CSS over a period of 48Hrs to reduce background growth to a minimum and thus phosphotyrosine levels.

5.1.2 Assay Methodology

To maintain *in situ* levels of phosphorylated tyrosine, the phosphatase inhibitor, sodium orthovanadate was immediately added to cell lysates at a concentration of 100 μM. Figure 5-1, demonstrates the effect of adding sodium orthovanadate to the cell lysates. It is clear that both the MCF-7WT and MCF-7Adr cell line show substantially reduced phosphotyrosine levels in the absence of phosphatase inhibitor, even in the short time scale of this experiment. Cells were harvested and run out on a gel then immunoblotted within a 24Hr period. The extent of tyrosine phosphorylation in the presence of phosphatase inhibitor is

Figure 5-1 Effect of Sodium Orthovanadate on Phosphotyrosine Levels



Western blot analysis (6% gel) of cell lysates (50 μg cellular protein per lane) probed withphosphotyrosine antibody. Gel A shows MCF-7WT cellular protein in presence of sodium orthovanadate (+) and the absence of sodium orthovanadate (-). Gel B shows MCF-7Adr cellular protein again in the presence (+) or absence (-) of sodium orthovanadate. Sodium orthovanadate added to fresh cell lysates at 0.1 mM.

Figures on the RHS represent MW markers.

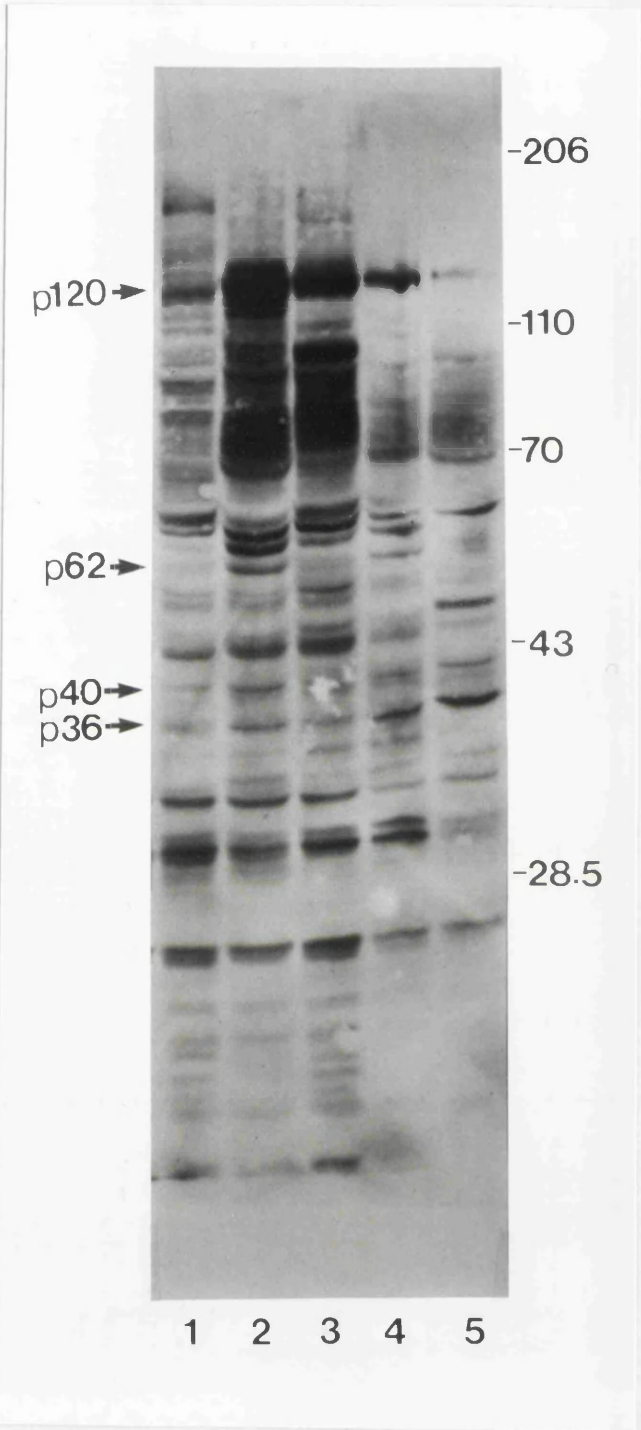
equal to the normal basal rate of phophorylation in the cell at any single point in time. In contrast, the level of phosphotyrosine activity upon mitogen activation has been measured as a cumulative amount over a 30 minute period, both mitogen and phosphatase inhibitor were added to the cells for 30 minutes before lysis. By treating controls with phosphatase inhibitor alone for an equal period of time, differences between phosphotyrosine levels in the controls and mitogen activated cells will represent tyrosine phosphorylation of signal pathways associated with the activated receptor.

5.2 RESULTS

5.2.1 Patterns of Phosphotyrosine Expression in Breast Cancer Cell Lines

Tyrosine phosphorylation was examined in each of the breast cancer cell lines and a breast fibroblast line (Fig. 5-2). Clearly each of the cell lines express quite different levels of tyrosine phosphorylation even when grown under the same conditions but the proteins involved follow a similar pattern in each cell line. The phosphorylated protein at 120kDa has been highlighted (p120) as a protein of particular interest because it is highly phosphorylated in the MCF-7Adr cell line in comparison with the MCF-7WT line (lanes 1 & 2) and work specifically looking at this protein is presented later in the chapter. A number of other proteins which are differentially phosphorylated in the two MCF-7 cell lines have also been highlighted (p62, p40 & p36) and these will be referred to in the discussion (5.3). The main interest in these two lines is that they are closely

Figure 5-2 Tyrosine Phosphorylation in Breast Cancer Cell Lines



Tyrosine phosphorylation in four breast cancer cell lines and a breast fibroblast line, shown using western blot analysis (6% gel) probed with phosphotyrosine antibody. MCF-7WT (lane 1), MCF-7Adr (lane 2), ZR-75-1 (lane 3), MDA-MB-231 (lane 4) and BF10 (lane 5). MW markers shown on right hand side of blot.

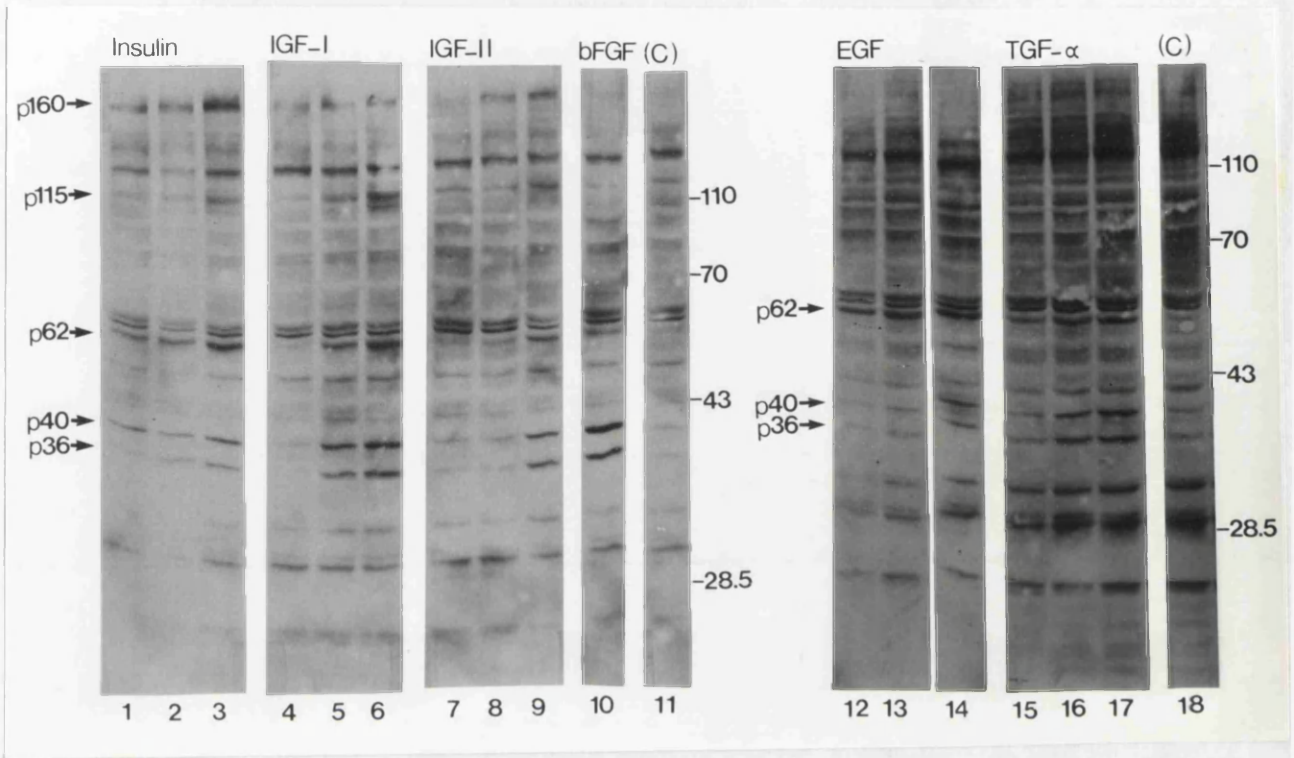
Blot is representative of four independent experiments.

related but show very different growth characteristics. Under basal growth conditions MCF-7WT cells show no growth unless stimulated by exogenous mitogens, whilst the MCF-7Adr cells continue to grow under basal conditions and are unaffected by the addition of exogenous growth factors. Since the MCF-7Adr line is derived from the MCF-7WT parent line, changes in the pattern of tyrosine phosphorylation may be able to tell us something of the changes within the cell which allow unregulated cell growth to occur.

5.2.2 Growth Factor Induced Phosphotyrosine Activity

Growth factor activated MCF-7WT cells were examined for phosphotyrosine activity (Fig 5-3). This cell line makes a very suitable model for examining changes in the level of phosphorylation since it expresses a relatively low background level of tyrosine phosphorylation and it exhibits a strong growth response to each of the factors tested. Each growth factor was examined at three concentrations roughly correlating with low, medium and high levels of mitogenic activity. Insulin was examined at 1,10 & 100 μg/ml, IGF-I and IGF-II at 1,10 & 100ng/ml, EGF at 1,10 & 100ng/ml, TGF-α at 1,10 & 80ng/ml and bFGF at 100ng/ml. bFGF was examined at only a single concentration but figure 5-4, lane 9 shows tyrosine phosphorylation in MCF-7WT cells after stimulation with a 10 fold lower concentration of bFGF (10ng/ml) and it also appears to show concentration dependent tyrosine phosphorylation. Insulin, IGF-I and IGF-II all show dose responsive increases in phosphorylation of a number of proteins marked p160, p115, p62, p40 and p36. The proteins at 160kDa and 115kDa marked p160 and p115 appear to be specific for this set of growth factors and are

Figure 5-3 Tyrosine Phosphorylation following Growth Factor Stimulation



Western blot (10%) gel showing tyrosine phosphorylation in MCF-7WT cells upon growth factor stimulation. Lanes are arranged as follows:
(1) Insulin 1 μg/ml , (2) Insulin 10 μg/ml , (3) Insulin 100 μg/ml ,
(4) IGF-I 1 ng/ml , (5) IGF-I 10 ng/ml , (6) IGF-I 100 ng/ml ,
(7) IGF-II 1 ng/ml , (8) IGF-II 10 ng/ml , (9) IGF-II 100 ng/ml ,
(10) bFGF 100 ng/ml , (11) Control , (12) EGF 1 ng/ml ,
(13) EGF 10 ng/ml , (14) EGF 100 ng/ml , (15) TGF-α 1 ng/ml ,
(16) TGF-α 10 ng/ml , (17) TGF-α 80 ng/ml , (18) Control.
MW markers are shown on the right hand side of each blot.

On each occasion cells were grown under conditions outlined in Chapter 2 (2.9.2). Growth factor + sodium orthovanadate were added for 30 mins before cellular protein was solubilized. Control lanes contain protein from cells treated for 30 mins with sodium orthovanadate alone. 50 μg cellular protein run out per lane.

not seen under bFGF, EGF or TGF-α stimulation, whereas 62kDa, 40kDa and 36kDa proteins marked p62, p40 and p36 show increased tyrosine phosphorylation when the cells are stimulated by any of the growth factors tested.

The insulin like growth factors IGF-I and IGF-II were compared for their abilites to stimulate tyrosine phosphorylation. IGF-I increased phosphorylation of p115, p62, p40 and p36 at a 10 fold lower molar concentration than IGF-II correlating with growth response (Fig.5-3). Insulin required a thousand fold higher molar concentration to stimulate tyrosine phosphorylation to the same extent as IGF-I. This may reflect the increased affinity of IGF-I to the particular receptor which is suspected to be the IGF-I receptor.

Similar dose response relationships occur with EGF and TGF-α which are known to activate the EGF receptor. The substrate proteins p62, p40 and p36 are all phosphorylated in response to TGF-α and EGF in a concentration dependent manner and it appears these particular proteins are common to the signal transduction pathways of each growth factor. The autoradiograph in Figure 5-3 gives no indication of phosphorylated EGF receptor which would be expected, since downstream tyrosine phosphorylation events can clearly be seen. This may simply reflect the low EGF receptor expression in this cell line which was unable to be detected using an EGF receptor antibody (Fig.4-2).

5.2.3 Mitogen Induced Phosphotyrosine Activity

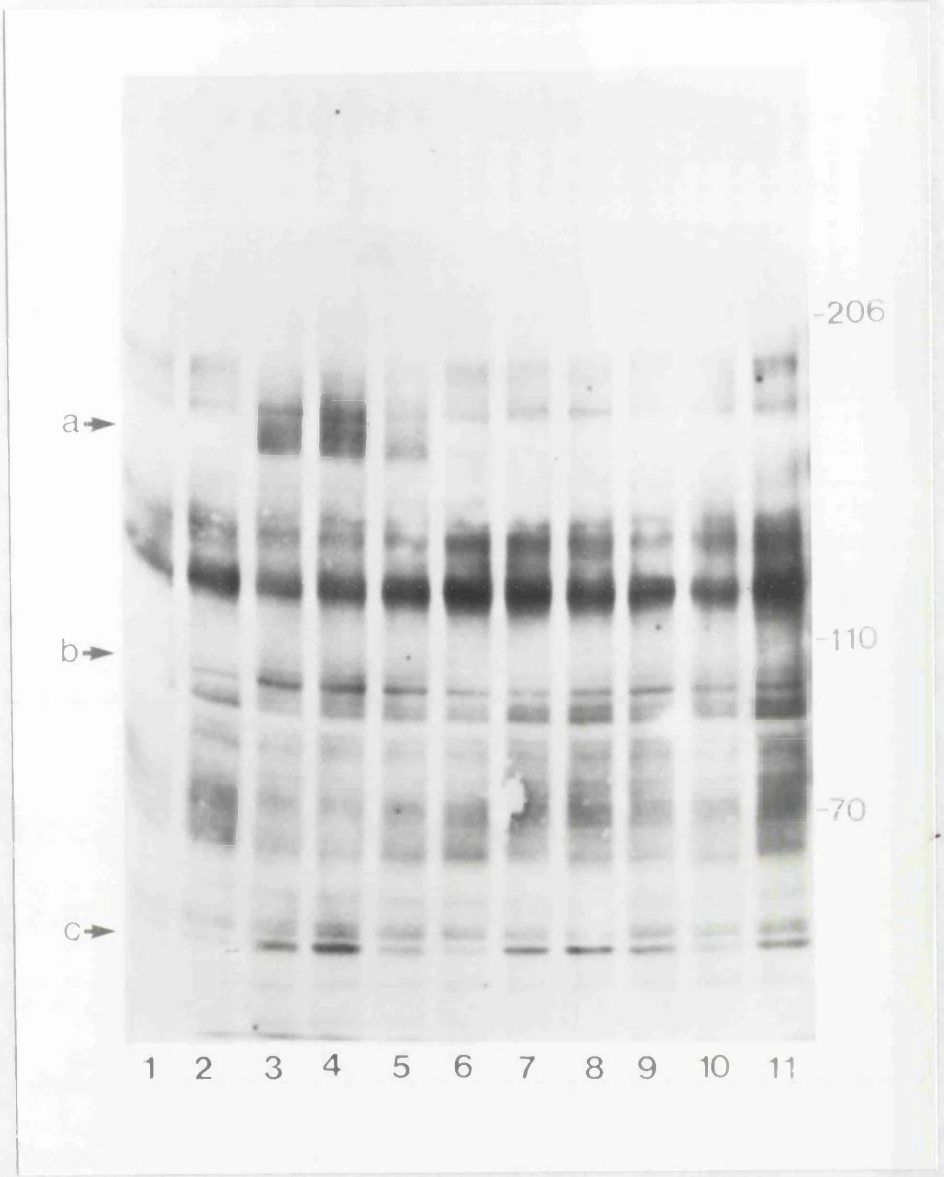
MCF-7WT cells were stimulated with a range of different mitogens and cellular proteins were western blotted for phosphotyrosine activity (Fig.5-4). This followed the same protocol as shown in Figure 5-3 and a number of the same

mitogenic conditions have been examined. Specific tyrosine phosphorylation has occurred in the case of insulin and IGF-I as in the phosphoprotein marked 'a' at 160kDa and may represent a specific phosphorylation event to the IGF-I receptor. The phosphoproteins marked 'b' and 'c' at approximate sizes of 100 and 56kDa respectively, appear to be in a higher phosphorylation state under a number of different growth factor conditions (lanes 3,4,7,8,9,& 11) which include insulin, EGF, TGF-α, and fibroblast conditioned medium. These bands do not show specificity to particular growth factors and probably represent phosphorylation events downstream of the receptors in the signal transduction pathway.

In lanes 2 and 10 of Figure 5-4, the effect of E_2 on the tyrosine kinase activity of the MCF-7WT cell line has been examined. Since it was proposed that E_2 partly regulates growth of MCF-7WT cells through increased synthesis of mitogenic growth factors which act upon the cell in an autocrine manner (see section, 1.2.2), it was thought such an autocrine stimulus should, if present, be detected as an increase in the level of tyrosine kinase phosphorylation. From the autoradiograph (Fig.5-4), it is clear no increase in the levels of phosphoproteins a, b or c are evident. At these two time points, E_2 causes no increase in the level of cellular tyrosine kinase phosphorylation. From similar experiments by Reddy *et al.*,(1992), no EGF receptor phosphorylation was evident after E_2 stimulation in the MCF-7 cell line but it was found in the ER positive breast cancer cell line T47D after a 16Hr exposure to E_2. The author suggested EGF receptor levels in the MCF-7 cell line were too low for detecting receptor phosphorylation.

It is interesting that conditioned medium from breast tumour derived fibroblast cultures shown in lane 11 also increases the level of cellular

Figure 5-4 Tyrosine Phosphorylation: Response to Mitogens



Tyrosine phosphorylation in MCF-7WT cells after mitogenic stimulation. Lanes run as follows: (1) Control , (2) E_2 10^{-9}M (30 mins) , (3) E_2+Ins 10 μg/ml , (4) Insulin , (5) IGF-1 10 ng/ml , (6) IGF-11 10 ng/ml , (7) EGF 10 ng/ml , (8) TGF-α 10 ng/ml , (9) bFGF 10 ng/ml , (10) E_2 (1 Hr) , (11) CM.

MW markers shown on right hand side of blot.

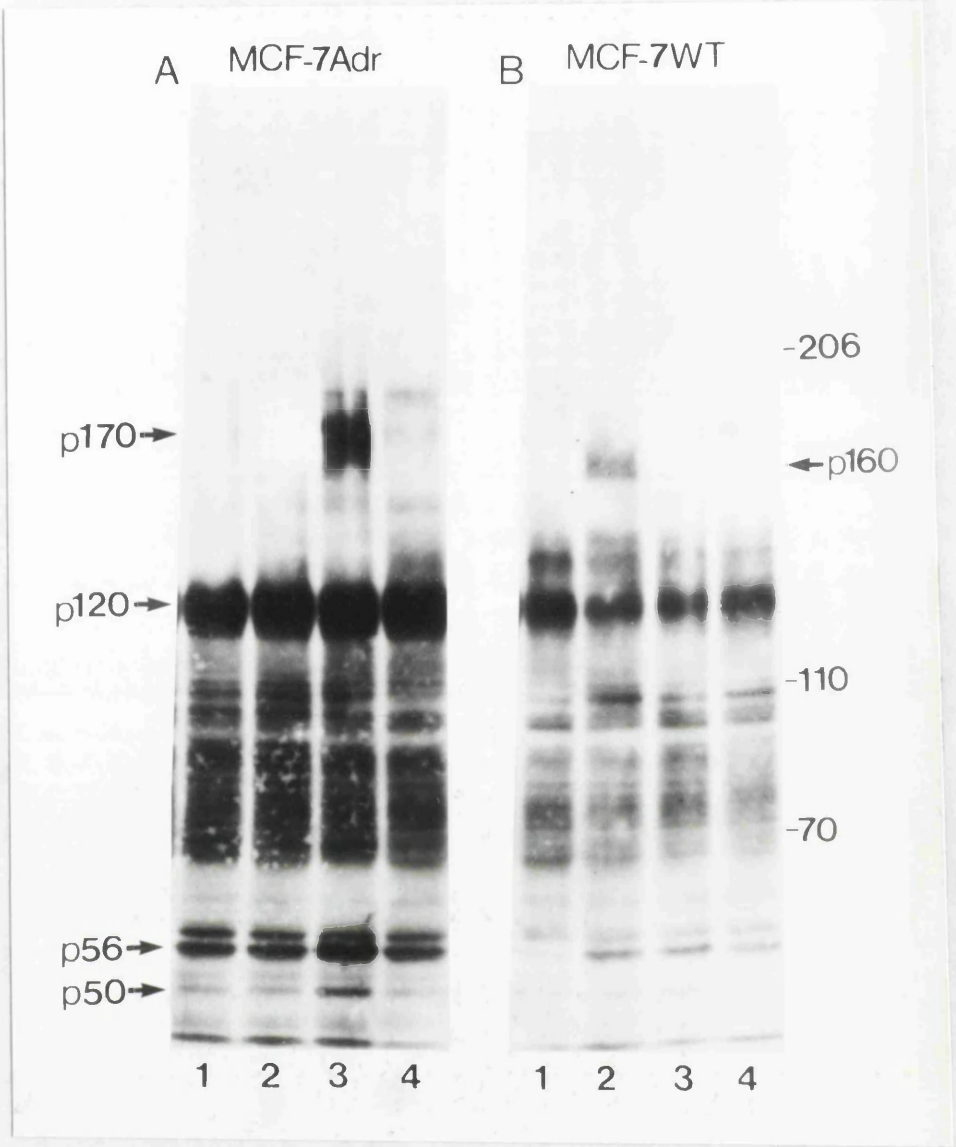
On each occasion cells were grown under conditions outlined in Chapter 2 (2.9.2). Growth factor + sodium orthovanadate were added for 30 mins before cellular protein was solubilized. Control lanes contain protein from cells treated for 30 mins with sodium orthovanadate alone. 50 μg cellular protein run out per lane. Lanes (2) and (10) show cells treated with E_2 10^{-9}M at 30mins and 1Hr, lane (11) show cells treated with fibroblast CM for 30mins.

phosphotyrosine correlating with growth response in this cell line (described in Chapter 6). This suggests that the conditioned medium may well contain a ligand or ligands to tyrosine kinase receptor proteins found in MCF-7WT cells.

5.2.4 Comparison of MCF-7WT & MCF-7Adr Cells Under Mitogen Stimulation

Because of the results presented in section 5.2.2 with the MCF-7WT cell line it was decided to carry out the same experiment in the MCF-7Adr line which show quite different growth characteristics to the parent line under mitogen stimulation. Each of the growth factors insulin, EGF and bFGF are known to work through different tyrosine kinase receptors IGF-I, EGF and FGF receptors respectively (reveiwed in sections 1.4.1 to 1.4.8). In the MCF-7Adr line the only clear increase in phosphorylation occurs upon EGF stimulation (Fig. 5-5A, lane 3). The band occurring at 170kDa and marked p170 on lane 3 almost certainly represents phosphorylation of the EGF receptor itself which as we have already seen in Figure 4-2 is highly expressed in this cell line. Increased phosphorylation of two bands marked p56 and p50 also appear in lane 3 and may represent phosphorylation of substrate proteins of the EGF receptor. The MCF-7WT cell line shows a slightly different pattern of increased tyrosine phosphorylation. All three growth factors increase the tyrosine phosphorylation of the p56 band which is also present in MCF-7Adr cells upon EGF stimulation, but there is no detectable phosphorylation of the p170 protein in MCF-7WT (Fig.5-5B lane 3) even though EGF is known to be mitogenic to these cells. The p50 phosphoprotein in the MCF-7WT cell line is only just detectable and it is

Figure 5-5 Mitogen Response Comparing MCF-7Adr and MCF-7WT Cells



Tyrosine phosphorylation in, MCF-7Adr cells (A), and MCF-7WT cells (B), after mitogen stimulation. Lane (1) Control, (2) Insulin 10 μg/ml, (3) EGF 10 ng/ml, (4) bFGF 10ng/ml. Western analysis using 6% gel, MW markers shown on right hand side of blot.

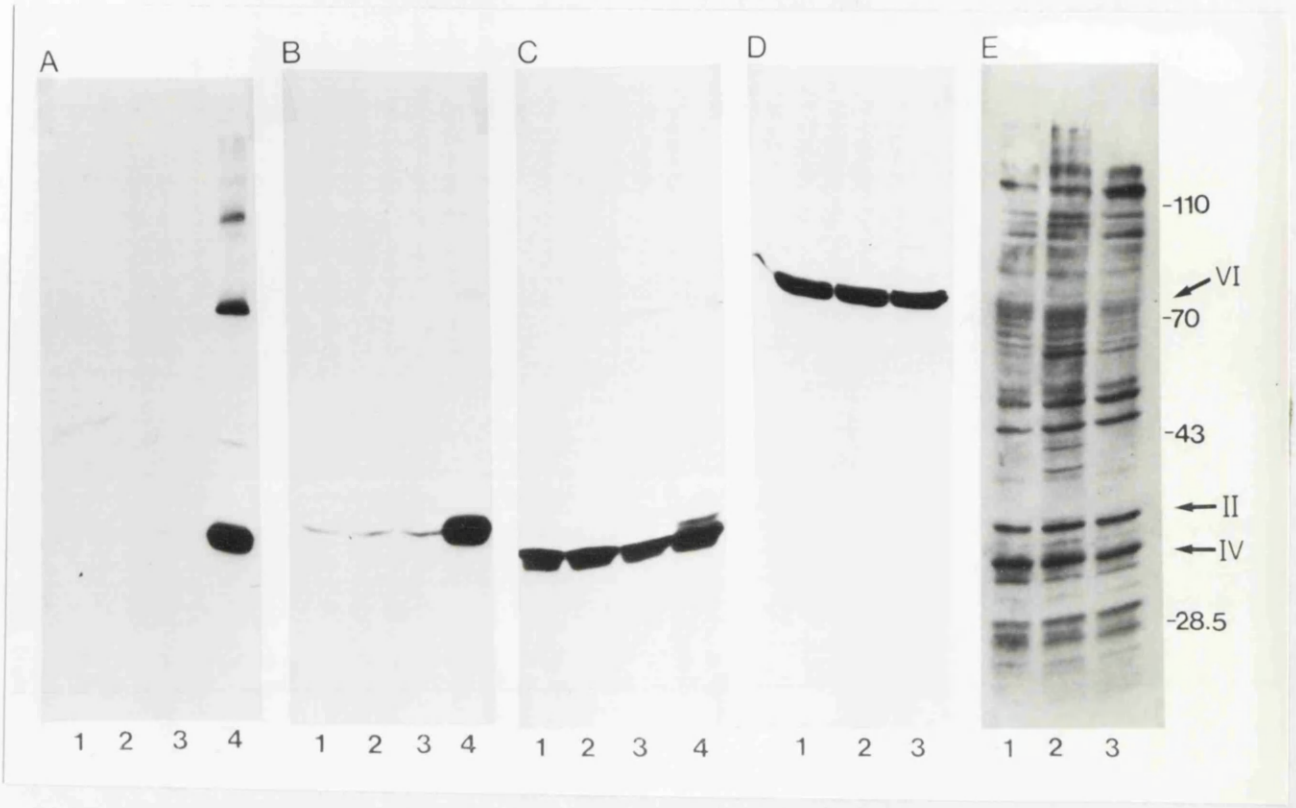
Growth factor + sodium orthovanadate added for 30 mins before protein solubilization. 50 μg of cellular protein ran out per lane.

impossible to see any stimulation between the different growth factor conditions. Insulin stimulates tyrosine phosphorylation of a protein p160 (lane 2) which may relate to the IGF-I receptor, this is not apparent on the MCF-7Adr line. These differences may simply represent the number of receptors present on the cells with low receptor levels showing phosphotyrosine activity below the level of assay sensitivity.

5.2.5 Identification of Proteins Phosphorylated on Tyrosine Residues

Phosphorylation events downstream of receptor activity may be of importance in signal transduction. By examining a number of candidate proteins for these downstream events it was hoped that some of the phosphotyrosine bands could be identified. The family of annexin proteins were chosen for examination since they are known to be involved in signal transduction (Ross *et al.*,1990), and have been identified previously as substrate proteins for the EGF receptor (Pepinsky & Sinclair, 1986). They are of a size corresponding to tyrosine phosphorylation events downstream of the receptor. Four annexins were selected as candidate proteins; annexin I, II, IV and VI. Both MCF-7WT and MCF-7Adr were immunoblotted for expression of these proteins. MCF-7Adr was found to express all four annexin proteins at high levels (Fig.5-6[ii]), whilst the MCF-7WT cell line (Fig.5-6[i]) did not express detectable levels of annexin I, and expressed low levels of annexin II, both annexins IV and VI were highly expressed. Annexins I,II, IV and VI were detected at 40, 39, 36 and 76kDa respectively and after reprobing the filters with phosphotyrosine antibody approximate positions

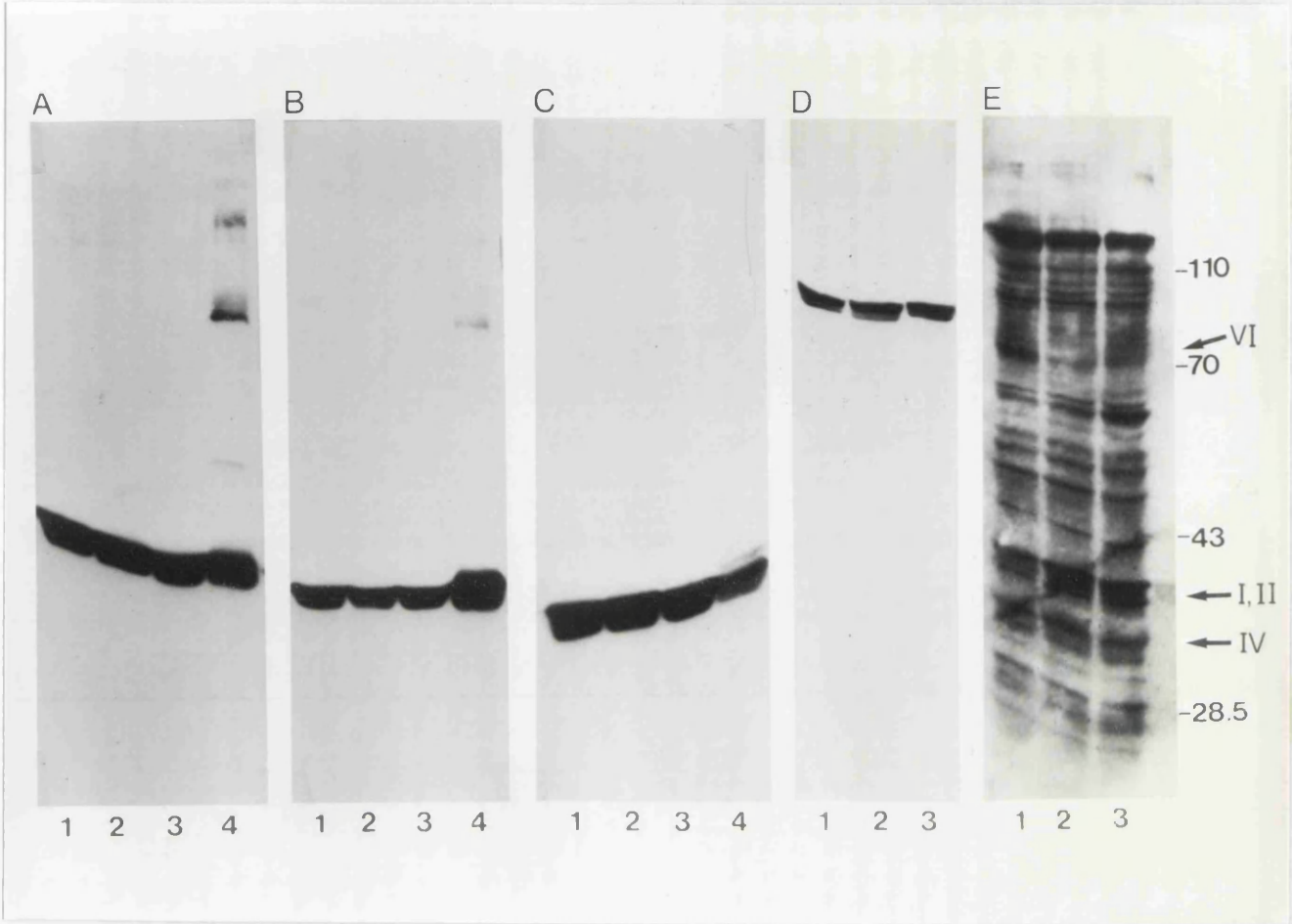
Figure 5-6 (i) Presence of Annexins in MCF-7WT Cells



Western blots (10% gel) probed for: Annexin I (A), Annexin II (B), Annexin IV (C), Annexin VI (D), and phosphotyrosine (E).

In each case Lane (1) is MCF-7WT control, (2) MCF-7WT stimulated with insulin 100 μg/ml, (3) MCF-7WT stimulated with EGF 10 μg/ml, (4) annexin protein standard (Affiniti). Autoradiograph E is C after stripping and reprobing with anti-phosphotyrosine. MW markers are shown on the right hand side of the gels.

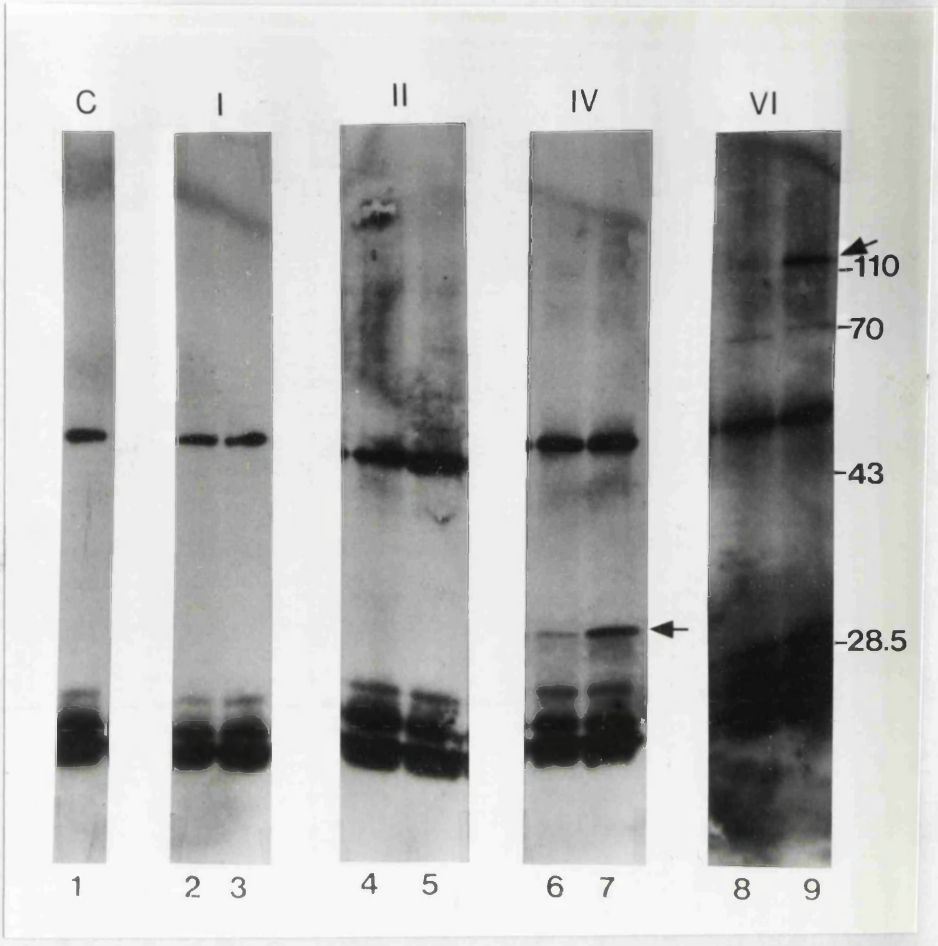
Figure 5-6 (ii) Presence of Annexins in MCF-7Adr Cells



Western blots (10% gel) probed for : Annexin I (A), Annexin II (B), Annexin IV (C), Annexin VI (D), and phosphtyrosine (E).

In each case Lane (1) is MCF-7Adr control, (2) MCF-7Adr stimulated with insulin 100μg/ml, (30 MCF-7Adr stimulated with EGGF 10μg/ml, (4) annexin protein standard (Affiniti). Autoradiograph E is A after stripping and reprobing with anti-phosphotyrosine. MW markers are shown on the right hand side of the gels.

Figure 5-7 Annexins Present in Phosphotyrosine Immunoprecipitation



Phosphotyrosine immunoprecipitations probed with annexin antibodies. Lane (1) - control preparation containing no cellular protein showing banding pattern of phosphotyrosineantibody.

All other lanes show MCF-7WT and MCF-7Adr phosphotyrosine immunoprecipitates. Lanes (2) and (3) probed for annexin I. Lanes (4) and (5) probed for annexin II. Lanes (6) and (7) probed for annexin IV. Lanes (8) and (9) probed for annexin VI. MW markers shown on right hand side of blot.

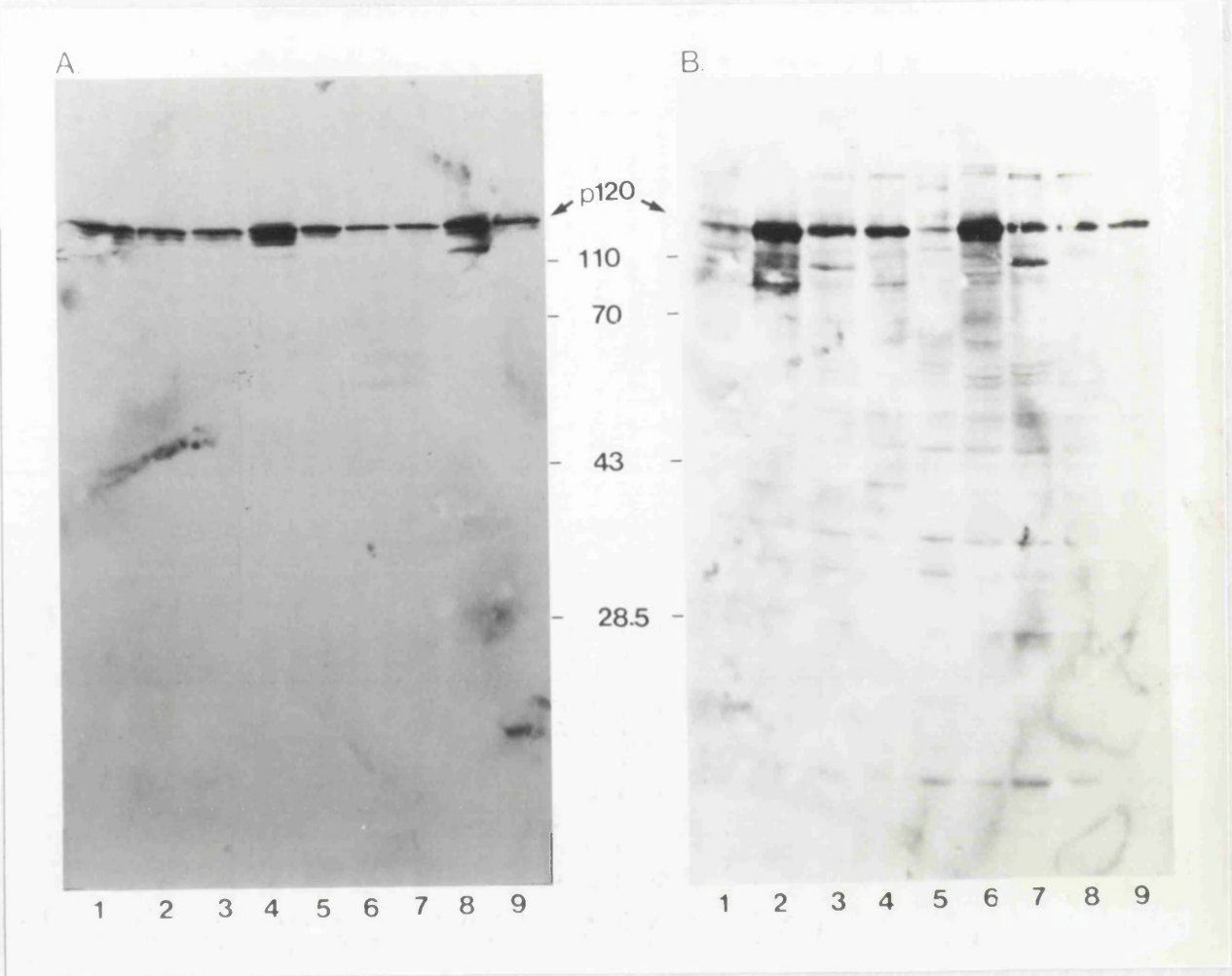
Lanes 2,4,6 & 8 represent MCF-7WT immunoprecipitates. Lanes 3,5,7 & 9 represent MCF-7Adr immunoprecipitates.

of phosphotyrosines relating to possible phosphorylated annexins were identified (Fig.5-6[ii],E). Phosphotyrosine immunoprecipitations were performed to verify which of the annexins were phosphorylated. After immunoprecipitating cell lysates with the phosphotyrosine antibody and resolving the proteins by SDS PAGE, western blotting was carried out and probed for each of the different annexins. Only annexins IV and VI were identified in the immunoprecipitates (Fig. 5-7). In each case the MCF-7Adr line showed a higher expression of phosphorylated annexins IV and VI.

5.2.6 GAP Expression in MCF-7 Cell Lines

Returning to the differentials between the two MCF-7cell lines it is clear the annexins vary in their expression and extent of phosphorylation. Of particular interest is the very strong band of phosphotyrosine expression seen at 120kDa, in the MCF-7Adr line, this has been highlighted in Figures 5-2 and 5-5. Clearly there is differential phosphorylation of this protein between the two MCF-7 cell lines which is not dependent upon exogenous stimuli. In Figure 5-8A a range of breast cell lines were probed for the GTPase activating protein, GAP, each was found to express the protein at an approximate size of 120kDa. The same filter was then stripped and reprobed with the phosphotyrosine antibody and a band occurred at 120kDa (Fig. 5-8B). To directly compare GAP expression (A) with phosphotyrosine expression (B) in each of the breast cell lines the two have been displayed in parallel in Figure 5-9. Lanes 1 and 5 both show expression levels in the MCF-7WT cell line and lanes 2 and 6 expression levels in the MCF-7Adr line. Looking at A, it is clear GAP expression is very similar between the two cell

Figure 5-8 Expression of GAP in Breast Cancer Cell Lines

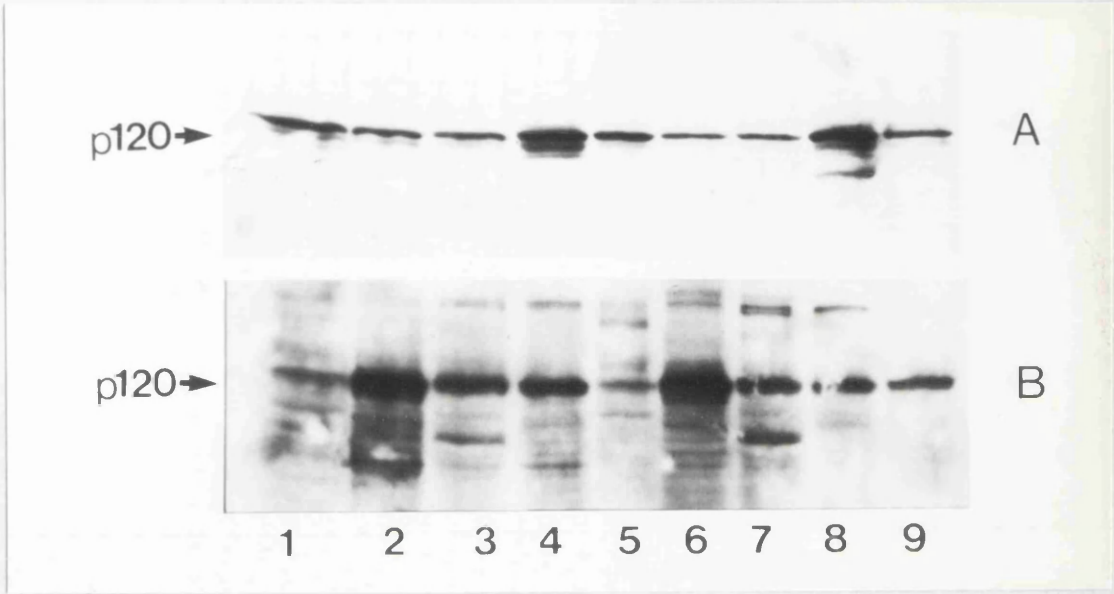


A. Western gel of breast cancer cell lysates probed with GAP antibody.

B. Same gel after stripping and reprobing with phosphtyrosine antibody.

Lane (1) shows MCF-7WT, (2) MCF-7Adr, (3) ZR-75-1,(4) MDA-MB-231.
Cells on lanes (1) to (4) grown in RPMI (pvf) + 2.5% CSS.
Lane (5) MCF-7WT, (6) MCF-7Adr, (7) ZR-75-1, (8) MDA-MB-231,
(9) BF10.
Cells on lanes 5 to 9 grown in RPMI + 5% FCS.

Figure 5-9 Comparison of p120 Probed for GAP and Phosphotyrosine



Gels A and B as in Figure 5-8 shown in parallel. Gel A probed for GAP protein, Gel B probed for phosphtyrosine.

lines and if anything may be slightly higher in the MCF-7WT line, when lanes 5 & 6 are compared. Looking at B, the phosphotyrosine expression is quite different, MCF-7Adr shows a much higher level of phosphotyrosine expression at 120kDa. There is the suggestion that GAP may be in a higher phosphorylated state in the MCF-7Adr line under unstimulated culture conditions, than its parent MCF-7WT line, although the work done so far is not able to conclusively prove this. To examine whether the increased tyrosine phosphorylation of the p120 protein is actually GAP, it would be necessary to immunoprecipitate cellular proteins with the GAP antibody then probe them with the phosphotyrosine antibody to lok for differences in the level of tyrosine phosphorylation.

5.3 DISCUSSION

The examination of mechanisms of signal transduction induced by growth factors is likely to prove useful to our understanding of why cancer cells are able to proliferate in an unrestrained manner. The two MCF-7 cell lines make an excellent choice for the study of growth control as a cellular model for malignant progression. Evidence suggests that growth factor binding allows receptor autophosphorylation on tyrosine residues which causes a conformational change enhancing kinase activity toward other substrates. Mutated receptors which enhance ligand independent autophosphorylation have been described by Yarden & Ullrich,(1988[b]), these allow constitutive protein-tyrosine kinase activity in the absence of ligand. Following patterns of phosphotyrosine expression in the two MCF-7 cell lines in both growth factor stimulated and unstimulated conditions may identify whether tyrosine kinase activity is an important factor in the

unregulated growth of MCF-7 cell lines.

When all the breast cancer cell lines were examined for phosphotyrosine content under normal growth conditions, levels varied considerably between cell lines. MCF-7Adr and ZR-75-1 cells had much higher expression levels than the MCF-7WT or MDA-MB-231 cells. Comparison of the paired MCF-7 cells showed there to be large differences in tyrosine phosphorylation common to a range of soluble proteins, particularly in the molecular weight range 60 to 120kDa.

One protein in particular with an approximate size of 120kDa shows a striking differential in tyrosine phosphorylation between the MCF-7Adr line and the MCF-7WT line. From its size a guess was made at its identity and the role it may play in growth regulation. The GTPase-activating protein (GAP) has a molecular mass of 120kDa and has been shown to phosphorylate on tyrosine in cells transformed by cytoplasmic and receptor linked tyrosine kinases (Ellis *et al.*,1990). GAP contains two copies of the SH2 domain thought to play a role in intermolecular interactions with tyrosine kinases (Koch *et al.*,1991). For these reasons GAP was felt to be a good candidate protein for the 120kDa protein. All the breast cancer cell lines expressed GAP, however on reprobing the same filters with an antibody against phosphotyrosine, the level of tyrosine phosphorylation was greatly increased in the MCF-7Adr line at a position on the filter concordant with the migration of GAP. GAP is certainly a strong candidate for the 120kDa protein, if it were to exist in a highly phosphorylated state in MCF-7Adr cells then unregulated growth could be the result. Further work immunoprecipitating cell lysates with the GAP antibody and examining levels of tyrosine phosphorylation are required to substantiate identity of the protein.

A number of tyrosine phosphorylated proteins which appear strongly in MCF-7Adr unstimulated cultures but not MCF-7WT unstimulated cultures, namely p62 and to a lesser extent p40 and p36 (Fig. 5-2,lanes 1 & 2) do show growth factor regulated expression in the MCF-7WT cell line. When tyrosine phosphorylation is examined in the MCF-7WT line (Fig.5-3), these three proteins are regulated by all the growth factors tested and are likely to be common substrate proteins to growth factor signal transduction pathways. By comparison of the growth factor stimulated tyrosine phosphorylation in MCF-7WT cells and unstimulated MCF-7Adr cells, it would appear p62, p40 & p36 represent constitutive tyrosine phosphorylation of growth factor receptor substrate proteins in the MCF-7Adr cell line. It is possible therefore that deregulated growth of the MCF-7Adr line may be caused through inappropriate expression of signalling pathways in the MCF-7WT line rather than acquisition of alternative pathways. The identity of p62, p40 & p36 is uncertain but some possibilities have emerged from the examination of annexin expression in the two MCF-7 cell lines.

Ligand activation of growth factor receptors with intrinsic protein tyrosine kinase activity leads to receptor autophosphorylation and phosphorylation of a number of cytoplasmic substrate proteins. These substrate proteins are likely to be involved in the transduction of mitogenic signals making them suitable targets for drug intervention.

In the MCF-7WT cell line, p62, p40 & p36 are growth factor responsive substrate proteins which phosphorylate on tyrosine residues, showing no specificity to any receptor type, responding to each growth factor tested. Three of the annexin proteins make good candidates for the two lower molecular weight

phosphorylated proteins. The annexins also known as calpactins or lipocortins have been shown to be substrate proteins for the EGF receptor (Pepinsky & Sinclair,1986), but so far their tyrosine phosphorylation has no association with cell transformation (Cantley *et al.*,1991). Annexin I is not expressed in MCF-7WT cells as determined by western analysis and so cannot be a substrate. Annexin II has a molecular weight of 40kDa however levels of Annexin II are low in the MCF-7WT line, although the size is in agreement with the phosphoprotein p40. Annexin II was not detected in phosphotyrosine immunoprecipitations, however recovery of phosphoproteins was very low. Annexin IV presents the strongest case for being a substrate protein. It appears as a 36kDa protein on SDS Page gels and is strongly expressed in both MCF-7WT and MCF-7Adr cells therefore it may correspond to phosphoprotein p36, a substrate protein stimulated by all growth factors in MCF-7WT cells and in unstimulated MCF-7Adr cells. Annexin IV was also easily detected in phosphotyrosine immunoprecipitations from both cell lines, although the level of detection was much higher in the MCF-7Adr line. This would correspond to an unregulated high level of phosphorylation of this protein.

The p62 band which appears as a common substrate protein maybe the *src* oncogene product. The *src* protein has previously been found to associate with the PDGF receptor (Kypta *et al.*,1990) and it is a likely substrate for other growth factor receptors. Another possibility is the p62 GAP associated protein which has been found to phosphorylate upon activation by *src* (reviewed in Koch *et al.*,1991). Further investigation is required to establish the identity of the p62 substrate protein.

5.4 SUMMARY AND CONCLUSIONS

Examination of the tyrosine phosphorylation in breast cancer cell lines has revealed levels of activity which reflected the nature of growth regulation in that cell line. That is to say, the MCF-7WT line which requires the presence of mitogens for growth exhibited very low levels of phosphorylation under basal growth conditions, whilst the MCF-7Adr line able to grow in an autonomous manner showed high phosphorylation levels.

Under growth factor regulation the MCF-7WT cell line increased levels of tyrosine phosphorylation in a range of proteins. Some of these relate by size to proteins phosphorylated in unstimulated MCF-7Adr cell lysates. Many of these substrate proteins are common to a range of growth factors tested and may represent common steps in the signal transduction process leading to cell proliferation. They may well serve as targets for the control of unregulated growth common to many transformed cells by intervention with new anticancer agents.

The approach described in this chapter may provide a new way of identifying components in the signal transduction pathway activated in response to known mitogens. It may also be of use in characterising the growth response of cells to unknown factors. In addition it may prove a valuable test system for new anticancer agents designed to inhibit tyrosine kinases.

CHAPTER 6

STROMAL EPITHELIAL INTERACTIONS IN BREAST CANCER

CHAPTER 6

STROMAL EPITHELIAL INTERACTIONS IN BREAST CANCER

6.1 INTRODUCTION

Serum free medium conditioned by breast tumour derived fibroblasts has been examined for its ability to regulate the growth of each of the breast cancer cell lines already characterised in thesis. Conditioned medium from each of the fibroblast lines tested, increased the growth of each of the breast cancer cell lines. The active factor(s) present in the conditioned medium have been partially characterised.

6.1.1 Background

Growth factors are known to influence the growth of many human breast cancer cell lines. In chapter 4 the growth response to a range of growth factors was examined in each of the four human breast cancer cell lines, clearly the two ER positive cell lines MCF-7WT and ZR-75-1 were more responsive to the addition of exogenous growth factors than either of the ER negative lines when measured *in vitro*. This chapter will deal with the paracrine influence of breast tumour fibroblasts on the growth of breast cancer cell lines. There is some evidence to suggest fibroblasts have an influence on the growth of breast tumours and many aspects of this phenomenon have been reviewed in section 1.6.3 of the introduction.

6.1.2 Derivation of Breast Tumour Derived Fibroblasts

A number of fibroblast cell lines were derived from fresh breast tumour biopsy material. Each tumour was disaggregated in a collagenase containing medium then the fibroblast cells were isolated by a filtration process, detailed in section 2.2.3. The fibroblasts were cultured through 2 to 3 passages before storage in liquid nitrogen where they remained until experimental use.

To verify that each of the breast fibroblast lines were indeed pure and free of epithelial contamination cultures were carefully screened by light microscopy and analyzed by immunofluorescence. Monoclonal antibodies to vimentin (Ab-1) and cytokeratin pan were used as primary label on the fixed preparations of monolayer fibroblast cultures (methods 2.8.1 & 2.8.2). Figure 6-1, shows immunofluorescent staining patterns of two of the fibroblast cell lines and for comparison two epithelial cell lines. Both the fibroblast lines show strong staining for vimentin (Fig.6-1A&B), an intermediate filament protein normally expressed in non-epithelial cells and often used as a marker to identify cells of mesenchymal origin. A monoclonal antibody able to recognise all cytokeratins, anti-cytokeratin pan was used to ensure no epithelial characteristics were present in the derived fibroblast lines. Staining patterns of the breast carcinoma cell line MCF-7WT are shown in the Figure 6-1C. Clearly the two breast fibroblast cell lines conform to normal fibroblast associated intermediate filament protein patterns. Interestingly in Figure 6-1D, MCF-7Adr cells showed uncharacteristic epithelial staining since they strongly express vimentin as well as cytokeratin intermediate filament proteins. This phenomenon has been noted before, in hormone independent breast cancer cell lines (Sommers *et al.*,1989) where it was suggested vimentin

Figure 6-1 Immunofluorescent staining

A. BF10 breast fibroblasts	(i) Vimentin (ii) Cytokeratin-pan
B. BF11 breast fibroblasts	(i) Vimentin (ii) Cytokeratin-pan
C. MCF-7WT	(i) Vimentin (ii) Cytokeratin-pan
D. MCF-7Adr	(i) Vimentin (ii) Cytokeratin-pan

Cellular staining for vimentin and cytokeratin as described in methods. Magnification (x400).

Figure 6-1 **Vimentin and Cytokeratin Fluorescence Staining**

Ai

Aii

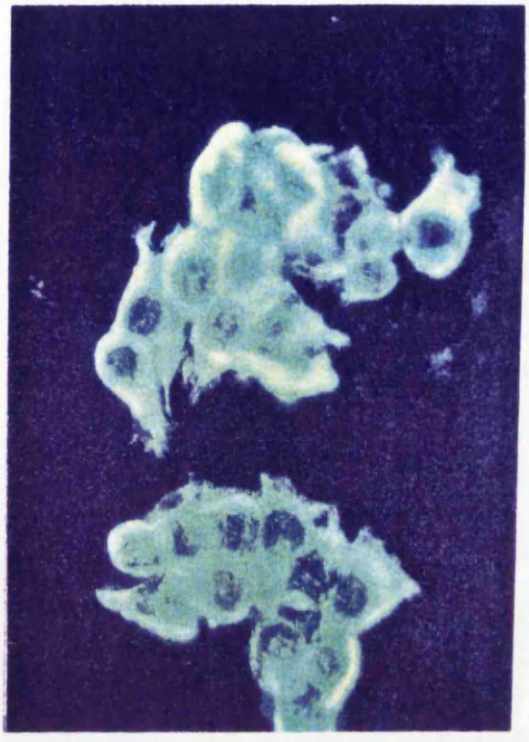
Bi

Bii

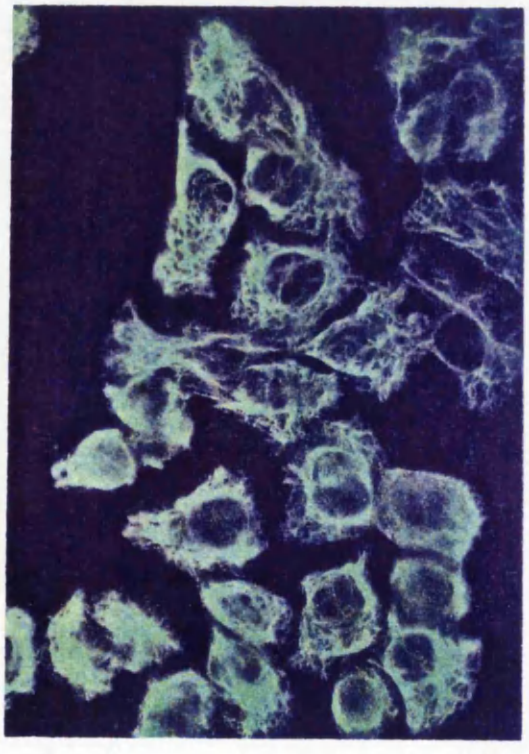
Figure 6-1 **Vimentin and Cytokeratin Fluorescence Staining**

Ci

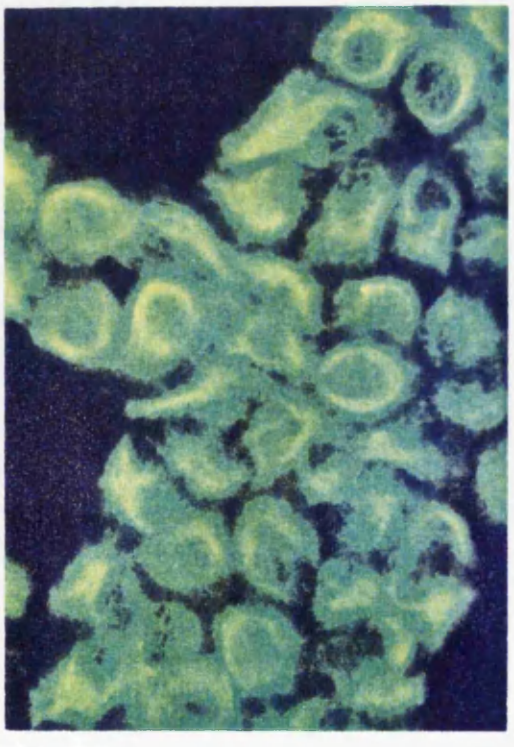
Cii



Dii



Di



expression may correlate with a high degree of malignancy.

6.1.3 Characterisation of Breast Fibroblast Cell Lines

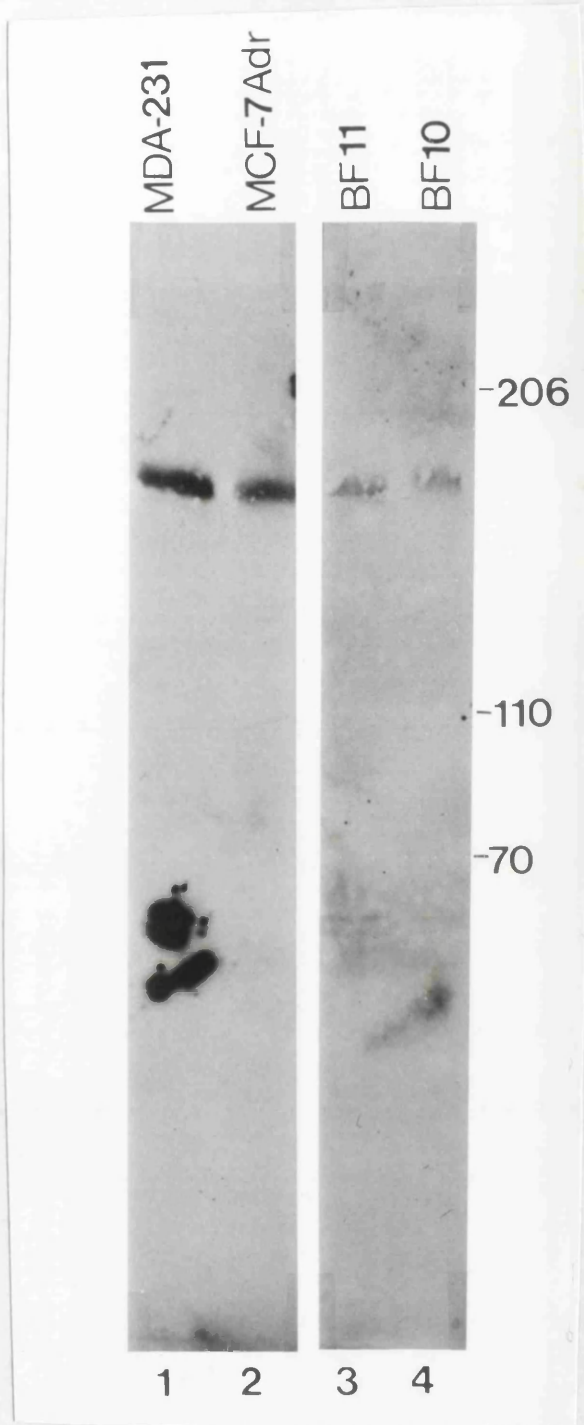
The human breast fibroblast cultures had relatively slow doubling times compared to epithelial cell lines, approximating to 80Hrs in the optimal growth conditions of 15% FCS. Evidence of EGF receptor expression was found in the two fibroblast cell lines tested, BF11 and BF12. Figure 6-2, shows receptor expression on a western blot of whole cell lysates using the rabbit polyclonal antibody BG48. The bands are only just detectable using this method and the levels are much lower than those found in the MDA-MB-231 cell line which was used here as a positive control but are higher than the EGF responsive MCF-7WT cell line which appears negative for the EGF receptor using this method. This tends to indicate fibroblasts will respond to TGF-α, a growth factor known to be produced by breast carcinoma cell lines and shown to be under oestrogen control in a number of ER positive breast cancer cell lines (see section 1.4.1).

6.2. RESULTS

6.2.1 Activity of Conditioned Medium

Serum free medium was conditioned by subconfluent cultures of breast tumour fibroblast lines over a period of 96Hrs (section 2.3). The conditioned medium was then applied to breast cancer cell lines growing in the MTT assay in 5% CSS. Figure 6-3(A-D) shows the growth response in each of the breast cancer cell lines to fibroblast conditioned medium. It is represented as a percentage

Figure 6-2 EGF Receptor Expression in Breast Fibroblasts



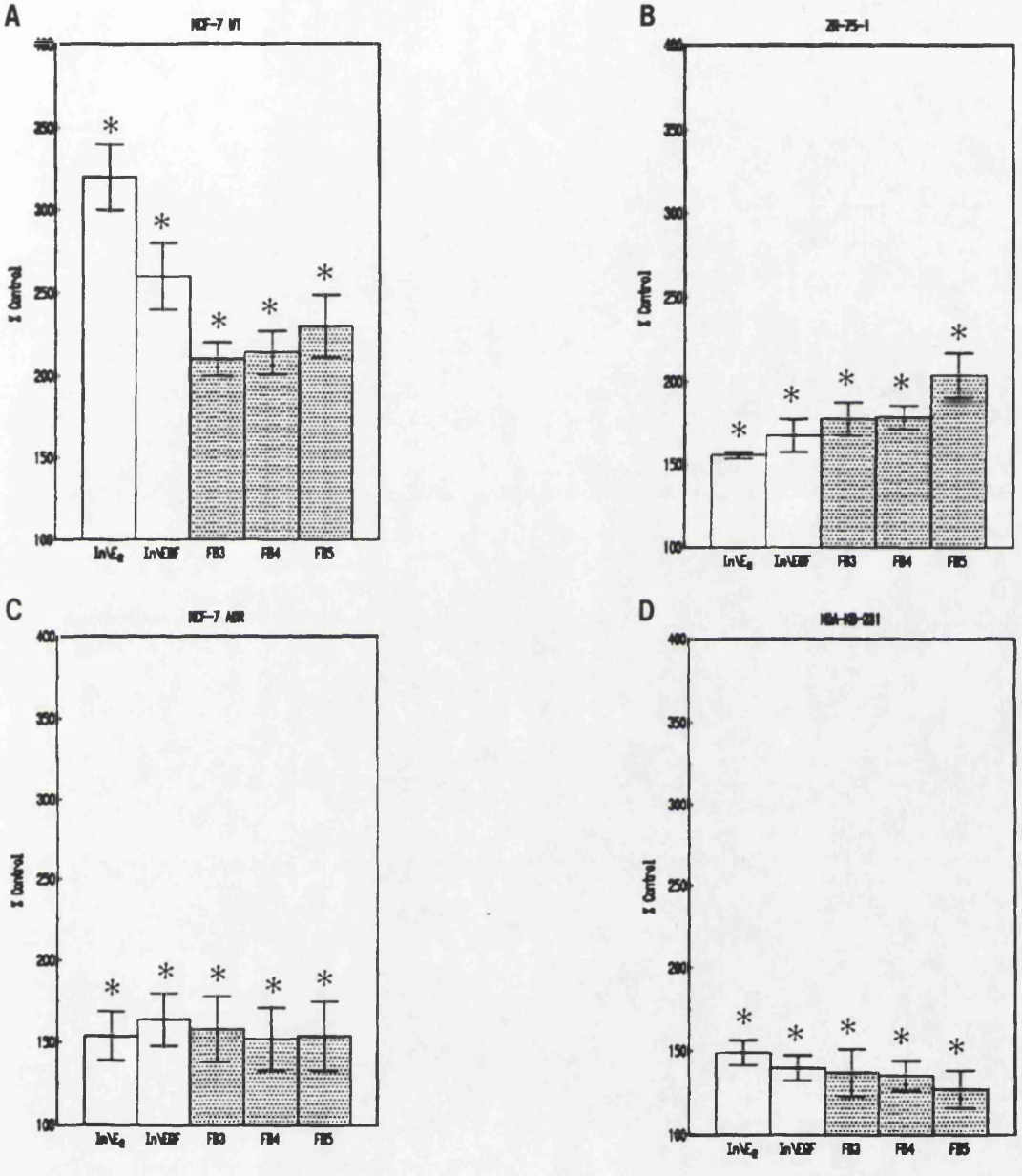
Immunoblot of SDS - Page Gel showing EGF receptor expression in whole cell lystates. MDA-231 and MCF-7Adr cells have been run on same gel for comparison (lanes 1 & 2). EGF receptor detected in both BF11 and BF10 cells.

increase in growth over control, where the controls are grown in RPMI(prf) + 5%CSS and taken to be 100%. For each cell line the growth response to optimal concentrations of insulin and E_2, as well as insulin and EGF are also shown. These were measured simultaneously in the MTT assay.

The MCF-7WT cell line (Fig.6-3A) shows the greatest increase in growth rate in response to the conditioned medium, more than doubling the rate in control wells but not achieving optimal growth as represented by growth in the presence of insulin and E_2. ZR-75-1 cells (Fig.6-3B) also show a clear response to the fibroblast conditioned medium but this time the overall response was marginally greater than the response achieved in optimal growth conditions with insulin and E_2. The conditioned medium also had a small growth effect on the two ER negative cell lines; MDA-MB-231 and MCF-7Adr. If the growth effect of conditioned medium is due to the presence of a growth factor or factors it should be possible to dilute out the factor with control medium, indeed this was the case. In Figure 6-4, conditioned medium from BF-11 cells increases the rate of growth of MCF-7WT cells to approximately 2.5(250%) times the control values, this rate steadily declines as the conditioned medium was diluted in control medium to 1.1 times (111%) at a dilution of 1:100 or 1%.

A number of physicochemical properties of the conditioned medium were tested to characterise the nature of the active factors involved, these included sensitivity to temperature, acid, trypsin and the ability to bind to heparin. The temperature sensitivity of the conditioned medium is detailed in Figure 6-5, whilst all other properties are listed below in Table 6-1.

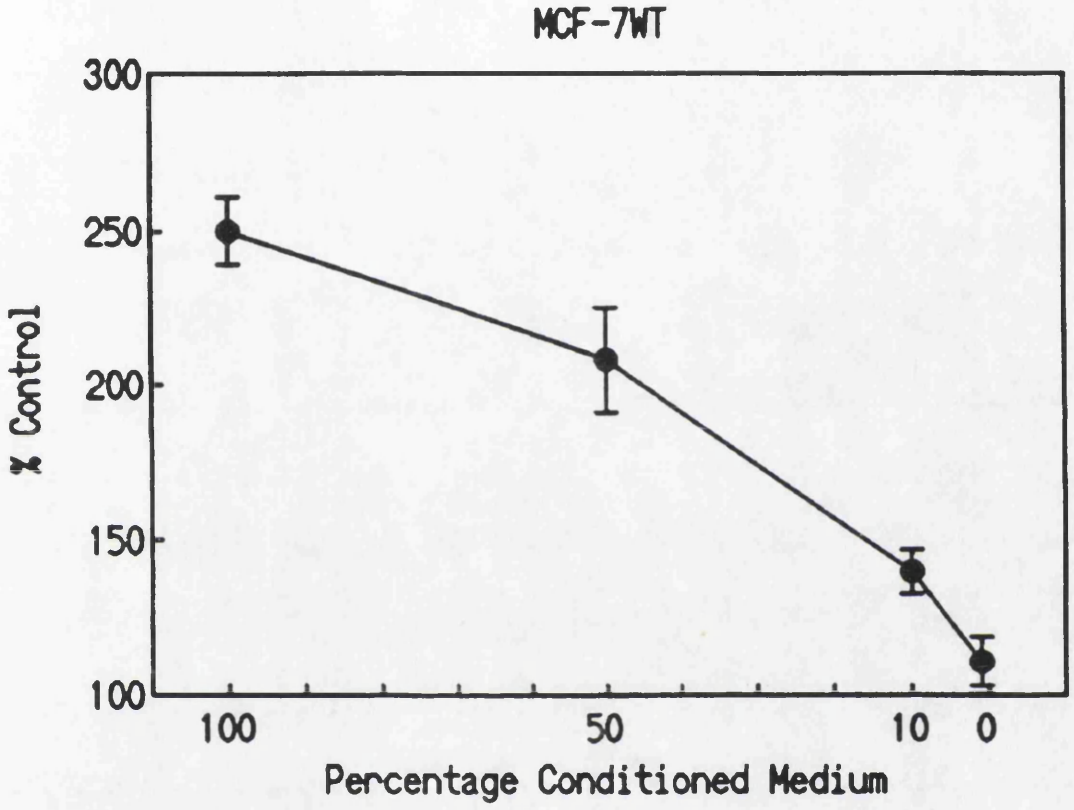
Figure 6-3 Growth Response to Fibroblast Conditioned Medium



Growth stimulation of four breast cancer lines by breast fibroblast conditioned medium. Shaded bars represent the growth response to conditioned medium from 3 different fibroblast lines : FB3, FB4, & FB5. The growth response to optimal concentrations of E_2 (10^{-9}M) + Insulin (10μg/ml) and Insulin(10μg/ml) + EGF(10μg/ml) were measured simultaneously for a direct comparison. All bars represent the mean of 3 samples. * represents significant difference from control growth ($P < 0.01$). Vertical lines represent one S.D.

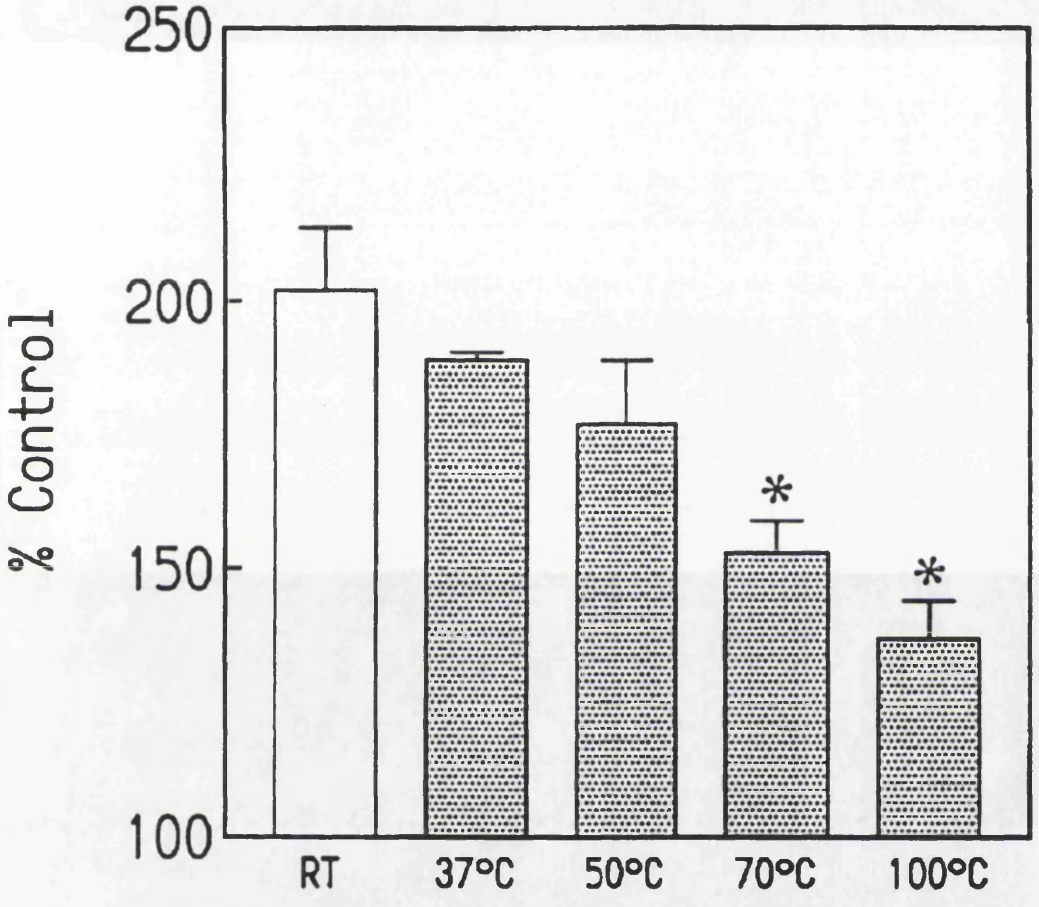
One representative experiment from a series of three.

Figure 6-4 Fibroblast Conditioned Medium Dilution Curve



Growth response in MCF-7WT cells to a number of dilutions of conditioned medium from BFII fibroblast cells. Conditioned medium was diluted in control medium and growth response measured over a 3 day MTT assay. Results are expressed as a percentage of control growth, each point represents the mean of 3 samples. Vertical bars represent one S.D.

Figure 6-5 Temperature Sensitivity of Conditioned Medium



Growth response in MCF-7WT cells to conditioned medium from BF-II fibroblasts after a number of temperature treatments detailed in Table 6-1. Results are expressed as a percentage of control growth. Each bar represents the mean of 3 samples. * represents a significant difference from growth with conditioned medium held at room temperature (RT) at $P < 0.01$. Vertical lines denote one S.D.

Experiment carried out twice with BF11 CM and twice with BF10 CM. On each occasion results were comparable.

Table 6-1

Physical and chemical characteristics of breast fibroblast CM.

Temperature sensitivity- CM held at temperature for stated time then immediately cooled on ice. Control CM was held at room temperature for the same period of time then put on ice.

Acid sensitivity- CM acidified for 30mins before neutralization. In control acid and alkali were added simultaneously.

Trypsin sensitivity- trypsin added at stated concentration for 2Hrs before the addition of soyabean trypsin inhibitor. In control both were added simultaneously.

Heparin binding- measured using FPLC heparin binding column. Unbound material reconstituted to original volume. Salt eluted bound material also reconstituted to original volume.

In each case activity shown as a percentage of untreated CM activity

TABLE 6-1 CONDITIONED MEDIUM CHARACTERISTICS

PROPERTY	DETAIL	% CM ACTIVITY	METHOD
TEMPERATURE SENSITIVITY	37°C 50°C 70°C 100°C	87% 75% 52% 36%	2Hrs 37oC 30mins 50°C 10mins 70°C 2mins 100°C
ACID SENSITIVITY	Acid Acid control	98% 97%	5 µl 6N HCl:200 µl CM (30mins). Neutralize NaOH
TRYPSIN SENSITIVITY	Trypsin Trypsin control	26% 94%	2.5mg/ml trypsin (2Hrs 37°C). Trypsin inhibitor 520 µg/ml
HEPARIN BINDING	Bound Unbound	116% 61%	FPLC heparin binding column followed by salt elution

The factor or factors, show acid stability but temperature and trypsin sensitivity suggesting they are of a proteinaceous nature. They also show a tendancy to bind to heparin which implies that some of the active component of the conditioned medium belongs to the heparin binding set of growth factors otherwise known as the fibroblast growth factors. 10mls of conditioned medium was passed through a heparin sepharose packed HR 10/10 FPLC column at a flow rate of 1ml/min in a buffer of 0.1M NaCl in 0.02M Tris (pH 7.6). The bound material was eluted in 2M NaCl in 0.02M Tris (pH 7.6) and finally desalted in RPMI. Further purification proved difficult because activity was quickly lost upon manipulation of the conditioned medium. Since there was evidence to suggest a heparin binding growth factor maybe important for the conditioned medium activity,

bFGF was checked for a possible growth promoting role in the conditioned medium using a neutralizing antibody. The bFGF antibody (British Biotechnology) was added to conditioned medium at 50 μg/ml before its addition to cells in the MTT assay. 50 μ/ml IgG had previously been found to completely neutralise the response of an optimal concentration of bFGF (10ng/ml). On both the occasions this antibody was tested, no reduction in the conditioned medium activity was found, suggesting bFGF was not itself responsible for increased growth of MCF-7WT cells, this does not rule out the possibility that other members of the FGF family may be involved.

6.2.2 E_2 and Tamoxifen Treatment of Fibroblast Conditioned Medium

The effects of E_2 and tamoxifen on the fibroblast conditioned medium were also examined by the resultant growth response in breast cancer cell lines. Fibroblast cultures were changed to serum free medium containing 10^{-9}M E_2, 10^{-6}M tamoxifen, or both. After 96Hrs the conditioned medium was removed and tested for activity using the MTT assay. Figure 6-6A shows the growth response in MCF-7WT cells to treated conditioned medium from BF10 breast fibroblast cells. The growth response to conditioned medium treated with E_2 or E_2 and tamoxifen is significantly higher ($P<0.01$) than untreated conditioned medium, tamoxifen treatment alone causes no change in the growth response. As a control, serum free medium with the addition of E_2 or tamoxifen was set up and incubated over a 96Hr period in a cell free enviroment, these were then checked for a growth response in MCF-7WT cells and the results are shown in Figure 6-6B. In the sham incubated medium there is a small response to E_2 ($P<0.02$)

Figure 6-6

A. Growth response of MCF-7WT cells to BF10 fibroblast conditioned medium. CM—untreated conditioned medium, CM(E_2)—CM with 96Hr E_2 10^{-9}M treatment, CM (E_2 T)—CM with 96Hr E_2 and tamoxifen treatment.

* represents significant difference from untreated CM ($P<0.01$).

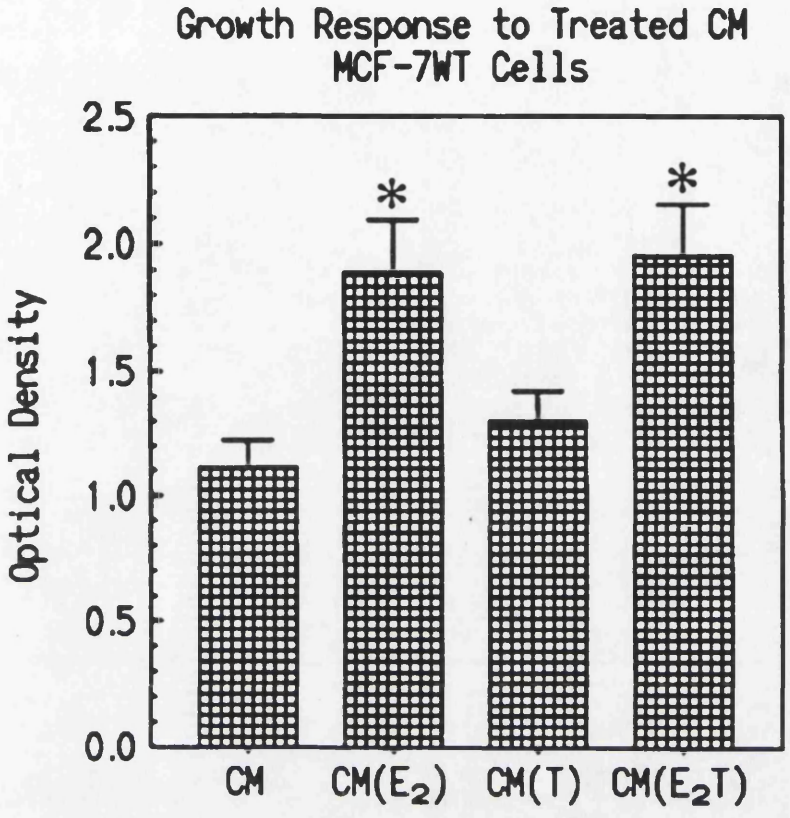
Experiment repeated on five occasions with consistent results. A different fibroblast line is examined in Fig.6-8.

B. Growth response of MCF-7WT cells to sham incubated medium, medium treated as in **A** then incubated for 96Hrs in a cell free environment.

* represents significant difference from control medium ($P<0.02$).
☆ represents significant difference from E_2 treated medium ($P<0.02$).

Each bar represents the mean of three separate MTT plates, growth measured as optical density. Vertical lines represent one S.D.

Figure 6-6 **Oestradiol and Tamoxifen Treatment of Conditioned Medium**



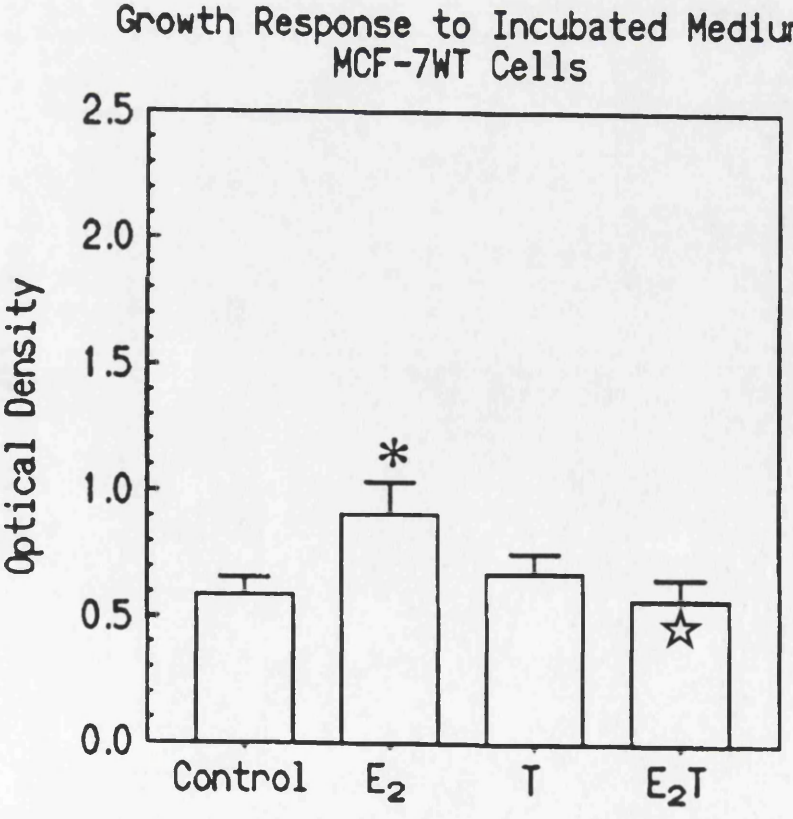
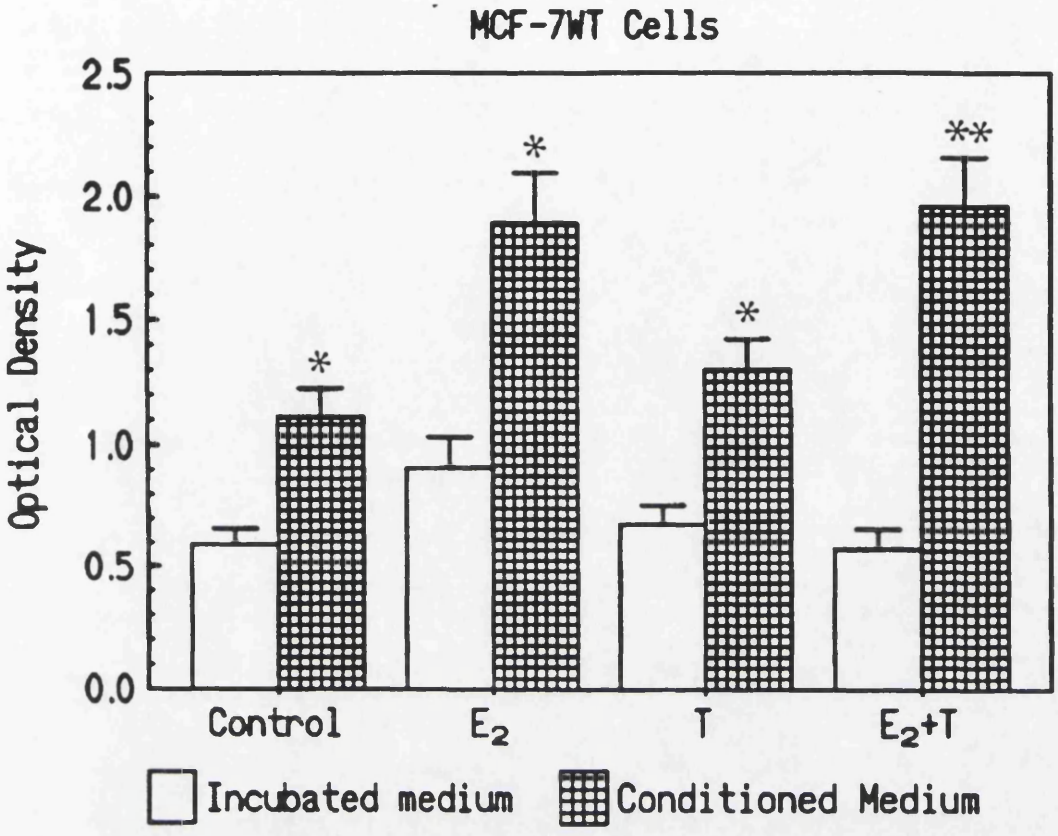


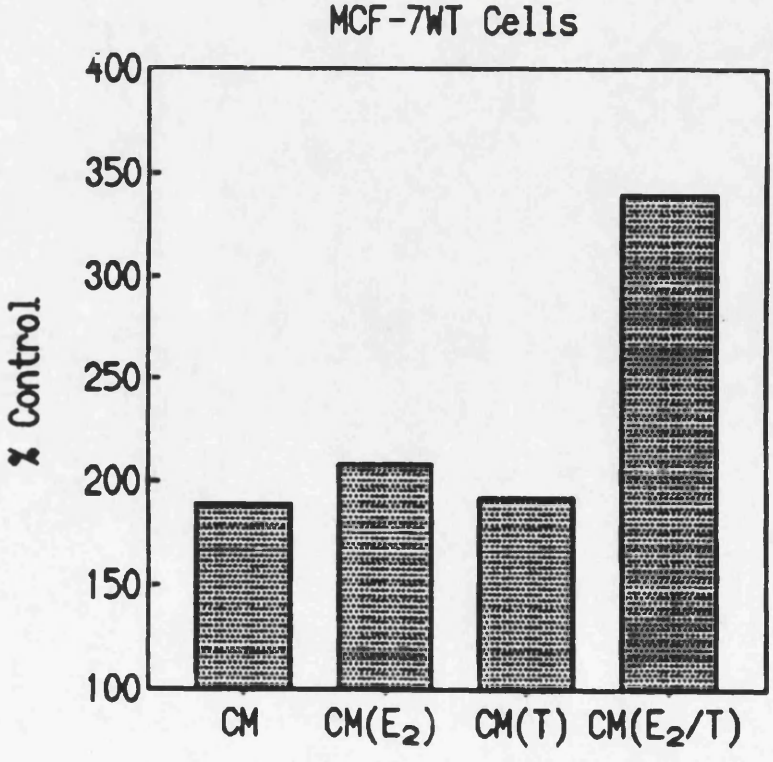
Figure 6-7

A. Figure represents same data as in **Figure 6-6 A & B** shown together for comparison. Each CM has been tested against specific sham incubated medium control. * represents significant difference $P < 0.01$,**$P < 0.001$.

B. Treated CM represented as a percentage of specific sham incubated medium controls. Control taken to be 100%.

Figure 6-7 **Growth Response to Treated Conditioned Medium**





above the control incubated medium. Tamoxifen causes no measurable change in response but in the presence of E_2 it brings growth response back to levels seen with control medium, this inhibitory effect is not seen in the treated conditioned medium. Figure 6-7A shows both sets of data on the one diagram and allows the growth effect of the conditioned medium factors to be seen, in each case the conditioned medium is significantly greater than its control ($P<0.01$). Finally the data is expressed as a percentage of specific sham incubated medium control where the are taken to be 100% (Figure 6-7B).

Figures 6-8 A & B show the growth response in MCF-7WT cells and ZR-75-1 cells respectively to treated conditioned medium from BF11 cells. The results are expressed as a percentage of basic control medium, the response of the cells to optimal concentrations of E_2 and insulin are also present for comparison. The overall pattern of growth response is similar between the two fibroblast lines as well as both the indicator breast cancer cell lines although the overall increase is much smaller for the ZR-75-1 cell line, as it was in response to E_2 and growth factors.

To further examine the effect of E_2 on the fibroblast cells through the conditioned medium, an experiment was set up to compete out the presence E_2 in the conditioned medium by adding fresh tamoxifen before testing in the MTT assay. Figures 6-9 A & B show the growth response in MCF-7WT and ZR-75-1 cells respectively. The first four hatched bars show a repeat of the treated conditioned medium experiment whilst the crosshatched bars represent further treatment of conditioned medium after incubation with fibroblasts. $CM(E_2)+T$ shows the response to E_2 treated conditioned medium with the addition of 10^{-6}M

tamoxifen, to remove the direct effects of E_2 present in the conditioned medium. This resulted in the growth response being reduced back to the level of untreated conditioned medium. To check the validity of this, untreated conditioned medium was tested in the presence of E_2 10^{-9}M and tamoxifen 10^{-6}M (CM+E_2+T). Again the total growth response was similar to conditioned medium alone. When fresh E_2 was added (CM+E_2), there was a growth response similar to E_2 treated conditioned medium [CM(E_2)]. Together these results seemed to suggest that the additional growth found with the treated conditioned medium was simply due to the presence of E_2 in the conditioned medium and an additive growth response was achieved. However, in the situation where fresh tamoxifen was added to the conditioned medium there was a decrease in the growth response to conditioned medium. This growth inhibition was independent of E_2 since no E_2 was present in the conditioned medium therefore tamoxifen was having an E_2 independent inhibitory effect at a concentration of 10^{-6}M in this assay. Both cell lines show similar growth responses overall but again these are reduced in the ZR-75-1 cell line.

The experiment shown in Figure 6-10 was set up to investigate the dose response to tamoxifen in this assay. In the presence of conditioned medium, tamoxifen had a small but significant, positive growth effect at 10^{-8} and 10^{-7}M, this was probably due to its partial agonist effects at 10^{-6}M tamoxifen, a small but significant ($P<0.01$) inhibition of growth was seen. In the presence of CM(E_2) tamoxifen concentrations of 10^{-8}, 10^{-7},10^{-6}M all brought about inhibition of the growth response, which was significant at 10^{-7} and 10^{-6}M. This suggests at least some of the inhibitory effects are due to competitive inhibition of E_2, whilst

Figure 6-8

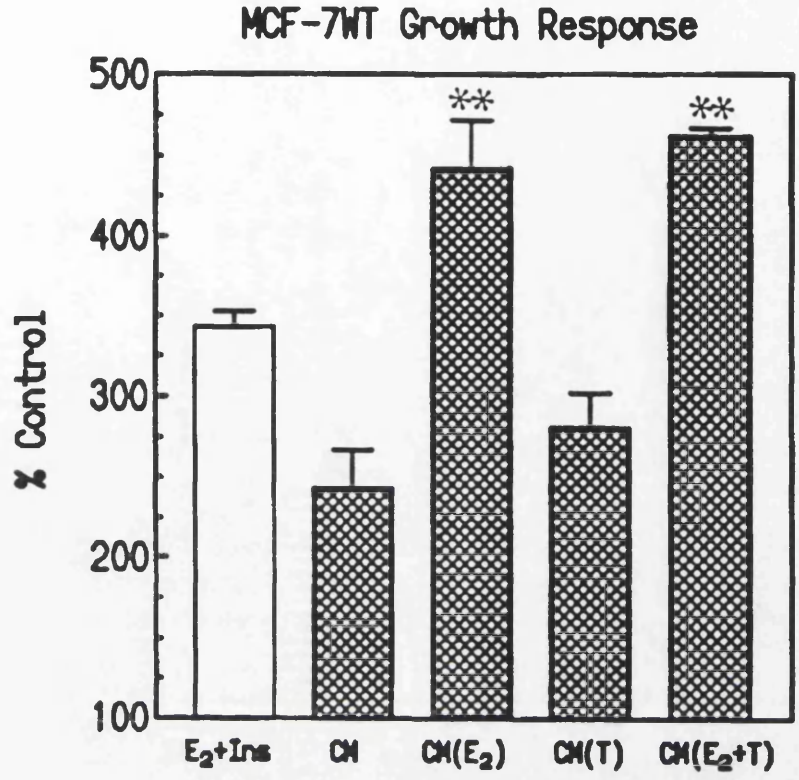
Graphs represent growth response to treated CM from BFII fibroblasts as a percentage of growth in control medium (control=100%). **A**; Growth response in MCF-7WT cells. **B**; ZR-75-1 cells.

E_2 + Ins - cells treated with E_2 10^{-9}M + Insulin 10μg/ml,
CM—untreated CM, CM (E_2)—CM treated E_2 10^{-9}M for 96 Hrs,
CM(T)— CM treated tamoxifen 10^{-6}M for 96 Hrs, CM (E_2+T) — CM treated E_2 and tamoxifen.

Significant difference of treated against untreated CM represented by
* $P<0.01$, ** $P<0.001$.

Each bar represents the mean of three separate MTT plates. Vertical lines represent one S.D.

Figure 6-8 **Growth Response to Treated Conditioned Medium**



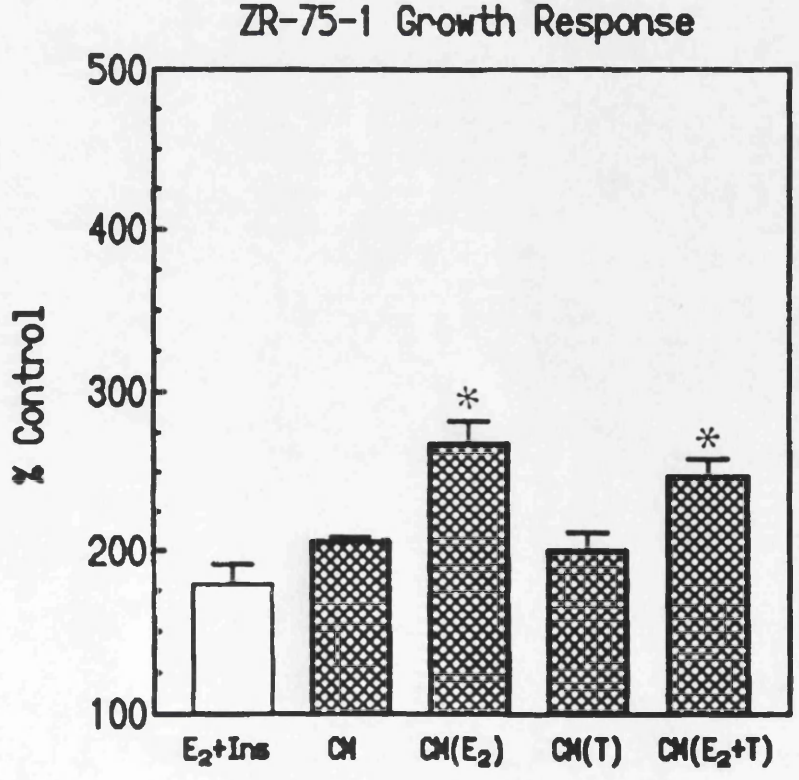


Figure 6-9

Graphs represent the growth response in **A**; MCF-7WT cells. **B**; ZR-75-1 cells, shown as a percentage of growth in control medium (control = 100%).

Single hatched bars represent similar experiment as in **figure 6-8** showing growth response to:

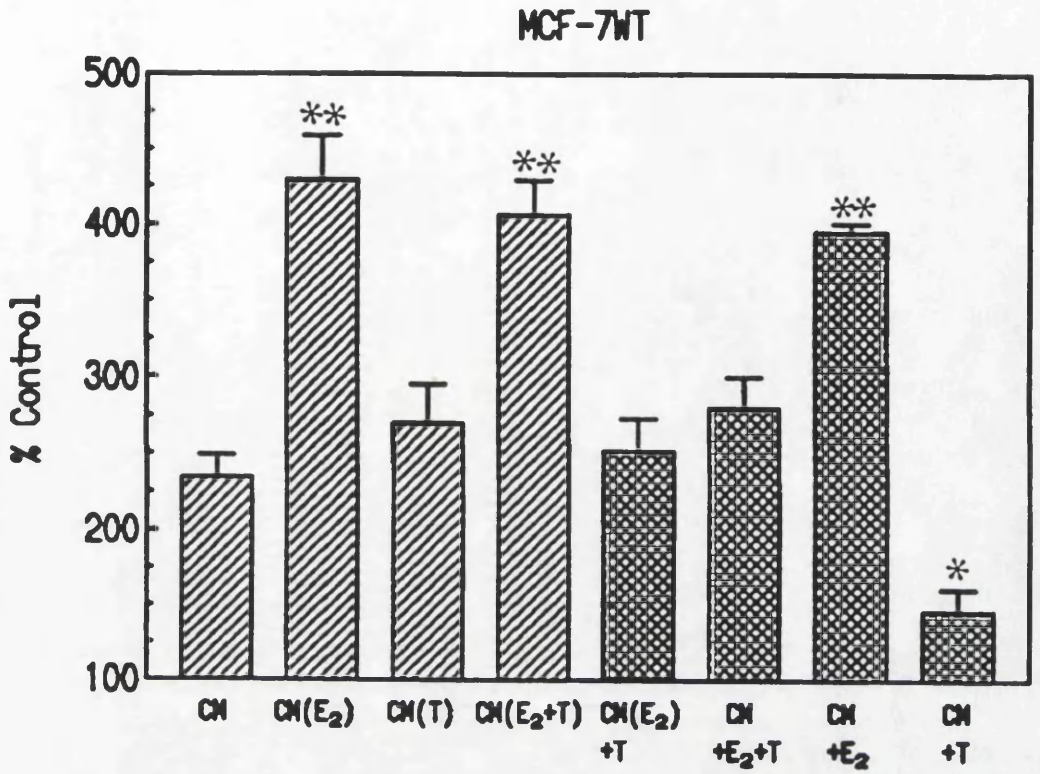
CM
CM(E_2) ; CM treated with E_2 10^{-9}M
CM(T) ; CM treated with tamoxifen 10^{-6}M
CM(E_2+T) ; CM treated with E_2 and tamoxifen.

Cross hatched bars represent additional treatment of CM after incubation:

CM(E_2)+T ; E_2 treated CM with tamoxifen added at 10^{-6}M
CM+E_2+T ; CM with the addition of E_2 10^{-9}M and tamoxifen 10^{-6}M
CM+E_2 ; CM with the addition of E_2 10^{-9}M
CM+T - CM with the addition of tamoxifen 10^{-6}M.

Significant difference from untreated CM represented by * $P<0.01$, ** $P<0.001$. Each bar represents the mean of three separate MTT plates. Vertical lines represent one S.D.

Figure 6-9 **Effects of E_2 and Tamoxifen on Treated Conditioned Medium**



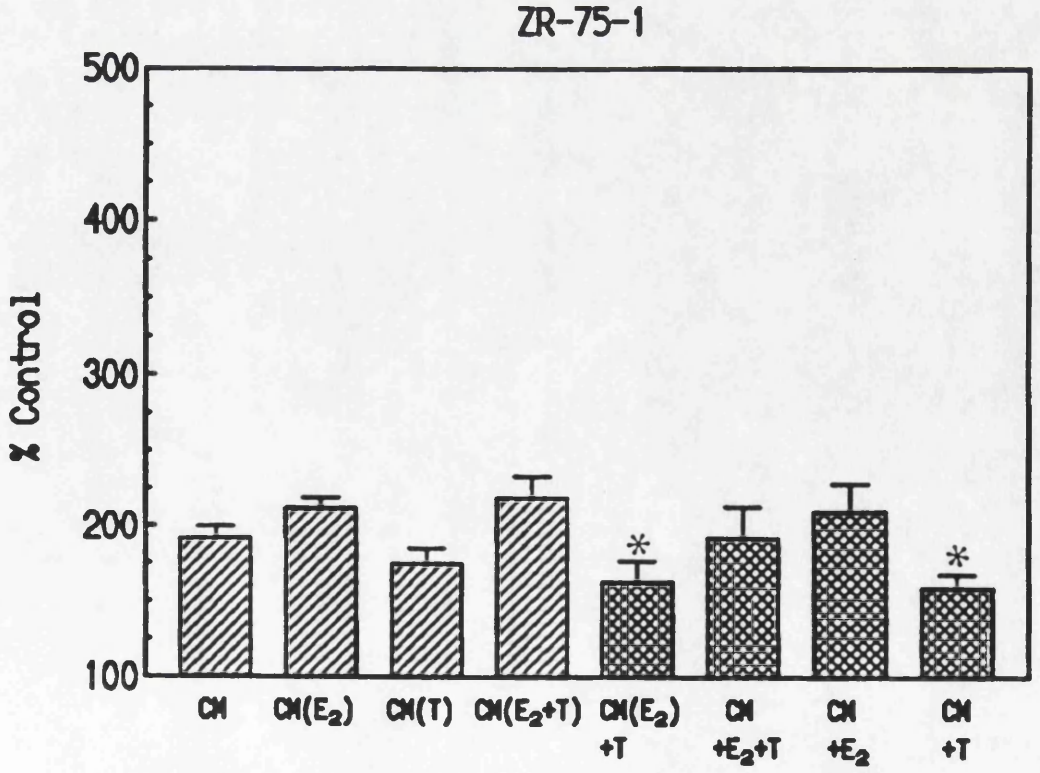
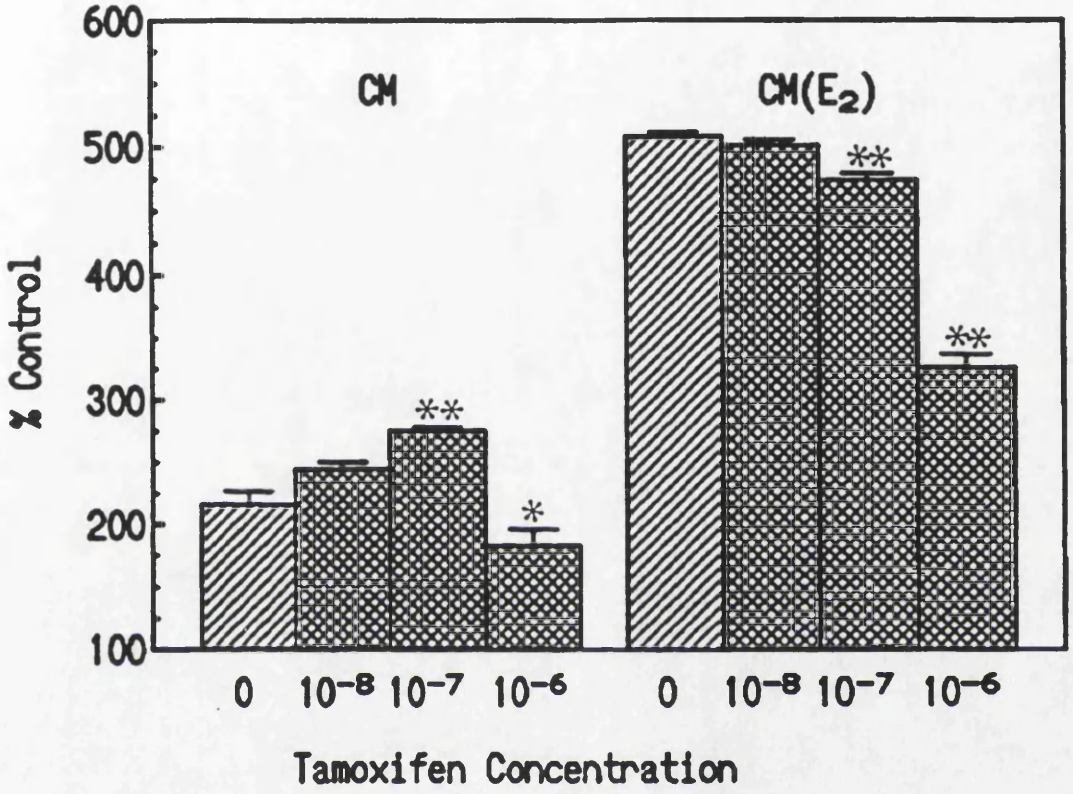


Figure 6-10 Tamoxifen Treatment of Fibroblast Conditioned Medium



Effect of tamoxifen (10^{-8}, 10^{-7}, & 10^{-6} M) on CM & E_2 treated CM, CM(E_2). Significant difference from CM (E_2) represented as * $P<0.01$, **$P<0.001$. Each bar represents the mean of three separate MTT plates. Vertical lines represent one S.D.

at 10^{-6}M tamoxifen E_2 independent inhibition may also play a role.

6.3 DISCUSSION

Growth promotional activity was found in all the fibroblast conditioned media investigated in this work. Five different lines of breast tumour derived fibroblasts were examined for growth promotional activity in breast carcinoma cell lines. For the ER positive MCF-7WT cell line growth promotional activity was present in all the conditioned media tested. Tyrosine phosphorylation was also examined in the MCF-7WT cell line after stimulation with breast fibroblast conditioned medium, which brought about a clear increase in the extent of tyrosine phosphorylation (section 5.2.3, Fig.5-4, lane 11). Many of the phosphoproteins which were increased upon stimulation by conditioned medium were of the same size as those stimulated by a range of growth factors. This suggests the activity of the conditioned medium is due to growth factors, although no specific growth factor could be identified.

In a similar study by van Roozendaal *et al*,(1992) fibroblasts cultures were derived from malignant breast tissue, normal breast tumour adjacent to a malignancy, normal breast tissue from reduction mammmoplasty and skin. Conditioned medium from both 'normal tissue and tumour derived fibroblasts were able to induce the growth of breast cancer cell lines but the extent of the proliferation was significantly higher in the conditioned medium from malignant sources. *In vivo*, Horgan *et al*,(1987) found fibroblasts from normal and malignant breast tissue were able to stimulate the growth and development of MCF-7 xenografts in nude mice.

All of these results suggest fibroblasts particularly those derived from a malignant tumour are able to induce the growth of breast cancer cells by a paracrine mechanism. The factors responsible for the proliferative response to the conditioned medium may be many and varied. There is evidence of the presence of mRNA of; PDGF A, bFGF, FGF-5, IGF-II and TGF-ß in fibroblast cell cultures derived from malignant breast lesions (Cullen *et al.*,1991). Many of these growth factors are able to activate the growth of epithelial cells. Many other active factors may be present but remain to be identified. It is quite possible that the proliferative response seen in the breast carcinoma cells is the overall result of a number of paracrine influences which individually will have inhibitory and mitogenic effects on the target epithelial cells but together add up to an overall positive growth response. This positive growth effect is highest in conditioned medium of malignant derived fibroblasts which may be due to an increased expression of mitogenic factors or decreased expression of inhibitory factors or both.

The work carried out in this chapter suggests fibroblasts can influence the cellular response to E_2 of ER positive breast cancer cells although the mechanism involved is unknown. Some closely related work by Cunha, examining the role of stroma in oestrogen induced epithelial proliferation in mice examines the specificity of fibroblast cells. Normal epithelia of the vagina was dependent on the presence of vaginal stromal cells to cause oestrogen induced proliferation. When the vaginal stroma was replaced with stromal cells from the urinary bladder, the oestrogen induced proliferation was lost. It was also shown that oestrogen could induce the proliferation of urinary bladder epithelium, which lack

ER, when combined with stroma of vaginal origin. These cells did not respond to oestrogen when associated with bladder derived stroma suggesting oestrogen responsive epithelial proliferation is dependent on the appropriate stromal environment. There is evidence to suggest these stromal cells express the ER (Cunha & Young, 1992). In the breast system, Haslam (1986) demonstrated that oestrogen influences on the growth of normal mouse mammary epithelia was dependent on the presence of mammary derived fibroblasts. The situation is different in neoplastic cells where direct E_2 stimulated proliferation can take place in ER positive cells. In the MCF-7WT line there did seem to be a direct response to E_2 from the treated fibroblast conditioned medium but the possibility of an indirect oestrogen response mediated through the fibroblast cells cannot be excluded. Removal of E_2 from the conditioned medium would be the only effective way to answer this question. Further work looking at a range of fibroblasts from various origins would ascertain whether the E_2 induced response is a feature of malignant breast tumour derived fibroblasts or a feature of all fibroblasts.

The fibroblast conditioned medium was used to examine the effects of tamoxifen since Colletta *et al.*,(1990) had found a number of antiestrogens were able to induce increases in the synthesis of active TGF-ß in fetal-fibroblasts despite a lack of ER in these cells. The results shown here measure an overall response to treated conditioned medium and no attempt has been made to measure individual growth factor expression, but it is clear tamoxifen treatment incurs no inhibitory growth effects on the resultant conditioned medium. This does not rule out the possibility that TGF-ß levels may be increased but it does suggest the overall growth effect is unaltered by tamoxifen treatment as measured

in MCF-7WT and ZR-75-1. It would appear tamoxifen does not play an important role in stromal-epithelial interactions of a breast tumour.

6.4 SUMMARY AND CONCLUSION

Breast tumour derived fibroblasts appear to produce a proteinaceous factor or factors, capable of promoting a growth response in the MCF-7WT breast cancer cell line and to a lesser extent the ZR-75-1 cell line. E_2 treatment of fibroblast cultures causes a large increase in the mitogenic capacity of the conditioned medium toward MCF-7WT and ZR-75-1 cells. It is uncertain whether this increased growth was due to an additive effect of residual E_2 and the conditioned medium or an indirect effect of E_2 on the fibroblasts and therefore the mitogenic capacity of the conditioned medium.

It is clear the presence of fibroblasts in the surrounding breast stroma are capable of increasing the growth of some breast cancer cell lines.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

Several aspects of the regulation of breast cancer growth have been explored during the course of this thesis: oestrogen regulation, peptide growth factor regulation, and activation of tyrosine kinase pathways within the cell. Each of these important aspects of cellular growth control have been examined in relative isolation, and it is important that the information is brought together in order to gain a greater insight into the growth of a tumour. Hormones, growth factors, and post receptor transmembrane signal transduction will summate or interact to produce the final proliferative response of the tumour. Here, the interplay between each of these different regulatory systems will be discussed.

The relationship between E_2 and TGF-α is a good example of interplay between hormones and growth factors. E_2 is a growth regulator of the ER positive cell line MCF-7WT, and has been shown by others (Dickson *et al.*,1986), to regulate synthesis of TGF-α. TGF-α in turn, can bind to and activate the EGF receptor of these cells in an autocrine manner. This brings about phosphorylation of the EGF receptor and a number of substrate proteins. There is a growth response to EGF in the MCF-7WT cell line when the cells are grown in oestrogen free culture. Both EGF and TGF-α brought about phosphorylation of a number of tyrosine containing proteins, although there was no evidence of phosphorylation of the 170kDa EGF receptor in the MCF-7WT cell line. Since EGF and TGF-α did stimulate phosphorylation of a number of substrate proteins, this suggested

that the receptor was present but at a concentration below assay detection.

Other growth factors may also be expressed in response to activation of the ER, and it is clear that a large range of growth factors are mitogenic to this cell line. In fact, other mechanisms must be involved in the growth response to E_2, since TGF-α can only account for a small proportion of the E_2 regulated growth. The growth response to optimal concentrations of EGF is small compared to the growth response to optimal concentrations of E_2. In the presence of insulin, EGF was unable to significantly increase the cell growth, whilst E_2 brought about an additive growth response.

What other mechanisms are involved in E_2 regulation of the growth of the MCF-7WT cell? Other growth factors expressed under ER regulation and acting on the cell in an autocrine manner could be responsible. Members of the IGF-1 family and bFGF are strongly mitogenic to the MCF-7WT cell line and therefore make suitable autocrine regulators. Secretion was found not to relate to E_2 stimulation in the MCF-7 cells (Lippman *et al.*, 1986), although IGF-I was secreted by a range of breast cancer cells. Work carried out here, examining the growth response to E_2 and insulin would also suggest the IGF family of growth factors do not play an important role in the autocrine regulation of E_2 stimulated growth. Insulin, through activation of the IGF-I receptor was able to significantly increase the growth of MCF-7WT cells beyond the level reached under optimal concentrations of E_2 alone. This suggested IGF-I receptors were not maximally stimulated under E_2 conditions. Other growth factors, such as members of the FGF family have not yet been investigated as ER regulators and it is possible they may a role.

It is important not to overlook the work of van der Burg *et al.* (1991), who proposed a direct mechanism for oestrogen regulated growth, dependent on the presence of insulin or IGF's for a direct stimulatory effect. This would also account for the missing factor in our E_2 regulated system.

For each growth factor able to stimulate the growth of MCF-7WT cells, there was a noticeable increase in the level of tyrosine phosphorylation in the cell. Proteins which were phosphorylated on tyrosine, showed growth factor specificity at high molecular weights (>150kDa), which corresponded with the phosphorylation of tyrosine residues on the growth factor receptor itself. The remainder of stimulated tyrosine phosphorylation showed no specificity between any of the growth factors. Each growth factor appears to show specific tyrosine phosphorylation at the receptor level but share the same early tyrosine phosphorylation responses. This lack of specificity has been discussed in some detail in PC12 cells where EGF and NGF share the same early signalling pathways but result in quite different biological effects (Chao, 1992). Clearly the cell must reach a point of progression which is specific to a particular growth factor.

The MCF-7WT cell line made an excellent model for examining growth responses to E_2 and growth factors since it showed large responses to exogenously applied factor, but it must be remembered that this is only a single example of a breast cancer cell. It was important to look at a range of characteristically different breast cancer cell lines, since a tumour will contain a heterogenous population of transformed cells. It is possible that the characteristics of populations of cancer cells within a single tumour may vary as much as the four

cell lines examined in this thesis. Using the MCF-7Adr cell line as an example, this ER negative cell line showed no growth response to E_2 or EGF, however these cells did show a much higher growth rate in basal media suggesting that they were able to regulate their own growth. Evidence from western blots would tend to substantiate this claim since the cells showed a high level of tyrosine phosphorylation when compared to the parent ER positive line.

It was interesting to note that the phosphoproteins which were only present in the parent MCF-7WT cell line under growth factor stimulation, were also present in unstimulated MCF-7Adr cells. The three phosphoproteins, named here as: p62, p40, and p36, the identity of which remains unknown appear to function as important regulators of cellular proliferation. This is assumed because MCF-7Adr cells which display unregulated growth in a depleted medium express the phosphoproteins, whilst the parent MCF-7WT cells which proliferate only in the presence of growth factors or E_2, will express the phosphoproteins only under growth factor stimulation. This gives clear evidence of an escape from normal regulated growth control in the MCF-7Adr cell line. There are a number of possible explanations for loss of growth control. Growth factor stimulated pathways may be permanently switched on in the ER negative cell line. A number of ER negative breast cancer cell lines are known to express high levels of TGF-α (Bates *et al.*,1988), although no particular study of the MCF-7Adr line has been made. If it is also true of the MCF-7Adr line, then the increased level of phosphotyrosine expression may be a reflection of unregulated autocrine growth factor stimulation. Loss of function of a specific tyrosine phosphatase, truncation of a growth factor receptor leading to constitutive activation of the

receptor, or oncogenic mutation of other molecules involved in mitogenic signalling eg. *ras* or *myc*, equally explain the increased levels of tyrosine phosphorylation.

It is important not to overlook the role of non tumourigenic cells in the growth of the tumour. Such cells will influence the growth of the tumour through: vascularization, structural support, or the synthesis of growth factors which act on the tumour cells in a paracrine manner. Many of these cells have been shown to be important to the overall growth of the tumour. Endothelial cells are required to form blood vessels in the tumour, whilst the fibroblast cells, often very prolific in breast tumours, appear to have an important regulatory function. Conditioned medium from breast tumour derived fibroblasts was examined for its mitogenic effects on breast cancer cell lines, in order to isolate the ability of fibroblasts to act as paracrine growth regulators. The two ER positive cell lines, MCF-7WT and ZR-75-1 both showed a significant growth response to the conditioned medium, whilst the two ER negative lines, MCF-7Adr and MDA-MB-231 showed a much lower level of growth response. This agrees with cell line growth responses to exogenous growth factors and it would appear fibroblast conditioned media is acting by a similar mechanism.

When all the data is brought together, throughout the full range of breast cancer cells. It is clear many factors can affect the tumour growth. In this study, growth effects have been found with E_2, a range of growth factors and fibroblast conditioned medium. It is also important to note the existence of autonomous unregulated growth in a number of breast cancer cell lines. If a single tumour was to contain a heterogenous population of cells with as wide a variation in

growth patterns as was found in the four cell lines examined here, where could a useful therapeutic target be found? Taking TGF-α, through activation of the EGF receptor, as the therapeutic target of choice, since it appears to be a growth mechanism common to both types of breast cancer cell, there is the danger that a cell will simply switch its growth response to another growth factor receptor. The cell may already be responding to a number of growth factors, and by blocking one mitogenic signal, it will simply increase its response to the other signals. A more useful target may actually be a much less specific one, such as: a general blockage of growth factor receptor activation, or therapy targeted to a substrate phosphoprotein common to all the growth factor signal transduction pathways. Until the identity and function of the tyrosine kinase receptor substrate proteins are known, it is difficult to predict at which point interference in the system will be most beneficial.

Although a reductionist approach has been adopted in this thesis, it is important to remember that cells growing within a tumour are open to the entire range of signals simultaneously. Autocrine, paracrine and hormonal factors are all likely to affect the growth of the tumour. Examination of the effects of E_2 treatment of fibroblast conditioned medium clearly indicates that these two factors together bring about a synergistic increase in the growth of the two ER positive cell lines tested, MCF-7WT and ZR-75-1. The mechanism by which E_2 and the fibroblasts are interacting is uncertain (see Chapter 6), but together they can increase the growth of ER positive cells beyond that achieved by either of the constituent parts. In most tumours the carcinoma cells will have access to both E_2 and fibroblast derived factors. Targeting therapy toward either one single

factor will have only a partial inhibitory effect on the tumour. To completely inhibit the growth of a tumour cell, all the factors involved in the growth of that cell need to be dealt with.

Tamoxifen, the widely used endocrine agent, working as an antiestrogen in ER positive breast cancer cells, is known to affect cell growth through a number of additional mechanisms. It has been found that tamoxifen can bind to and inhibit PKC (O'Brian *et al.*,1988) and inhibit cellular uptake of Ca^{2+} (Ferno *et al.*,1985), both of these are involved with cellular signal transduction. Other points in the membrane signal transduction cascade may also be affected, but as yet go unrecognised. The success of tamoxifen is due, in part, to its ability to affect cell growth regulation at a number of levels which together cause growth inhibition.

The tyrphostins, presently being developed as possible antitumour agents, are protein tyrosine kinase inhibitors that can inhibit growth factor stimulated proliferation. The tyrphostins can be designed to selectively inhibit activity of a single type of growth factor receptor, or they can exhibit a wide spectrum of activity against a range of protein tyrosine kinases (reviewed in, Levitzki & Gilon, 1991). It remains to be seen whether a specific or nonspecific approach will bring the greatest success. For the treatment of breast carcinoma where the tumour may often contain a heterogenous population of cancer cells a nonselective approach to the protein tyrosine kinases could be of greatest benefit. Where a tumour shows high expression of a growth factor receptor such as the EGF receptor, a tyrphostin showing increased activity against the EGF receptor but still able to inhibit other protein tyrosine kinases may be the most suitable therapeutic

agent.

As cellular mechanisms of signal transduction and growth control become more clearly understood, design of drugs targeting specific points in the signal transduction cascade will become more commonplace. The breast tumour which is growth regulated by a wide range of growth factors and hormonal agents would certainly be expected to benefit from such new types of treatment.

CHAPTER 8

REFERENCES

CHAPTER 8

REFERENCES

Adams,E.,Newton,C.,Brainsberg,H.,Shaikh,N.,Ghilchik,M. & James,V. (1988)
Effects of human breast fibroblasts on growth and 17ß-estradiol dehydrogenase activity of MCF-7 cells in culture
Breast Cancer Research and Treatment, Vol.11, pp165-172

Adriane,J.,Gaudray,P.,Dionne,C.,Crumley,G.,Jaye,M.,Schlessinger,J.,Jeanteur,P., Birnbaum,D.& Theillet,C. (1991)
BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers
Oncogene, Vol.6, pp659-663

Akiyama,T.,Ishida.J.,Nakagawa,S.,Ogawara,H.,Watanabe,S.,Hoh,N.,Shibuya,M.& Fukami,Y. (1987)
Genistein, a specific inhibitor of tyrosine-specific protein kinases
J.Biol.Chem., Vol.262, No.12, pp5592-5595

Arrick,B.,Korc,M.,Derynck,R. (1990)
Differential regulation of expression of three transforming growth factor ß species in human breast cancer cell lines by estradiol
Cancer Research, Vol.50, pp299-303

Arteaga,C.,Tandon,A.,VonHoff,D.,Osborn,C. (1988)
Transforming growth factor ß:potential growth inhibitor of estrogen receptor-negative human breast cancer cells
Cancer Research, Vol.48, pp3898-3904

Assoian,R.,Komoriya,A.,Meyers,C.,Miller,D.& Sporn,M. (1983)
Transforming growth factor-ß in human platlets
J. Biol. Chem., Vol.258, No.11, pp7155-7160

Barker,S.,Panahy,C.,Puddefoot,J.,Goode,A.,Vinson,G. (1989)
Epidermal growth factor receptor and oestrogen receptors in the non-malignant part of the cancerous breast
Br.J.Cancer, Vol.60, pp673-677

Barnes,D. & Sato,G. (1979)
Growth of a human mammary tumour cell line in a serum free medium
Nature, Vol.281, pp388-389

Baird,A. & Klagsbrun,M. (1991)
The fibroblast growth factor family
Cancer Cells, Vol.3, pp239-243

Basset,P.,Bellocq,J.,Wolf,C.,Stoll,I.,Hutin,P.,Limacher,J.,Podhajcer,O.,Chenard, M.,Rio,M. & Chambon,P. (1990)
A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas
Nature, Vol.348, pp699-704

Bates,S.,Davidson,N.,Valvarius,E.,Freter,C.,Dickson,R.,Tam,J.,Kudlow,J., Lippman,M. & Salomon,D. (1988)
Expression of transforming growth factor α and its messemger ribonucleic acid in human breast cancer: Its regulation by estrogen and its possible functional significance
Mol.Endo., Vol.2, No.6, pp543-555

Baulieu,E. (1987)
Steroid hormone antagonists at receptor level: a role for heat shock protein MW 90000 (hsp90)
J.Cell Biochem., Vol.35, pp161-174

Baxter,R.,Martin,J. (1989)
Binding proteins for the insulin-like growth factors:Structure regulation and function
Prog.Growth Factor Research, Vol.1,pp49-68

Beatson,G. (1896)
Suggestions for a new method of treatment, with illustrative cases
Lancet 2, 104-107, pp162-165

Bezwoda,W. & Meyer,K. (1990)
Effect of α-interferon, 17ß-estradiol; and tamoxifen of estrogen receptor concentration and cell cycle kinetics of MCF-7 cells
Cancer Research, Vol.50, pp5387-5391

Blackshear,P.,Haupt,D.,App,H.& Rapp,U. (1990)
Insulin activates the Raf-1 protein kinase
J.Biol.Chem., Vol.265, pp12131-12134

Borg,A.,Sigurdsson,H.,Clark,G.,Ferno,M.,Fuqua,S.,Olsson,H.,Killander,D.& McGuire,W. (1991)
Association of INT2/HST1 coamplification in primary breast cancer with hormone dependent phenotype and poor prognosis
Br. J. Cancer, Vol.63, pp136-142

Borgna,J. & Rochefort,H. (1981)
Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues
J.Biol.Chem., Vol.256, pp859-868

Bretscher,A. (1989)
Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A431 cells by epidermal growth factor
J.Cell Biol.,Vol.108, pp921-930

Bronzert,D.,Pantazis,P.,Antoniades,H.,Kasid,A.,Davidson,N.,Dickson,R. and Lippman,M. (1987)
Synthesis and secretion of platlet derived growth factor by human breast cancer cell lines
Proc. Natl. Acad. Sci., USA, Vol.84, pp5763-5767

Burg van der,B.,deGroot,R.,Isbrucker,L.,Kruyer,W.,deLaat,S. (1991)
Direct stimulationby estrogen of growth factor signal transduction pathways in human breast cancer cells
Abstract 406, 4th International Congress on Hormones and Cancer, Sept 15-19, 1991

Burgess,A. (1989)
Epidermal growth factor and transforming growth factor α.
British Medical Bulletin, Vol.45, No.2, pp401-424

Cailleau,R.,Young,R.,Olive,M. and Reeves,W.(1974)
Breast tumour cell lines from pleural effusions
Journal of the National Cancer Institute.,Vol.53,No.3.

Cantley,L.,Auger,K.,Carpenter,C.,Duckworth,B.,Graziani,A.,Kapeller,R. and Soltoff,S. (1991)
Oncogenes and signal transduction
Cell, Vol.64,pp281-302

Chao,M., (1992)
Growth factor signalling: where is the specificity?
Cell, Vol.68,pp995-997

Chen,L.,Shulman,L.& Revel,M. (1991)
IL-6 receptors and sensitivity to growth inhibition by IL-6 in clones of human breast carcinoma cells
J.Biol.Regulators & Homeostatic Agents, Vol.5(4), pp125-136

Chen,W.,Lazar,C.,Poenie,M.,Tsien,R.,Gill,G.& Rosenfeld,M. (1987)
Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor
Nature, Vol.328, pp820-823

Ciardiello,F.,Kim,N.,Liscia,D.,Bianco,C.,Lidereau,R.,Merlo,G.,Callahan,R., Greiner,J.,Szpak,C.,Kidwell,W.,Schlom,J.,Salomon,D. (1989)
mRNA expression of transforming growth factor alpha in human breast carcinomas and its activity in effusions of breast cancer patients

J.N.C.I., Vol.81, No.15, pp1165-1171

Clarke,R.,Dickson,R.& Brunner,N. (1990)
The process of malignant progression in human breast cancer
Annals of Oncology, Vol.1, pp401-407

Clarke,R.,Brunner,N.,Katz,D.,Glanz,P.,Dickson,R.,Lippman,M.,Kern,F. (1989)
The effects of a constitutive expression of trnasforming growth factor-α on the growth of MCF-7 human breast cancer cells in vitro and in vivo
Mol. Endo., Vol.3, No.2, pp372-380

Clemmons,D.,Underwood,L.,Van Wyke,J. (1981)
Hormonal control of immunoreactive somatomedin production by human fibroblasts
J. Clin. Invest., Vol.67, pp10-17

Coezy,E.,Borgna,J.,Rochefort,H. (1982)
Tamoxifen and metabolites in MCF-7 cells: Correlation between binding to estrogen receptor and cell growth inhibition
Cancer Research, Vol.42, pp317-323

Cohen.S., (1962)
Isolation of a mouse submaxillary gland protein accelarating incisor erruption and eyelid opening in the new born animal.
J.Biol.Chem., Vol.237, pp1555-1562

Colletta,A.,Wakefield,L.,Howell,F.,van Roozendaal,K.,Danielpour,D.,Ebbs,S., Sporn,M.& Baum,M. (1990)
Anti-estrogens induce the secretion of active transforming growth factor beta from human fetal fibroblasts.
Br.J.Cancer, Vol.62, pp405-409.

Cormier,E. & Jordan,V. (1989)
Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor.
Eur.J.Cancer Clin.Oncol., Vol.25, No.1, pp57-63

Cullen,K.,Smith,H.,Hill,S.,Rosen,N. & Lippman,M. (1991)
Growth factor messenger RNA expression by human fibroblasts from benign and malignant lesions
Cancer Research, Vol.51, pp4978-4985

Cullen,K.,Yee,D.,Sly,W.,Perdue,J.,Hampton,B.,Lippman,M.,Rosen,N. (1990)
Insulin like growth factor receptor expression and function in human breast
Cancer Research, Vol.50,pp48-53

Cunha,G. & Young,P. (1992)
Role of stroma in oestrogen-induced epithelial proliferation

Epith.Cell Biol., Vol.1, pp18-31

Czech, M. (1982)
Structural and functional homologies in the receptors for insulin and insulin-like growth factors.
Cell, Vol.31, pp8-10

Dabre,P.,Curtis,S.,King,R. (1984)
Effects of oestradiol and tamoxifen on human breast cancer cells in serum free culture
Cancer Research, Vol.44, pp2790-2793

Dabre,P. & Daly,R. (1989)
Effects of oestrogen on human breast cancer cells in culture
Proceedings of the Royal Society of Edinburgh, pp119-132 Vol.95.

Dabre,P., Yates,J.,Curtis,S. & King,R. (1983)
Effect of estradiol on human breast cancer cells in culture
Cancer Research, Vol.43, pp349-354

Daly,R. & Darbre,P. (1990)
Cellular and molecular events in loss of estrogen sensitivity in ZR-75-1 and T47D human breast cancer cells
Cancer Research, Vol.50, pp5868-5875

Dano,K.,Andreasen,P.,Grondahl-Hansen,J.,Kristensen,P.,Neilson,L.,Skriver,L. (1985)
Plasminogen activators, tissue degradation and cancer
Adv. Cancer Research, Vol.44, pp139-266

Dati,C.,Antoniotti,S.,Taverna,D.,Perroteau,I.,DeBortoli,M. (1990)
Inhibition of c-erbB2 oncogene expression by estrogens in human breast cancer cells
Oncogene, Vol.5, pp1001-1006

Davidson,J.,Klagsburn,M.,Hill,K.,Buckley,A.,Sullian,R.,Brewer,P. and Woodward,S. (1985)
Accelarated wound repair, cell proliferation and collagen accumulation are produced by a cartiledge-derived growth factor
J.Cell Biol., Vol.100, p1219

Davidson,N.,Gelmann,E.,Lippman,M.& Dickson,R. (1987)
Epidermal growth factor receptor gene expression in estrogen receptor positive and negative human breast cancer cell lines.
Mol. Endo., Vol.1, No.3, pp216-223

DeLarco,J. & Todaro,G. (1978)
Growth factors from murine sarcoma virus transformed cells
Proc.Natl.Acad.Sci., USA, Vol.75, pp4001-4005

Derynck,R.,Goeddel,D.,Ullrich,A.,Gutterman,J.,Williams,R.,Bringman,T. and Berger,W. (1987)
Synthesis of messenger RNAs for transforming growth factors α and ß and the epidermal growth factor in human tumours
Cancer Research, Vol.47, pp707-712.

[b] Dickson,R.,Bates,S.,McManaway,M.& Lippman,M. (1986)
Characterisation of estrogen responsive transforming activity in human breast cancer cell lines
Cancer Research, Vol.46, pp1707-1713

[a] Dickson,R.,McManaway,M.,Lippman,M. (1986)
Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumour growth
Science, Vol.232, pp1540-1542

Dionne,C.,Crumley,G.,Bellot,F.,Kaplow,J.,Searfoss,G.,Ruta,M.,Burgess,W.,Jaye, M.& Schlessinger,J. (1990)
Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors
EMBO, Vol.9, pp2685-2692

Dong,X.,Berthois,Y.& Martin,P. (1991)
Effect of epidermal growth factor on the proliferation of human epithelial cancer cell lines: Correlation with the level of occupied EGF receptor.
Anticancer Research, Vol.11, pp737-744

Duffy,M. (1990)
Plasminogen activators and cancer
Blood Coagulation and Fibrinolysis, Vol.1(6), pp681-687

Duffy,M.,Reilly,D.,O'Sullivan,C.,O'Higgins,N.,Fennelly,J.& Andreasen,P. (1990)
Urokinase-plasminogen activator, a new independent prognostic marker in breast cancer
Cancer Research, Vol.50, pp6827-6829

Dvorak,H.,Dvorak,A.,Manseau,E.,Wiberg,L.,Churchill,W. (1979)
Fibrin gel investment associated with line 1 and line 10 solid tumour growth, angiogenesis and fibroplasia in guinea pigs: Role of cellular Immunity, myofibroblasts, microvascular damage and infarction in line 1 tumour regression
J.N.C.I., Vol.62, pp1459-1472

Dvorak,H.,Senger,D.,Dvorak,A. (1983)
Fibrin as a component of the tumour stroma: Origins and biological significance
Cancer Metast.Rev., Vol.2(1), pp41-73

Early breast cancer trials collaborative group, (1988)
Affects of adjuvant tamoxifen and cytotoxic therapy on mortality in early breast cancer
New England Journal Medicine, Vol.319, pp1681-1692

Eisemann,A.,Ahn,J.,Graziani,G.,Tronick,S. and Ron,D. (1991)
Alternative splicing generates at least five different isoforms of the human basic-FGF receptor
Oncogene, Vol.6, pp1195-1202

Ellis,C.,Moran,M.,McCormick,F. and Pawson,T. (1990)
Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases
Nature Vol.343 pp377-380

Engel,L.,Young,N.,Tralka,T.,Lippman,M.,O'Brien,S.,Joyce,M. (1978)
Establishment and Characterisation of three new continuous cell lines derived from human breast carcinomas
Cancer Research, Vol.38, p3352

Ennis,B.,Valverius,E.,Bates,S.,Lippman,M.,Bellot,F.,Kris,R.,Shlessinger,J.,Masui, H.,Goldenberg,A.,Mendelsohn,J.,Dickson,R. (1989)
Anti-epidermal growth factor receptor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells
Mol.Endo., Vol.3, No.11, pp1830-1838

Ervin,P.,Kaminski,M.,Cody,R.,Wicha,M. (1989)
Production of mammostatin, a tissue specific growth inhibitor by normal human mammary cells
Science, Vol.244, pp1585-1587

Evans,R. (1988)
The steroid and thyroid hormone receptor superfamily
Science, Vol.240, pp889-895

Favoni,R.,Cubbage,M.,Powell,D.,Lippman,M.,Rosen,N. & Yee,D. (1989)
Multiple insulin-like growth factor binding proteins are produced by human breast cancer cell lines
Br.Cancer Res.Treat., Vol.14:A, pp168

Ferno,M. & Borg,A. (1985)
Antiestrogen binding sites in human breast cancer biopsies
Anticancer Research, Vol.5, p307

Finch,P.,Rubin,J.,Miki,T.,Ron,D. and Aaronson,S. (1989)
Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth
Science, Vol.245, pp752-755

Fisher,B.,Costantino,J.,Redmond,C.,Poisson,R.,Bowman,D.,Couture,J.,Dimitrov,N., et al. (1989)
A randomized clinical trial evaluating tamoxifen in the treatment of patients with node negative breast cancer who have estrogen receptor positive tumours
New England Journal Medicine, Vol.320, pp479-484

Folkman,J. & Klagsbrun,M. (1987)
Angiogenic Factors
Science, Vol.235, pp442-447

Fuqua,S.,Fitzgerald,S.,Allred,D.,Elledge,R.,Nawaz,Z.,McDonnell,D.,O'Malley,B. ,Greene,G. & McGuire,W. (1992)
Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumours
Cancer Research, Vol.52, pp483-486

Fuqua,S.,Fitzgerald,S.,Chamness,G.,Tandon,A.,McDonnell,D.,Nawaz,Z., O'Malley,B. & McGuire,W. (1991)
Variant human breast tumour estrogen receptor with constitutive transcriptional activity
Cancer Research, Vol.51, pp105-109

Furlanetto,R. and DiCarlo,J. (1987)
Somatomedin-C receptors and growth effects in human breast cancer cells maintained in long term tissue culture
Cancer Research Vol.44,pp2122-2128

Furlanetto,R.,DiCarlo,J. and Wisehart,C. (1987)
The type II Insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts
J. Clinical Endocrin. and Metab., Vol.64, No.6, pp1142-1149

Giard,D.,Aaronson,S.,Todaro,G.,Arnstein,P.,Kersey,J.,Dosik,H.&Parks,W.(1973)
In vitro cultivation of human tumours: Establishment of cell lines derived from aseries of solid tumours
J.Natl.Cancer Inst., Vol.51, pp1417-1423

Glenney,J.,Tack,B. & Powell,M. (1987)
Calpactins: Two distinct Ca+ + regulated phospholipid and actin binding proteins isolated from lung and placenta
J.Cell Biol., Vol.104, pp503-511

Glover,J.,Irwin,J.,Dabre,P. (1988)
Interaction of phenol red with estrogenic and antiestrogen action on growth of human breast cancer cells ZR-75-1 and T47-D
Cancer Research, Vol.48, p3693,

Gomm,J.,Smith,J.,Ryall,G.,Baillie,R.,Turnbull,L. and Coombes,R. (1991)
Localization of basic fibroblast growth factor and transforming growth factor beta in the human mammary gland
Cancer Research, Vol.51,pp4685-4692

Graham,M.,Krett,N.,Miller,L.,Leslie,K.,Gordon,D.,Wood,W.,Wei,L.&Horwitz,K. (1990)
T47Dco cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancer to hormone resistance
Cancer Research, Vol.50, pp6208-6217

Gregory,H.,Thomas,C.,Willshire,I.,Young,J.,Anderson,H.,Baildam,A.,Howel,A. (1989)
Epidermal and transforming growth factor α in patients with breast tumours
Br.J.Cancer, Vol.59, pp605-609

Grey,A.,Schor,A.,Rushton,G.,Ellis,I.,Schor,S. (1989)
Purification of the migration stimulating factor produced by foetal and breast cancer patient fibroblasts
Proc.Natl.Acad.Sci., USA, Vol.86(7), pp2438-2442

Gullick,W. (1988)
A comparison of the structures of single polypeptide chain growth factor receptors that possess protein tyrosine kinase activity, in 'Hormones and their actions' eds. 'Cooke,B.,King,R. & van de Molen,H.' Elsevier Amsterdam, pp348-360

Gullick,W.,Love,S.,Wright,C.,Barnes,D.,Gusterson,B.,Harris,A.,Altman,D. (1991)
c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes
Br.J.Cancer, Vol.63, pp434-438

Habib,F. and Leake,R. (1987)
Quantitative assay of steroid receptors, in 'Steroid Hormones: A Practical Approach'
Eds., B.Green and R.Leake, IRL Press, Oxford, pp67-92

Haggie,J.,Sellwood,R.,Howell,A.,Birch,J.,Schor,S. (1987)
Fibroblasts from relatives of patients with hereditary breast cancer show fetal-like behaviour in vitro
The Lancet, June 27, pp1455-1457

Hamm,J. & Allegra,J. (1991)
New hormonal approaches to the treatment of breast cancer
Critical reviews in Oncology/Hematology,(II), pp29-41

Haslam,S. (1986)
Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen in vitro. Cancer Research, Vol.46, pp310-316

Heldin,C. (1992)
Structural and functional studies on platelet-derived growth factor.
EMBO Journal, 11(12),pp4251-4259.

Heldin C.,Westermark,B.,Wasteson,A. (1981)
Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia
Proc. Natl. Acad. Sci.,USA, Vol.78, pp3664-3668

Heldin,C.,Westermark,B.,Wasteson,A. (1980)
Chemical and biological properties of a growth factor from human-cultured osteosarcoma cells: Resemblance with platelet-derived growth factor
J.Cellular Physiol., Vol.105, pp235-246

Henry,J.,Piggot,N.,Mallick,U.,Nicholson,S.,Farndon,J.,Westley,B.& May,F.(1991)
pNR-2/pS2 immunolocalisation staining in breast cancer: correlation with prognostic factors and endocrine response.
B.J.C. Vol.63, pp152-156

Horgan,K.,Jones,D. & Mansel,R. (1987)
Mitogenicity of human fibroblasts in vivo for human breast cancer cells
Br.J.Surg., Vol.74, pp227-229

Horwitz,K. (1992)
Cellular heterogeneity and mutant oestrogen receptors in hormone resistant breast cancer
Cancer Surveys, Vol.14 'Growth regulation by nuclear hormone receptors' ICRF, pp41-53

Horwitz,K. & McGuire,W. (1978)
Estrogen control of progestrone receptor in human breast cancer
J.Biol.Chem., Vol.253, No.7, pp2223-2228

Howell,A.,Dodwell,D.,Laidlaw,I.,Anderson,H.,Anderson,E. (1990)
Tamoxifen as an agonist for metastatic breast cancer, in 'Endocrine therapy of breast cancer IV', eds.,A.Goldhirsch,
Springer-Verlag, London, pp49-58

Huff,K.,Kaufman,D.,Grabbay,K.,Spencer,E.,Lippman,M. and Dickson,R. (1986)
Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells
Cancer Research, Vol.46,pp4613-4619

Hunter,T. (1989)
Protein-tyrosine phosphatases: The other side of the coin
Cell, Vol.58, pp1013-1016

Ignetz,R.,Massague,J. (1986)
Transforming growth factor-ßstimulates the expression of fibronectin adn collagen and their incorporation into the extracellular matrix

J.Biol.Chem.,Vol.261, No.9, pp4337-4345

Isaacs,J. (1988)
Clonal heterogeneity in relation to response
In:Endocrine management of cancer, Vol.1, Ed.Stoll,B.
Basel:Karger, pp125-140

Jammes,H.,Peyrat,J.,Ban,E.,Vilain,M.,Haour,F.,Dijiane,J.andBonneterre,J.(1992)
Insulin-like growth factor I receptors in human breast tumour: Localisation and quantification by histo-autoradiographic analysis
Br.J.Cancer, Vol.66,pp248-253

Jordan,V. (1990)
Estrogen receptor mediated direct and indirect antitumor effects of tamoxifen
J.N.C.I., Vol.82, No.21, pp1662-1663

Jordan,V.,Phelps,E.,Lingren,J. (1987)
Effects of antioestrogens on bone in castrated and intact female rats
Breast Cancer Research & Treatment, Vol.10, pp31-35

Kaibuchi,K.,Fukumoto,Y.,Oku,N.,Hori,Y.,Yamamoto,T.,Toyoshima,K.&Takai,Y. (1989)
Activation of the serum response element and 12-0-tetradecaoylphobol-13-acetate response element by activated c-raf-1 protein ina manner independent of protein kinase C
J.Biol.Chem.,Vol.264, pp20855-58

Kao,R.,Hall,J.,Engel,L. & Stem,R. (1984)
The matrix of human breast tumor cells is mitogenic for fibroblasts
Am.J.Pathol., Vol.115, pp109-116

Kaplan,D.,Morrison,D.,Wong,G.,McCormick,F.& Williams,L. (1990)
PDGFß receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex
Cell, Vol.61, pp125-133

Katzenellenbogen,B.,Kendra,K.,Norman,M.,Berthois,Y. (1987)
Proliferation,hormonal responsiveness and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens
Cancer Research, Vol.47, pp4355-4360

Kawamoto,T.,Mendelsohn,J.,Le,A.,Sato,G.,Lazar,C.,Gill,G. (1984)
Relationship of epidermal growth factor receptor concentration to growth of human epidermoid carcinoma A431 cells
J.Biol.Chem., Vol.259, pp7761-7766

Keski-Oja,J.,Lyons,R. and Moses,H. (1987)
Inactive secreted form(s) of transforming growth factor-beta: activation by proteolysis

J.Cell Biochem.Suppl., Vol.11A, p60

Kidereau,R.,Callahan,R.,Dickson,C.,Peters,G.,Escot,C. & Ali,I. (1988)
Amplification of the INT-2 gene in primary human breast tumours
Oncogene Research, Vol.2, pp285-291

Koenders,P.,Beex,L.,Geunts-Moespot,A.,Heuval,J.,Kienhuis,C.& Benraad,T. (1991)
Epidermal growth factor receptor negative tumours are predominantly confined to a subgroup of estradiol receptor positive human primary breast cancers
Cancer Research, Vol.51, pp4544-4548

Knabbe,C.,Lippman,M.,Wakefield,L.,Flanders,K.,Kasid,A.,Derynck,R.& Dickson,R. (1990)
Evidence that transforming growth factor-ß is a hormonally regulated negative growth factor in human breast cancer cells
Cell, Vol.48, pp417-428

Koch,C.,Anderson,D.,Moran,M.,Ellis,C.& Pawson,T. (1991)
SH2 andSH3 Domains: Elements that control interactions of cytoplasmic signalling proteins
Science Vol.252, pp668-674

Kraus,M.,Popescu,N.,Amsbaugh,S.,King,C. (1987)
Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumour cell lines by different molecular mechanisms
EMBO, Vol.6, pp605-610

Kypta,R.,Goldberg,Y.,Ulug,E. and Courtreidge,S. (1990)
Association between the PDGF receptor and members of the src family of tyrosine kinases
Cell, Vol.62,pp481-92

Kyriakis,J.,App,H.,Zhang,X.,Banerjee,P.,Brautigan,D.,Rapp,U.&Avruch,J.(1992)
Raf-1 activates MAP kinase-kinase
Nature, Vol.358, pp417-421

Leake,R.,Freshney,R. & Munir,I. (1987)
Steroid response in vivo and in vitro, in 'Steroid hormones a practical approach',ed. B.Green & R.Leake.
IRL Press Ltd, Oxford, England, pp213-214

Levitzki,A. & Gilon,C. (1991)
Tyrphostins as molecular tools and potential antiproliferative drugs
Trends in Pharmaceutical Science, Vol.12, pp171-174

Li.P.,Wood,K.,Marnon,H.,Haser,W.& Roberts,T.(1991)
Raf-1: A kinase currently without a cause but not lacking in effects
Cell, Vol.64, pp479-482

Lippman,M. & Aitken,S. (1981)
Estrogen and antiestrogen effects on thymidine utilization by human breast cancer cells in tissue culture, in 'Progress in cancer research and therapy' eds. Lacobelli,S.,King,R.,Linder,H. & Lippman,M.
New York, Raven Press, pp1-10

Lippman,M. (1981)
Hormonal regulation of human breast cancer cells in vitro
Banbury Rep., Vol.8, pp171-181

Lippman,M. (1991)
Growth factor control of normal and malignant mammary call growth
Abstract 310, 4th International Congress on Hormones and Cancer, Sept. 15-19, Amsterdam

Lippman,M.,Dickson,R.,Bates,S.,Knabbe,C.,Huff,K.,Swain,S.,McManaway,M., Bronzert,D.,Kasid,A.,Gelmann,E. (1986)
Autocrine and paracrine growth regulation of human breast cancer
Breast Cancer Research and Treatment, Vol.7, pp59-70

Li,S. and Shipley,G. (1991)
Expression of multiple species of basic fibroblast growth factor mRNA and protein in normal and tumour derived mammary epithelial cells in culture
Cell Growth and Differentiation, Vol.2, pp195-202

Livneh,E.,Dull,T.,Prynes,R.& Schlessinger,J. (1988)
Release of phorbol ester-induced mitogenic block by mutation at Thr 654 of epidermal growth factor receptor
Mol.Cell Biol., Vol.8, pp2302-2308

Long,B.,McKibben,B.,Lynch,M.& van den Berg,H. (1992)
Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75-1 human breast cancer cell line.
Br.J.Cancer, Vol.65, pp865-869

Love,R.,Newcomb,P.,Wiebe,D.,Surawicz,T.,Jordan,V.,Carbone,P.,DeMets,D. (1988)
Lipid and lipoprotein effects of tamoxifen therapy in postmenopausal women with node negative breast cancer
Breast Cancer Res. Treat., Vol.12, pp307-310

Madsen,M.,Briand,P. (1990)
Relationship between tumorigenicity,in vitro invasiveness and plasminogen activator production of human breast cancer cell lines
Euro.J.Cancer, Vol.26(7), pp793-797

Malden,L.,Novak,U.,Burgess,A. (1989)
Expression of transforming growth factor alpha messenger RNA in normal and neoplastic gastrointestinal tract

Int.J.Cancer, Vol.43(3), pp380-384

Mansukhani,A.,Moscatelli,D.,Talarico,D.,Levytska,V.& Basilico,C.
A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF
Proc. Natl. Acad. Sci.,USA, Vol.87, pp4378-4382

Margolis,B.,Rhee,S.,Felder,S.,Mervic,M.,Lyall,R.,Levitzki,A.,Ullrich,A.,Zilberstein,A.& Schlessinger,J. (1989)
EGF induces phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signalling
Cell, Vol.57, pp1101-1107

Masiakowski,P.,Breathnach,R.,Bloch,J.,Gannon,R.,Krust,A.,Chambon,P. (1982)
Cloning of cDNA sequences of hormone regulated genes from the MCF-7 human breast cancer cell lines
Nucleic Acids Res., Vol.10, p7895

May,F.,Westley,B. (1988)
Identification and characterization of estrogen-regulated RNA's in human breast cancer cells
J.Biol.Chem., Vol.263, No.26, pp12901-12908

McCormick,F. (1989)
ras GTPase activating protein: signal transmitter and signal terminator
Cell, Vol.56, pp5-8

Meisenhelder,J.,Suh,P.,Rhee,S.& Hunter,T. (1989)
Phospholipase C-y is a substrate for the PDGF and EGF receptor protein tyrosine kinases in vivo and in vitro
Cell, Vol.57, pp1109-1122

Merlino,G. (1990)
Epidermal growth factor receptor regulation and function
Cancer Biology, Vol.1, pp277-284

McGuire,W.,Carbone,P.,Sears,M. & Escher,G. (1975)
Estrogen receptors in breast cancer: an overview, in 'Oestrogen receptors in breast cancer'
eds. McGuire,W.,Carbone,P.,Vollmer,E., New York Raven Press, pp1-7

Mira-y-Lopez,R.,Osborne,M.,DePalo,A.,Ossowski,L. (1991)
Estradiol modulation of plasminogen activator production in organ cultures of human breast carcinomas: Correlation with clinical outcome of anti-estrogen therapy
International J.Cancer, Vol.47(6), pp827-832

Montcourrier,P.,Mangeat,P.,Salazar,G.,Morisset,M.,Sahuquet,A.,Rochefort,H. (1990)
Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic

vesicles
Cancer Research, Vol.50(18), pp6045-6054

Moscatelli,D.,Quarto,N. (1989)
Transformation of NIH 3T3 cells with basic fibroblast growth factor or the hst/K-fgf oncogene cause downregulation of the fibroblast growth factor receptor: Reversal of morphological transformation and restoration of receptor number by suramin
J. Cell Biol. Vol.109, pp2519-2527

Moses,H.,Tucker,R.,Leof,E.,Coffey,R.,Halper,J.& Shipley,G. (1985)
Type beta transforming growth factor is a growth stimulator and a growth inhibitor
Cancer Cells, Vol.3, pp65-67

Moses,H.,Yang,E.& Pietenpol,J.(1990)
TGF-ß stimulation and inhibition of cell proliferation:New mechanistic insights
Cell, Vol.63, pp245-247

Mosmann,T. (1983)
Rapid colorimetric assay for cell growth and survival: Application to proliferation and cytotoxicity assays
J.Immunol.Methods, Vol.65, pp55-63

Nicholson,S.,Richard,J.,Sainsbury,C.,Halcrow,C.,Kelly,P.,Angus,B.,Wright,C., Henry,J.,Farndon,J. & Harris,A. (1991)
Epidermal growth factor receptor (EGFr): Results of a 6 year follow-up study in operable breast cancer with emphasis on the node negative subgroup
Br.J.Cancer, Vol.63, pp146-150

Nister,M.,Heldin,C.,Wasteson,A. & Westermark,B. (1984)
A glioma derived analog to platelet derived growth factor: Demonstration of receptor competing activity and immunological cross reactivity
Proc. Nat. Acad.Sci., USA, Vol.81, pp926-930

O'Brian,C.,Housey,G.,Wienstein,I. (1988)
Specific and direct binding of protein kinase C to an immobilized tamoxifen analogue
Cancer Research, Vol.48, pp3626-3629

Osborne,C.,Boldt,D.,Estrada,P. (1984)
Human breast cancer cell synchronization by estrogens and antiestrogens in culture
Cancer Research, Vol.44, pp1433-1439

Owens,O.,Stewart,C., Brown,I.& Leake,R.(1991)
Epidermal growth factor receptors (EGFR) in human ovarian cancer
Br. J. Cancer 64,pp907-910

Pepinsky,R. and Sinclair,L. (1986)
Epidermal growth factor dependent phosphorylation of lipocortin
Nature Vol.321 pp81-84

Perez,R.,Pascual,M.,Macias,A.,Lage,A. (1984)
Epidermal grwoth factor receptors in human breast cancer
Breast Cancer Research & Treatment, Vol.4, pp189-193

Perez,R.,Betsholtz,C.,Westermark,B.& Heldin,C. (1987)
Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines.
Cancer Research, Vol.47 pp3425-3429

Popliker,M.,Shartz,A.,Avivi,A.,Ullrich,A.,Schlessinger,J.,Webb,C. (1987)
Onset of endogenous synthesis of epidermal growth factor in neonatal mice
Dev.Biol., Vol.119, pp38-44

Rechlar,M.,Zapf,J.,Nissley,S.,Froesch,E.,Moses,A.,Podskalny,J.,Schilling,E., Humbel,R. (1980)
Interactions of insulin-like growth factors I and II and multiplication - Stimulating activity with receptors and serum carrier proteins.
Endocrinology, Vol.107, p1451

Reddy,K.,Mangold,G.,Tandon,A.,Yoneda,T.,Mundy,G.,Zilberstein,A. & Osborne,C. (1992)
Inhibition of breast cancer cell growth in vitro by a tyrosine kinase inhibitor
Cancer Research, Vol.52, pp3636-3641

Rios,M.,Macias,A.,Perez,R.,Lage,A.,Skoog,L. (1988)
Receptors for epidermal growth factor and estrogen as predictors of relapse in patients with mammary carcinoma
Anticancer Research, Vol.8, p173

Roberts.R.,Gallagher,J.,Spooncer,E.,Allen,T.,Bloomfield,F. (1990)
Heparan sulphate bound growth factors: amechanism for stromal cell mediated haemopoiesis
Nature, Vol.332, pp376-378

Roberts,A.,Anzano,M.,Wakefield,L.,Roche,N.,Stern,D.,Sporn,M. (1985)
Type ß transforming growth factor: A bifunctional regulator of cellular growth
Proc.Natl.Acad.Sci., USA, Vol.82, pp119-123

Rochefort,H. (1990)
Cathepsin-D in breast cancer
Breast Cancer Research and Treatment, Vol.16, pp3-13

Rochefort,H.,Borgna,J.,Evans,E. (1983)
Cellular and molecular mechanisms of action of antiestrogens
J.Steroid.Biochem., Vol.19(1A), pp69-74

Rochefort,H.,Cavailles,V.,Augereau,P.,Capony,F.,Maudelonde,T.,Touitou,I., Garcia,M. (1989)
Overexpression and hormonal regulation of pro-cathepsin D in mammary and endometrial cancer
J.Steroid Biochem., Vol.34(1-6), pp177-182

Rohlik,Q.,Adams,D.,Kall,F.,Jacobs,S. (1987)
An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7WT cells in tissue culture
Biochem. Biophy. Research Comm. Vol.149, No.1, pp276-281

Roozendaal van,C.,vanOoijen,B.,Klijn,J.,Claussen,C.,Eggermont,A., Henzen-Logmans,S.,Foekens,J. (1992)
Stromal Influences on Breast Cancer Cell Growth
Br.J.Cancer, Vol.65, pp79-81

Ross,T.,Tait,J.,Majerus,P. (1990)
Identity of inositol 1,2-cyclic phosphate 2-phosphohydrolase with lipocortin III
Science, Vol.248, pp605-607

Rozengurt,E. (1992)
Growth factors and cell proliferation
Current Opinion in Cell Biology, Vol.4, pp161-165

Rubin,J.,Osada,H.,Finch,P.,Taylor,W.,Rudikoff,S.,Aaronson,S. (1989)
Purification and characterisation of a newly identified growth factor specific for epithelial cells
Proc. Natl. Acad. Sci., Vol.86, pp802-806

Sainsbury,J.,Farndon,J.,Needham,G.,Malcolm,A.,Harris,A. (1987)
Epidermal growth factor receptor status as predictor of early recurrence of and death from breast cancer
Lancet (i), p1398

Sainsbury,J.,Sherbert,G.,Farndon,J.,Harris,A. (1985)
Epidermal growth factor receptors and oestrogen receptors in human breast cancer
Lancet (i), p364

Salomon,D.,Zwiebel,J.,Bano,M.,Losonczy,I.,Fehnel,P.& Kidwell,W. (1984)
Presence of transforming growth factors in human breast cancer cells
Cancer Research, Vol.44, pp4069-4077

Schlessinger,J. (1988)
Signal transduction by allosteric receptor oligomerization
Trends Biochem.Sci., Vol.13 pp443-447

Schor,S.,Schor,A.,Grey,A.,Rushton,G.
Feotal and cancer patients fibroblasts produce an autocrine migration stimulating factor not made by normal adult cells

J.Cell Sci., Vol.90(Pt3), pp391-399

Segatto,O.,Lonardo,F.,Helin,K.,Fazioli,F.,Rhee,S.,Difiore,P. (1992)
erbB-2 autophosphorylation is required for mitogenic action and high affinity substrate coupling
Oncogene, Vol.7, pp1339-1346

Serger,D.,Galli,S.,Dvorak,A.,Perrozzi,C.,Harvey,V.,Dvorak,H. (1983)
Tumour cells secrete a vascular permeability factor that promotes accumulation of ascites fluid
Science, Vol.219, pp983-985

Silberstein,G.& Daniel,C. (1987)
Reversible inhibition of mammary gland growth by transforming growth factor-ß
Science, Vol.237, pp291-293

Sommers,C.,Walker-Jones,D.,Heckford,S.,Worland,P.,Valverius,E.,Clark,R.,Mc Cormick,F.,Stampfer,M.,Abularach,S.& Gelmann,E. (1989)
Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells.
Cancer Research, Vol.49, pp4258-4263.

Soule.H.,Vazquez,J.,Long,A.,Albert,S.& Brennan,M. (1973)
A human cell line from a pleural effusion derived from a breast carcinoma
J.Natl.Cancer Inst. Vol.51, pp1409-1416

Sporn,M. & Todaro,G. (1980)
Autocrine secretion and malignant transformation of cells
The New England Journal of Medicine, Oct.8, Vol.303, pp878-880

Sporn,M.,Roberts,A.,Wakefield,L.& de Crombrugghe,B. (1987)
Some recent advances in the chemistry and biology of transforming growth factor-beta
J.Cell.Biol., Vol.105, pp1039-1045

Sporn,M.,Roberts,A.,Wakefield,L.& Assoian,R. (1986)
Transforming growth factor-ß: Biological function and chemical structure
Science, Vol.233, pp532-34

Stahl,M.,Ferenz,C.,Kellener,K.,Kriz,R. & Knopf,J. (1988)
Sequence similarity of phospholipase C with the non-catalytic region of src
Nature, Vol.332, pp269-272

Starkey,R.,Cohen,S.,Orth,D. (1975)
Epidermal growth factor: Identification of a new hormone in human urine
Science, Vol.189, pp800-802

Sutherland,R.,Green,M.,Hall,R.,Reddel,R.,Taylor,I. (1983)
Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the Go/G1 phase of the cell cycle
Eur.J.Clon.Oncol., Vol.19, No.5, pp615-621

Sutherland,R.,Watts,C.,Hall,R.,Ruenitz,P. (1987)
Mechanisms of growth inhibition by nonsteroidal antiestrogens in human breast cancer cells
J.Steroid.Biochem., Vol.27, No.4-6, pp891-897

Sutherland,R.,Watts,C.,Ruenitz,P. (1986)
Definition of two distinct mechanisms of action of antiestrogens on human breast cancer cell proliferation using hydroxytriphenylethylenes with hi gh affinity for the estrogen receptor
Biochem.Biophys.Res.Comm., Vol.140, p523

Takahashi,K.,Suzuki,K.,Kawahara,S. and Ono,T. (1989)
Growth stimulation of human breast epithelial cells by basic fibroblast growth factor in serum-free medium
Int. J. Cancer, Vol.43, pp870-874

Tamin,I.,Cardinale,I.&Sehgal,P.(1991)
Interleukin-6 and 12-O-tetradecanoyl phorbol-13-acetate act synergistically in inducing cell-cell separation and migration of human breast carcinoma cells
Cytokine, Vol.3(3), pp212-223

Taylor,I.,Hodson,P.,Green,M.,Sutherland,R. (1983)
Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells
Cancer Research, Vol.43, pp4007-4010

Theillet,c.,Leroy,X.,DeLapeyriere,O.,Grosgeorges,J.,Adriane,J.,Raynaud,S., Simony-Lafontaine,J.,Goldfarb,M.,Escot,C.,Birnbaum,D. & Gaudray,P. (1989)
Amplification of FGF-related genes in human tumours: Possible involvement of HST in breast carcinoma
Oncogene, Vol.4, pp915-922

Thompson,A.,Kerr,D.& Steel,C. (1991)
Transforming Growth Factor ß1 is implicated in the failure of tamoxifen therapy in human breast cancer
Br.J.Cancer, Vol.63, pp609-614

Thompson,A.,Steel,C.,Foster,M.,Kerr,D.,Paterson,D.,Deane,D.,Hawkins,R.,Cart er,D.& Evans,H. (1990)
Gene expression in oestrogen-dependent human breast cancer xenograft tumours
Br.J.Cancer, Vol.62, pp78-84

Todaro,G.& Green,H. (1963)
Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines
J.Cell Biol., Vol.17, pp299-313

Todaro,G.,Fryling,C.,DeLarco,J. (1980)
Transforming growth factors produced by certain human tumour cells: Polypeptides that interact with epidermal grwoth factor receptors

Proc.Natl.Acad.Sci., USA, Vol.77, pp5258-5262

Richmond,A.,Lawson,D.,Nixon,D.,Stevens,J.,Chawla,R. (1983)
Extraction of a melanoma growth-stimulatory activity from culture medium conditioned by the Hs0294 human melanoma cell line
Cancer Research, Vol.43, pp2106-2112

Ullrich,A.& Schlessinger,J. (1990)
Signal transduction by receptors with tyrosine kinase activity
Cell, Vol.61, pp203-212.

Valverius,E.,Ciardiello,F.,Heldin,N.,Blondel,B.,Merlo,G.,Smith,G.,Stampfer,M., Lippman,M.,Dickson,R. & Salomon,D. (1990)
Stromal influences on transformation of human mammary epithelial cells overexpressing c-myc and SV40T
J.Cellular Physiol., Vol.145, pp207-216

Vickers,P.,Dixon,R.and Cowan,K.(1988)
A pleiotropic response associated with resistance of breast cancer cells to antineoplastic drugs and hormonal agents
TIPS Vol.9 pp443-445

Wakefield,L.,Smith,D.,Masui,T.,Harris,C.& Sporn,M. (1987)
Distribution and modulation of the cellular receptor for transforming growth factor-beta
J. Cell Biol., Vol.105, pp965-975

Wakeling,A. (1987)
In: Pharmacology and clinical uses of inhibitors of hormone secretion and action.
eds. Furr,B.,Wakeling,A.,
Eastbourne:Bailliere Tindall,(1).

Wakeling,A. (1989)
Comparitive studies on the effects of steroidal and nonsteroidal oestrogen antagonists on the proliferation of human breast cancer cells
J.Steroid Biochem., Vol.34, Nos.1-6, pp183-188

Wakeling,A. (1988)
Cellular mechanisms in tamoxifen action on tumours
Reviews on Endocrine Related Cancer, Vol.30, pp27-33

Wakeling,A.,Newboult,E.,Peters,S. (1989)
Effects of antiestrogens on the proliferation of MCF-7 human breast cancer cells
J.Mol.Endocrinol., Vol.2, pp1-10

Weaver,C.,Springer,P.& Katzenellenbogen,B. (1988)
Regulation of pS2 gene expression by affinity labelling and reversibly binding estrogens and anti-estrogens: comparison of effects on native gene and on pS2-chloramphenenicol acetyltransferase fusion genes transfected into MCF-7 human breast cancer cells.

J.Mol.Endo., Vol.2, pp936-945

Weidner,N.,Semple,J.,Welch,W. & Folkman,J. (1991)
Tumour angiogenesis and metastasis- Correlation in invasive breast carcinoma
New England J.Med., Vol.324, No.1, pp1-8

Welshons,W. & Jordan,V. (1987)
Adaptation of estrogen dependent MCF-7 cells to low estrogen (phenol red-free) culture
Eur.J.Cancer Clin. Oncol., Vol.23, No.12, pp1935-1939

Wilson,A.,Weatherill,P.,Nicholson,R.,Davies,P.,Wakeling,A. (1990)
A comparitive study of the interaction of oestradiol and the steroidal anti-osetrogen ICI 164,384 with the molybate-stabilized oestrogen receptor
J.Steroid.Biochem., Vol.35, pp421-428

Winstanley,J.,Cooke,T.,Murray,G.,Platt-Higgins,A.,George,W.,Holt,S., Myskor,M.,Spedding,A.,Barraclough,B.,Rudland,P. (1991)
The long term prognostic significance of c-erbB-2 in primary breast cancer
Br.J.Cancer, Vol.63, pp447-450

Wiseman,L.,Wakeling,A.,May,F. & Westley,B. (1989)
Effects of the antioestrogen ICI 164,384 on oestrogen induced RNAs in MCF-7 cells
J.Steroid Biochem., Vol.33, pp1-6

Yaish,P.,Gazit,A.,Gilon,C.,Levitzki,A. (1988)
Blocking of EGF dependent cell proliferation by EGF receptor kinase inhibitors.
Science, Vol.242, pp933-935

[b] Yarden,Y.& Ullrich,A., (1988)
Growth factor receptor tyrosine kinases
Annual Rev.Biochem., Vol.57, pp443-478

[a] Yarden,Y.and Ullrich,A. (1988)
Molecular analysis of signal transduction by growth factors
Biochemistry Vol.27, No.9,pp3113-3119

Yee,D., Cullen,K.,Paik,S.,Perdue,J.,Hampton,B.,Schwartz,A.,Lippman,M. and Rosen,N. (1988)
Insulin-like growth factor II mRNA expression in human breast cancer
Cancer Research, Vol.48,pp6691-6696

Yee,D.,Paik,S.,Lebovic.G.,Marcus,R.,Favoni,R.,Cullen,K.,Lippman,M.,Rosen,N. (1989)
Analysis of IGF-I gene expression in malignancy, evidence for a paracrine role in human breast cancer
Mol.Endocr., Vol.3,pp509-517

Yee,D.,Rosen,N.,Favoni,R.,Cullen,K. (1991)
Chap.5,:The insulin-like growth factors, their receptors and their binding proteins in human breast cancer. eds.Lippman,M.& Dickson,R.
Regulatory Mechanisms in Breast Cancer, Kluwer Academic Publishers, Boston.

Yoshida,S.,Kasuga,S.,Hirao,Y.,Fuwa,T.,Nakagawa,S. (1987)
Effect of biosynthetic human epidermal growth factor on the synthesis and sectretion of mucin glycoprotein from primary culture of rabbit fundal mucosa cells
In Vitro Cell Dev.Biol., Vol.23, pp460-464

*Nelson,J.,M^{c}Givern,M.,Walker,B.,Bailie,J.& Murphy,R. (1989)
Growth-inhibitory and growth-stimulatory effects of epidermal growth factor on human breast cancer cell line, MDA.MB.436: Dependence on culture conditions.
Eur.J.Cancer, Vol.25, No.12, pp1851-1855.

*Osborne,C.,Hamilton,B.,Titus,G.& Livingston,R. (1980)
Epidermal growth factor stimulation of human breast cancer cells in culture.
Cancer Research, Vol.40, pp2361-2366.

*Imai,Y.,Leung,C.,Friesen,H.& Shiu,R. (1982)
Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture.
Cancer Research, Vol.42, pp4394-4398.