The Pro-invasive Role of MMP-9 and c-Met in Faslodex-Resistant Breast Cancer

A thesis presented for the degree of
Doctor of Philosophy at Cardiff University by

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To My Parents

Abstract

The pure anti-oestrogen faslodex presents a valuable therapeutic option for post-menopausal women with endocrine-sensitive advanced breast cancer. However, emergence of resistance following long-term treatment constitutes a major clinical problem as faslodex-refractory disease is associated with poor prognosis. Consequently, elucidation of the mechanisms underlying resistance is imperative. An *in vitro* MCF-7 cell model of acquired resistance to faslodex (FAS-R) has been developed in our laboratory. Previous studies using this model revealed endocrine insensitivity to be accompanied by development of an invasive phenotype. Since proteolytic degradation of extracellular matrix components by matrix metalloproteinases (MMPs) is a prerequisite for tumour invasion and metastasis, the objective of this project was to explore the role of these proteases and tissue inhibitors of matrix metalloproteinases (TIMPs) in the aggressive behaviour of faslodex-resistant breast cancer cells. Additional studies were performed to identify the dominant growth factor signalling pathway regulating these pro-invasive events.

MMP and TIMP mRNA expression in FAS-R cells was variable. MMP-2 mRNA was increased in FAS-R cells compared with WTMCF-7 cells. Significantly, treatment with a broad-spectrum MMP inhibitor significantly reduced the invasive behaviour of FAS-R cells suggesting a central role for MMPs in FAS-R invasion. Importantly, FAS-R cells were found to overexpress c-Met receptor tyrosine kinase which, when activated by HGF/SF, induced latent MMP-9 protein expression and considerably augmented the motile, migratory and invasive capacities of these cells. Both ERK1/2 and PI3K/AKT pathways were activated by HGF/SF, and signalling through both resulted in increased secretion of latent MMP-9 protein. However, HGF/SF-enhanced FAS-R cell invasion was only suppressed by inhibition of the PI3K/AKT pathway or following treatment with the MMP inhibitor.

Collectively, these data suggest that in FAS-R cells HGF/SF/c-Met signalling enhances aggressive behaviour via PI3K-mediated MMP-9 secretion. c-Met may therefore present a therapeutic target in faslodex resistance.

Publications and Presentations

Published Abstracts

- Khirwadkar, Y., Jordan, NJ., Hiscox, SE. and Nicholson, R.I. Increased matrix metalloproteinase (MMP) expression in anti-oestrogen-resistant breast carcinoma cell lines. *Eur J Cancer 2005; 3 Suppl P19.*

- Khirwadkar, Y., Jordan, NJ., Hiscox, SE. and Nicholson, R.I. HGF/SF promotes an aggressive phenotype in Fulvestrant-resistant MCF-7 cells – evidence for MMP-9 and PI3K involvement. *Ann Oncol 2007; 18(Suppl. 4).*

Oral Presentations

- Khirwadkar, Y., Jordan, N., Hiscox, S. and Nicholson, R.I. (2005) Increased Matrix Metalloproteinase Expression in Anti-Oestrogen Resistant Breast Carcinoma Cell Lines. *9th Nottingham International Breast Cancer Conference.* Nottingham, UK.

Poster Presentations

- Khirwadkar, Y., Jordan, N., Hiscox, S. and Nicholson, R.I. (2007) HGF/SF Promotes an Aggressive Phenotype in Fulvestrant-Resistant MCF-7 Cells – Evidence for MMP-9 and PI3K Involvement. *5th International Symposium on Targeted Anticancer Therapies.* Amsterdam, The Netherlands.

- Khirwadkar, Y., Jordan, N., Hiscox, S. and Nicholson, R.I. (2006) Matrix Metalloproteinases in Endocrine Resistant Breast Cancer Cell Invasion. *2nd Tenovus/Astra Zeneca Annual Workshop.* Cardiff, UK.

- Khirwadkar, Y., Jordan, N., Hiscox, S. and Nicholson, R.I. (2006) Matrix Metalloproteinases in Endocrine Resistant Breast Cancer Cell Invasion. *Welsh School of Pharmacy Postgraduate Research Day.* Cardiff University, UK.

- Khirwadkar, Y., Jordan, N., Hiscox, S. and Nicholson, R.I. (2005) Increased Matrix Metalloproteinase Expression in Anti-Oestrogen Resistant Breast Carcinoma Cell Lines. *9th Nottingham International Breast Cancer Conference.* Nottingham, UK.

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Abbreviations

μ	micro-
A	amp
ADP	adenosine diphosphate
AF	activator function
AI	aromatase inhibitor
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
cDNA	complementary cDNA
C-terminal	carboxy-terminal (*cf.* protein structure)
DAPI	4'6-diamidino-2-phenylindole dihydrochloride
DCCM	defined cell culture medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	di-thiothreitol
E_2	17β-oestradiol
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMT	epithelial-mesenchymal transition

ER	oestrogen receptor
ERE	oestrogen response element
ERK	extracellular signal regulated kinase
EtBr	ethidium bromide
EtOH	ethanol
FAS-R	faslodex-resistant MCF-7 cell line
FCS	foetal calf serum
g (*cf.* centrifugal force)	gee *or* gravity
g (*cf.* weight)	gram
Gefitinib	4-(3-chloro-4-fluoroanilino)- 7-methoxy- 6-(3-morpholino propoxy) quinazoline
HCl	hydrochloric acid
HER	human epidermal growth factor receptor
HGF/SF	hepatocyte growth factor/scatter factor
HRP	horseradish peroxidase
HSP	heat shock protein
ICC	immunocytochemistry
IF	immunofluorescence
IgG	immunoglobulin G
K_2HPO_4	dipotassium hydrogen orthophosphate anhydrous
kDa	kilo Daltons
KH_2PO_4	potassium dihydrogen orthophosphate
L	litre
LY	Lilly
M	molar
m (as prefix)	milli-
MAPK	mitogen activated protein kinase
MCF	Michigan Cancer Foundation
MEK	MAP kinase extracellular signal regulated kinase

$MgCl_2$	magnesium chloride
min	minute
MMLV RT	Molony murine leukaemia virus reverse transcriptase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
mRNA	messenger RNA
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
$NaVO_4$	sodium orthovanadate
N-terminal	amino terminal (*cf.* protein structure)
OD	optical density
p-	phospho- (*c.f.* protein signalling activity)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	Parke Davies
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
RH	random hexamers
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation

Ser or S	Serine
SERD	selective oestrogen receptor downregulator
SERM	selective oestrogen receptor modulator
TAE	tris-acetate-EDTA buffer
TAM-R	tamoxifen-resistant MCF-7 cell line
TAM/TKI-R	tamoxifen-/gefitinib-resistant MCF-7 cell line
Taq	*Thermus aquaticus*
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-α	transforming growth factor alpha
TIMP	tissue inhibitor of metalloproteinase
TKI	tyrosine kinase inhibitor
TPA	12-*O*-tetradecanoylphorbal-13-acetate
Tris	tris(hydroxymethyl)aminomethane
Tween-20	polyoxyethylene-sorbitan monolaurate
Tyr or Y	Tyrosine
UV	ultra violet
V	volts
v/v	volume per volume
weight/volume	weight per volume
ZD1839	*see Gefitinib*

Chapter One

Introduction

"No problem can stand the assault of sustained thinking."

Francis Marie Arouet de Voltaire (1694-1778)

1 Introduction

1.1 Breast Cancer Incidence and Mortality Rates

The past decade has witnessed a sustainable decline in breast cancer mortality rates in Western Europe, Australia and North and South America; a development which is primarily due to the introduction of routine mammographic screening, more precise diagnosis and appropriate treatment regimes which notably includes extensive use of tamoxifen (Veronesi *et al.*, 2005, Boyle, 2005, Peto and Mack, 2000). In the UK, breast cancer mortality rates have declined steadily; and it is estimated that 8 out of 10 patients survive beyond five years as opposed to only 5 out of 10 in the 1970s (http://info.cancerresearchuk.org/cancerstats/types/breast/).

In contrast, the rates for breast cancer incidence have been rising steadily. Worldwide, more than 1 million women are diagnosed with breast cancer every year. The highest incidence rate is found in the USA (Jemal *et al.*, 2008). In the UK, over 45,500 women were diagnosed in 2006, which equals to about 125 women a day. (http://info.cancerresearchuk.org/cancerstats/types/breast/).

Breast cancer is now the most common female cancer in the UK. 1 in 9 women will contract the disease at some point in their lifetime (Quinn *et al.*, 2008) with approximately 80% of cases occuring in post-menopausal women. Furthermore, breast cancer is the second most common cause of death from cancer in women, second only to lung carcinoma. (http://info.cancerresearchuk.org/cancerstats/types/breast/).

As these statistics clearly demonstrate, breast cancer continues to constitute a major global health issue despite the not insubstantial advancement in terms of disease control. The failure to prevent incidence rates from rising is predominantly due to a failure to understand the underlying mechanisms of breast carcinogenesis and illustrates that breast cancer remains a complex

disease with an aetiology that is only poorly understood.

1.2 Risk Factors

Several risk factors thought to contribute to the development of breast cancer have been identified (**table 1.1**), however, many remain ill-defined and further epidemiological studies are needed to clarify their role. One of the most well-established risk factors is familial history of the disease, especially if linked with mutations in high penetrance breast cancer susceptibility genes such as BRCA1, BRCA2 and the tumour suppressor p53 (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). However, this genetic predisposition accounts for only a small percentage of all cases. The majority of breast tumours are caused by hormonal factors; and prolonged exposure to oestrogens, caused by early menarche, late first full-term pregnancy and late menopause or the use of exogenous hormones such as hormone replacement therapy and oral contraceptives have been identified as key risk factors (Beral, 2003; Berkey *et al.*, 1999; Hankinson *et al.*, 2004; Medina, 2004). The single greatest risk factor, however, is age; with the relative risk of developing the disease increasing significantly after the menopause (McPherson *et al.*, 2000; Veronesi *et al.*, 2005). Another established risk determinant is geographic location; as demonstrated by epidemiological studies that showed breast cancer to be more prevalent in Western populations such as the US and the UK as opposed to in countries such as India, Japan and China (McPherson *et al.*, 2000; Morton *et al.*, 2002; Parkin & Fernandez, 2006). Interestingly, studies of migrants who moved from low-incidence countries to high-incidence countries revealed that the rate of incidence of the host country was assumed within just one or two generations (Parkin, 2004; Ziegler *et al.*, 1993). Such findings emphasise that socio-economic status along with environmental and life-style factors such as weight, poor diet, increased alcohol consumption and lack of physical exercise also contribute to the risk of developing breast cancer (Boyle, 2005; Dumitrescu & Cotarla, 2005).

Risk factors	Relative risk
Family history, e.g. BRCA1 and BRCA2 gene mutations	≥ 2
Menarche before age 11	3
First full term pregnancy after age 40	3
Nulliparity or absence of breastfeeding	Relative risk falls by 4.3% for every 12 months of breastfeeding in addition to a 7% reduction for every birth
Onset of menopause after age 54	2
Use of hormone replacement therapy	1.66
Use of oral contraception	1.2
Age	Relative risk increases significantly after menopause
Geographic location, i.e. living in developed countries	5
Diet	1.5
Postmenopausal body weight, e.g. body mass index >35	2
Alcohol consumption	1.07

Table 1.1 Established and probable risk factors for breast cancer development. (Adapted from Veronesi *et al.*, 2005 and McPherson *et al.*, 2000).

1.3 Endocrine Therapy in the Treatment of ER-positive Breast Cancer

The functional link between oestrogens and breast cancer was first established by Albert Schinzinger who had observed that cessation in ovarian function resulted in atrophy of the breast (Schinzinger *et al.*, 1889). In 1896, George Beatson demonstrated mammary tumour regression following oophorectomy and thus introduced the first successful endocrine manipulation of advanced breast cancer (Beatson, 1896). It was the work of these two pioneers that established the concept of manipulation of the endocrine system as a treatment for breast cancer. In 1936, this hypothesis was further developed by Antoine Lacassagne who reasoned that, if breast cancer was due to an inherent sensitivity to oestrogen, the disease could be prevented through the use of agents that would antagonise the effects of oestrogen (Lacassagne, 1936). This essentially presents the rationale for present-day anti-oestrogen therapy which will be delineated in more detail in section 1.3.3. However, first an overview of oestrogens and their receptor and signalling functions in breast cancer is required before the mode of action of anti-hormone agents can be fully appreciated.

1.3.1 Breast Cancer and Oestrogens

Oestrogens are a group of sex steroid hormones synthesized from cholesterol and secreted by the ovaries and, to a lesser extent, by adrenal glands, adipose tissue and placenta. They occur naturally in several structurally related forms, the predominant intracellular oestrogen being 17β-estradiol (E_2) (**figure 1.1**) (Lewis & Jordan, 2005).

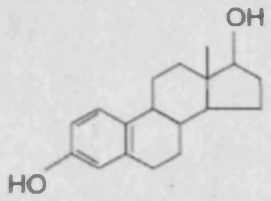


Figure 1.1 Structure of 17β-estradiol.
(from Nicholson and Johnston, 2005)

E_2 is secreted primarily by the ovaries and its synthesis is controlled by the pituitary gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Ali & Coombes, 2002). Despite the many beneficial effects oestrogens have on womens' health including cardiovascular protection and maintenance of bone mineral density, there is a wealth of evidence that supports an important role for E_2, in particular, in the stimulation and progression of malignant cancer of the breast (Morris & Wakeling, 2002). Oestrogens mediate most, if not all, of their functions via two receptors, ERα and ERβ (Gustafsson, 2000; Toft *et al.*, 1967), which belong to the nuclear receptor gene superfamily and function as ligand-activated transcription factors (Weinberg *et al.*, 2005). Whilst ERα (henceforth referred to as ER) is considered to be the predominant regulator of oestrogen-induced genes in breast cancer (Fuqua *et al.*, 2003), the role of ERβ in oestrogen-signalling is currently ill-defined and will therefore not be considered further in this thesis.

1.3.2 ER Structure and Signalling

Briefly, ER is a modular protein, consisting of 6 distinct functional domains, designated A-F, which are depicted in **figure 1.2** (Tsai & O'Malley, 1994). The A/B domain refers to the N-terminus and encodes a hormone-independent transcriptional activation function (AF-1), which is involved in transcriptional activation of target gene expression. Domain C corresponds to the highly conserved DNA binding domain (DBD). The DBD consists of two functionally distinct zinc-finger motifs which are responsible for the recognition and binding of oestrogen-response-elements (EREs) located in the promoter sequences of

oestrogen-regulated genes (Schwabe *et al.*, 1993). Domain D, the hinge region, separates the DBD from the ligand-binding domain (LBD). The flexibility resulting from this structure is thought to allow the conformational changes that occur in the receptor during its activation and is also important in receptor dimerisation. Domain E/F at the C-terminus of the ER molecule encodes the LBD, a hydrophobic pocket responsible for E_2 binding (Brzozowski *et al.*, 1997). The LBD additionally harbours a second transcriptional activation function (AF-2) which is hormone-dependent and activates target gene transcription in response to E_2 by recruiting and tethering co-activators to the ER.

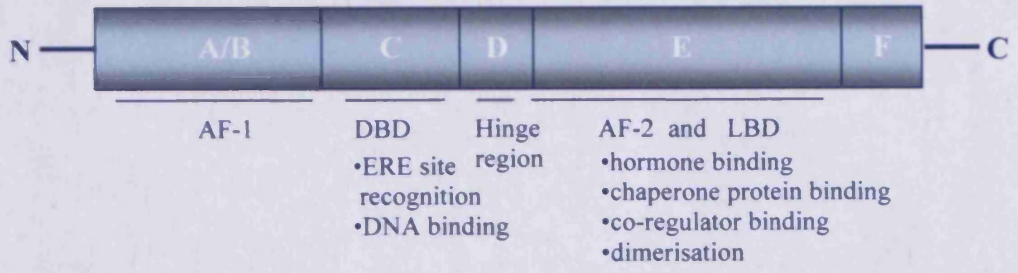


Figure 1.2 Oestrogen receptor domain structure.
Schematic diagram depicting the 6 domains of the ER and their respective functions.

ER signalling mechanisms are more complex than initially supposed with three distinct modes identified so far (Lewis & Jordan, 2005; Nicholson & Johnston, 2005; Osborne & Schiff, 2005); these are briefly outlined below.

1.3.2.1 'Classical' Genomic ER Signalling

In the classical mechanism of ER action, E_2 diffuses through the cell membrane and into the nucleus where it binds to the LBD of the ER which is associated with the molecular chaperone heat shock protein90 (hsp90). The binding of E_2 to the LBD induces a conformational change in the receptor which results in hsp90 dissociation, ER phosphorylation and dimerisation. The stable E_2:ER dimer

complex then binds the EREs located in the 5'-promoter regions of oestrogen-responsive genes. Once bound to the EREs, the ligand-occupied receptor complex recruits additional proteins, i.e. nuclear receptor co-activators and co-repressors, which respectively promote or repress oestrogen-regulated gene transcription. Whilst binding of E_2 leads to co-activator recruitment such as AIB1 (amplified in breast cancer 1), binding of Selective Estrogen Receptor Modulators (SERMS) typically results in co-repressor recruitment such as NCoR1, NCoR 2 and SMRT (silencing mediator for retinoid and thyroid receptor), and consequently in the inhibition of transcription of genes that regulate cell-cycle progression, proliferation and apoptosis. It is thought that the ratio of co-activator to co-repressor is one of the many factors that impact on the resistance of some breast cancers to therapy with SERMs (Schiff *et al.*, 2003).

1.3.2.2 'Non-Classical' Genomic ER Signalling

Interestingly, approximately one third of genes regulated by the ER do not contain ERE-like sequences (Weinberg *et al.*, 2005), with the ER modulating gene expression at alternative regulatory sites such as AP-1 (activator protein 1), SP-1 (specificity protein 1) and other poorly defined non-ERE sites (Kushner *et al.*, 2000; Safe, 2001; Saville *et al.*, 2000). In these instances, ERs do not bind to the DNA directly and function as transcription factors but instead act as co-activators themselves, stabilising the tethering of transcription factors such as the AP-1 complex Fos/Jun and SP-1 to the DNA and recruiting other co-activators to the binding site. Several genes essential to growth factor signalling, including the genes encoding insulin-like growth factor 1, cyclin D1, myc and the antiapoptotic factor Bcl-2, are regulated in this manner and it is thought that these alternative signalling pathways may play a contributory role in the development of resistance to therapy with SERMs.

1.3.2.3 Non-Genomic ER Signalling

In addition to nuclear or genomic ER signalling, E_2 can also result in very rapid effects that occur too quickly to involve transcriptional mechanisms; this phenomenon is referred to as 'non-genomic' ER signalling or membrane-initiated steroid signalling (MISS) and is mediated by extranuclear cytoplasmic or plasma membrane-associated ER (Kelly & Levin, 2001), which directly binds to growth factor signalling elements such as HER-2, EGFR, IGFR1, the p85 subunit of phosphatidylinositol 3 kinase (PI3K), Src and Shc. This leads to AKT/PKB and MAPK pathway activation resulting in powerful survival and proliferation responses.

Importantly, this cross-talk between the ER and growth factor receptors operates in both directions; hence growth factor and intracellular kinase pathways can in turn enhance ER signalling. This bi-directional molecular communication is also thought to be involved in the development of resistance against SERMs (refer to section 1.4).

1.3.3 Antihormonal Agents

Prior to endocrine therapy initiation, the hormone receptor status of the tumour needs to be established. ER and progesterone receptor (PR) presence is an important predictor of response to endocrine therapy (McGuire & Clark, 1992), and is usually associated with a lower rate of cell proliferation, evidence of tumour differentiation and consequently more favourable prognosis (Platet *et al.*, 2004; Weinberg *et al.*, 2005). Approximately 70% of breast cancers are ER-positive at diagnosis. In contrast, ER-negativity is a significant clinical feature present in 30% of breast cancer patients at presentation and also in some previously ER-positive tumours at the point of tamoxifen relapse (Gee *et al.*, 2005). Negative receptor status is generally linked to poor prognosis, precluding response to endocrine therapy and resulting in a more proliferative and aggressive phenotype (Kinne *et al.*, 1987; Kuukasjarvi *et al.*, 1996; Maynard *et al.*, 1978; Nicholson *et al.*, 2005; Parl *et al.*, 1984; Putti *et al.*, 2005). Since the

initial recognition of breast cancer as an oestrogen-dependent disease, three main strategies have been developed to block the action of oestrogen on tumour cells:

- Inhibition of oestrogen binding to the ER by using Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen (Lewis & Jordan, 2005)
- Prevention of oestrogen synthesis using the aromatase inhibitors (AIs) anastrozole, letrozole and exemestane (Coombes *et al.*, 2003)
- Downregulation of cellular ER levels using the Selective Estrogen Receptor Downregulator (SERD) fulvestrant (Johnston, 2004).

These three avenues of oestrogen signalling inhibition are further explored below.

1.3.3.1 Tamoxifen (Nolvadex™)

The non-steroidal triphenylethylene derivative tamoxifen (Nolvadex™, AstraZeneca Pharmaceuticals Ltd.) (**figure 1.3**) was the first anti-oestrogen to be developed.

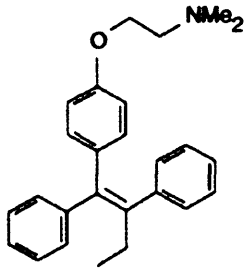


Figure 1.3 Structure of the SERM tamoxifen.
(from Nicholson and Johnston, 2005).

It is classed as a 'SERM' based on its ability to act as both an ER agonist and antagonist (Lewis & Jordan, 2005). Tamoxifen achieves its antineoplastic effect by competition for binding to the ER with endogenous circulating oestrogen. As opposed to E_2, which facilitates interaction of the ER with nuclear co-activators, tamoxifen induces a conformational change in the ER which prevents co-activator recruitment and leads to the recruitment of co-repressors

instead, thus inhibiting transcription of oestrogen-responsive genes (Lewis & Jordan, 2005). However, tamoxifen impairs only AF-2, and as a consequence AF-1 activation, ER dimerisation and nuclear translocation of the tamoxifen-ER complex to the EREs of target genes still occur, resulting in incomplete inactivation of ER-regulated transcription (Adamo *et al.*, 2007). This partial agonist effect is thought to explain the serious adverse events often associated with tamoxifen therapy such as endometrial cancer, thromboembolic events and gynaecological complications (Nicholson & Johnston, 2005).

1.3.3.2 Aromatase Inhibitors

While tamoxifen has been the gold standard adjuvant treatment for postmenopausal women following primary surgery, this unfavourable side effect profile as well as *de novo* and acquired resistance necessitated the development of alternative therapeutic agents.

In postmenopausal women, conversion of androgens to oestrogens occurs through the action of the cytochrome P450 enzyme aromatase in peripheral tissues such as adipose tissue and muscle, as detailed in **figure 1.4** below.

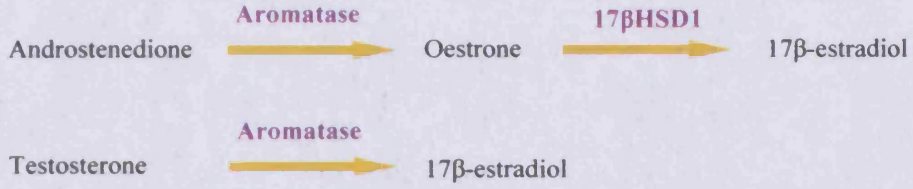


Figure 1.4 Aromatase-mediated conversion of androgens to oestrogens.
The androgens androstenedione and testosterone are converted into the oestrogens oestrone and 17β-estradiol through the actions of the enzymes aromatase and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) (Suzuki *et al.*, 2008).

Therefore, AIs have been developed to suppress cytochrome P450 enzyme aromatase activity which results in reduced circulating 17β-estradiol levels. This in turn inhibits oestrogen-regulated gene transcription via nuclear and non-nuclear pathways and thus removes the growth stimulus for ER-positive breast

carcinomas (Adamo *et al.*, 2007; Smith & Dowsett, 2003). Third generation AIs can be divided into two groups, the nonsteroidal AIs anastrozole (Arimidex®, AstraZeneca Pharmaceuticals Ltd.) and letrozole (Femara®, Novartis Pharmaceuticals Corp.), and the steroidal AI exemestane (Aromasin®, Pharmacia Corp.). They can be further subclassified according to their mode of action. While anastrozole and letrozole bind the aromatase cytochrome P450 moiety reversibly, exemestane has an irreversible action on the enzyme (Lonning, 2004). AIs possess both favourable efficacy and tolerability profiles. Moreover, due to the lack of cross-resistance between non-steroidal and steroidal AIs, these agents can be used sequentially which results in prolonged clinical benefit for the patient (Bertelli, 2005).

Recently, large, randomised trials such as the ATAC (Anastrozole, Tamoxifen Alone or in Combination), BIG 1-98 (Breast International Group 1-98) and MA.17 trials have demonstrated that therapy with third generation AIs leads to superior results compared to five years of therapy with tamoxifen, and AIs are now challenging the 25-year reign of tamoxifen as the first-line adjuvant of choice in early breast cancer. As a consequence, third-generation AIs are now an important therapeutic option for the adjuvant treatment of ER-positive early breast cancer in postmenopausal women, and are recommended as the preferred therapy by ASCO (American Society for Clinical Oncology), St. Gallen International Expert Consensus and NICE (National Institute for Health and Clinical Excellence) guidelines (Goldhirsch *et al.*, 2005; Winer *et al.*, 2002). Where tamoxifen is given as first-line treatment, treatment with this agent is routinely discontinued after five years, as recommended by the National Cancer Institute guidelines. However, the optimal treatment schedule for the sequential use of tamoxifen and AIs in the adjuvant setting is yet to be established; and whilst the overall tolerability of AIs is similar to that of tamoxifen, further safety studies are required to assess the long-term toxicities associated with AI therapy.

1.3.3.3 Fulvestrant (Faslodex™)

Both resistance to existing antihormonal agents and the partial agonist action of tamoxifen made necessary the development of novel ER antagonists that lack partial agonist properties, leading to the synthesis of fulvestrant (7α-[9-(4,4,5,5,5-pentafluoropentylsulphinyl)nonyl]estra-1,3,5-(10)-triene-3,17, β-diol), a steroidal 7α-alkylsulphinyl analogue of 17β-estradiol (Howell *et al.*, 2000). Like tamoxifen, fulvestrant (Faslodex™, AstraZeneca Pharmaceuticals Ltd.) (**figure 1.5**) competitively inhibits binding of 17β-estradiol to the ER.

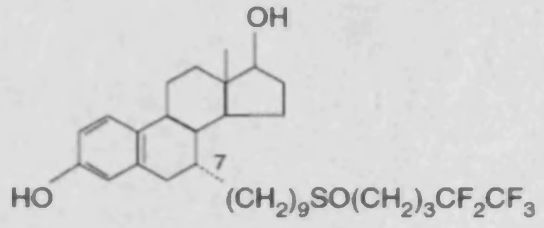


Figure 1.5 Structure of the SERD fulvestrant.
(from Nicholson and Johnston, 2005).

However, fulvestrant has a much stronger affinity for the ER than tamoxifen. While tamoxifen binds to the ER with a binding affinity which is only 2.5% that of 17β-estradiol, fulvestrant displays an ER-binding affinity of 98% (Wakeling & Bowler, 1987; Wakeling *et al.*, 1991). In terms of chemical structure, fulvestrant is distinctly different from the non-steroidal tamoxifen, raloxifene and other SERMs. Due to the steric hindrance caused by its long alkylsulphinyl side chain, fulvestrant induces a different conformational shape in the ER to that achieved by SERMs (Johnston, 2004). This results in inhibition of ER dimerisation (Parker, 1993), reduced nuclear localisation of the drug-receptor complex (Dauvois *et al.*, 1993; Fawell *et al.*, 1990) and subsequent prevention of ER binding to EREs in the promoters of oestrogen-responsive genes (Gibson *et al.*, 1991; Osborne *et al.*, 1995). Additionally, any fulvestrant-ER complex that does enter the nucleus is transcriptionally inactive as fulvestrant disables both transcriptional activation functions, AF-1 and AF-2, which results in complete abrogation of oestrogen-sensitive gene transcription. Furthermore, the drug-ER

complex is unstable which leads to accelerated degradation of the ER protein and downregulation of cellular ER levels (Dauvois *et al.*, 1992; Fawell *et al.*, 1990; Nicholson *et al.*, 1995); this downregulation of cellular ER protein occurs without reduction in ER mRNA levels (Osborne *et al.*, 2004). Thus, fulvestrant binds, blocks and accelerates degradation of the ER, resulting in pure anti-oestrogenic action (Carlson, 2005; Wardley, 2002); fulvestrant is hence referred to as a 'pure' anti-oestrogen and classed as a 'selective oestrogen receptor downregulator' (SERD) (Howell *et al.*, 2000).

Following the development of fulvestrant, numerous Phase II and Phase III clinical trials were initiated to establish clinical efficacy and appropriate positioning within the endocrine sequence cascade. A brief overview of the largest and most significant trials is given below.

The efficacy of fulvestrant in tamoxifen-resistant advanced breast cancer was demonstrated in two Phase III clinical trials, trials 0020 and 0021, which compared the tolerability and efficacy of fulvestrant with those of anastrozole (Howell *et al.*, 2002; Osborne *et al.*, 2002). A prospectively planned combined analysis of the data from both trials showed fulvestrant to be at least as effective as anastrozole in terms of time to progression (TTP); moreover, objective response (OR), clinical benefit (CB) and median duration of response were also similar. A subsequent combined analysis of survival data found that there was no significant difference in the median overall survival between tamoxifen and fulvestrant treatment (Howell & Buzdar, 2005). Based on these findings, fulvestrant received regulatory approval as second-line treatment for postmenopausal women with hormone-sensitive advanced breast cancer after progression or relapse on tamoxifen therapy in several countries.

A double-blind, randomized Phase III trial, trial 0025, subsequently compared fulvestrant with tamoxifen as first-line treatment for postmenopausal women with ABC (Howell *et al.*, 2004). However, although fulvestrant is at least as effective as anastrozole in the second-line setting (Robertson *et al.*, 2003), the

pure anti-oestrogen did not meet the criteria for non-inferiority to tamoxifen in the intent-to-treat population.

New Phase II and III clinical trials investigating additional roles for fulvestrant following prior non-steroidal AI treatment or as first line-therapy in combination with monoclonal antibodies or small molecule inhibitors are currently in progress or have already yielded results (Robertson, 2007) (see **table 1.2**). In addition, various loading-dose (LD) and high-dose (HD) regimens of fulvestrant are being evaluated in order to establish an optimum treatment plan.

	Population	Phase
Fulvestrant + bevacizumab	MBC, post-AI	II
Fulvestrant + trastuzumab	ER+ and/or PR+, HER2-overexpressing stage IV BC	II
Fulvestrant ± lapatinib ditosylate	Stage III or IV HER2-expressing hormone-receptor-positive BC	III
Fulvestrant + gefitinib	ER+ and/or PR+ ABC or MBC	II
Fulvestrant + tipifarnib	ER+ and/or PR+, inoperable LABC or MBC progressing after prior first-line endocrine therapy	II

Table 1.2 Details of current trials of fulvestrant plus monoclonal antibodies and small molecule inhibitors.
(Adapted from Adamo *et al.*, 2007).
MBC, metastatic breast cancer; BC, breast cancer; ABC, advanced breast cancer; LABC, locally advanced breast cancer.

Two randomised, controlled Phase III trials, EFECT (Evaluation of Faslodex™ and Exemestane Clinical Trial) and SoFEA (Study of Faslodex with or without concomitant Arimidex *vs* Exemestane following progression on non-steroidal Aromatase inhibitors), compared the tolerability and efficacy of fulvestrant *vs* exemestane in postmenopausal women who showed disease progression or recurrence following prior non-steroidal AI therapy (**table 1.3**). The EFECT trial is now closed and the results show fulvestrant LD and exemestane to be equally active and well-tolerated in a meaningful proportion of postmenopausal women with ABC who have experienced progression or recurrence during treatment with a nonsteroidal AI (Chia & Gradishar, 2008; Chia *et al.*, 2008). The SoFEA trial, in contrast, is currently on-going and recruiting participants; trial results are awaited with great interest (http://www.clinicaltrials.gov/ct2/show/record/ NCT00253422; (Dodwell *et al.*, 2008; Robertson, 2007).

Two additional trials, FACT (Fulvestrant and Anastrozole Clinical Trial) and SWOG 226 (Southwest Oncology Group 226), to compare the efficacy of fulvestrant plus anastrozole with anastrozole alone as first-line therapy are currently on-going (**table 1.3**). Both clinical trials are presently recruiting patients, and trial outcomes are eagerly anticipated (http://www.clinicaltrials. gov/ct2/show/record/NCT00075764).

	Phase	Population	Treatment regime	Patients	Status
EFECT	III	Post-nonsteroidal AI	Fulvestrant LD 250mg *vs* exemestane	660	Closed
SoFEA	III	Post-nonsteroidal AI	Fulvestrant LD 250mg ± anastrozole *vs* exemestane	750	Currently recruiting
SWOG 226	III	First-line	Fulvestrant 250mg + anastrozole *vs* anastrozole	690	Currently recruiting

Table 1.3 Details of trials of fulvestrant in comparison with AIs and of fulvestrant plus an AI in comparison to AI monotherapy. (Adapted from Johnston *et al.*, 2004). EFECT, Evaluation of Faslodex and Exemestane Trial; SoFEA, Study of Faslodex without or without concomitant arimidex *vs* Exemestane following progression on non-steroidal Aromatase inhibitors; SWOG 226, Southwest Oncology Group 226.

In summary, while not quite as effective in hormone-receptor-positive advanced breast cancer as tamoxifen, fulvestrant showed the same efficacy as anastrozole as second-line therapy and also the same efficacy as exemestane following progression on first-line non-steroidal AI therapy. Furthermore, when comparing the side effects of fulvestrant with those of tamoxifen and anastrozole respectively, fulvestrant was found to be well tolerated and associated with a lower incidence of joint disorders (Vergote & Abram, 2006). Importantly, it has also been demonstrated that sensitivity to further endocrine therapy following progression on first-line or second-line fulvestrant therapy is retained due to its lack of cross-resistance with existing endocrine agents (Buzdar, 2004). This observation is of particular importance as it allows flexible positioning of fulvestrant within the sequential endocrine agent cascade. This in turn will enable clinicians to achieve the aim of extending effective duration of well-tolerated treatment and will also allow patients to derive maximum benefit before cytotoxic chemotherapy with all its associated side effects becomes necessary (Carlson, 2002). As these data illustrate, fulvestrant is a versatile new treatment option for post-menopausal women with advanced or metastatic breast cancer who have progressed on prior endocrine therapy.

1.4 Resistance to Endocrine Therapy

Resistance to anti-oestrogen therapy is classified as 'intrinsic', also known as *de novo*, or 'acquired'. *De novo* resistant tumours are either ER-negative or do not respond to therapy despite positive ER status. Acquired resistance, in contrast, describes a state in which initially responsive ER-positive disease ceases to respond to antihormonal treatment after long-term therapy. While only 50% of all ER-positive tumours are responsive to endocrine agents such as tamoxifen at first presentation, in the metastatic setting most initially responsive tumours eventually become non-responsive; this results in rapid disease progression and high patient mortality (Osborne, 1998). Clearly, both resistant states severely limit the effectiveness of endocrine agents and therefore present a major

therapeutic challenge in the clinical management of breast cancer (Gee, 2006; Nicholson & Johnston, 2005).

While the causative mechanisms for endocrine resistance are multi-factorial and so far only partially comprehended, co-activator to co-repressor ratio in the tumour cell milieu (refer to section 1.3.2.1) and bi-directional cross-talk between oestrogen and growth factor signalling (refer to section 1.3.2.3) have been identified as important contributory factors that induce and sustain non-responsiveness to anti-hormone therapy (Nicholson *et al.*, 2005; Nicholson *et al.*, 2007; Osborne & Schiff, 2003; Schiff *et al.*, 2003). Interestingly, it is envisaged that increased growth-factor-driven kinase activity does not only directly promote cell proliferation but also efficiently phosphorylates key serine residues within the AF-1 domain of the ER. Phosphorylation of these serine residues, in particular serine 118 which presents a target for MAPK, re-activates ligand-independent ER signalling which results in re-expression of oestrogen-regulated genes and consequently in continued growth in the presence of anti-oestrogens (Bunone *et al.*, 1996; Kato *et al.*, 1995). Also, growth factor signalling pathways can promote ER co-activator phosphorylation which again results in the re-expression of oestrogen-regulated genes and continued proliferation (Font de Mora & Brown, 2000). Furthermore, as delineated in section 1.3.2.3, non-genomic ER signalling links the ER to growth factor receptors which also leads to sustained cell survival and proliferation.

As with tamoxifen, patients unfortunately do eventually progress on fulvestrant, and with increased use, fulvestrant resistance will pose a considerable clinical problem, potentially limiting the benefits of this novel ER antagonist (Buzdar, 2004; Cheung *et al.*, 2006; Robertson *et al.*, 2005).

In order to investigate the mechanisms underlying such acquired resistance, several *in vitro* breast cancer models displaying acquired resistance to antihormones have been established at the Tenovus Centre for Cancer Research (TCCR); these include tamoxifen-resistant, tamoxifen- and gefitinib-resistant and faslodex-resistant MCF-7 cells (TAM-R, TAM-/TKI-R and FAS-R cells,

respectively). Significantly, characterisation studies carried out on these cell lines have demonstrated that inappropriate activation of growth factor signalling cascades is one of the key factors in antihormone resistance (Jones *et al.*, 2005; Knowlden *et al.*, 2003), with resistant cells displaying remarkable flexibility in recruiting alternative signalling pathways to circumnavigate the need for oestrogen for continued growth.

Intriguingly, while the role of altered growth factor signalling in endocrine resistance has been firmly established, more recent in-house studies on the TAM-R model have demonstrated that such drug-resistant growth in these cells is associated with a gain in migratory and invasive behaviour, driving a very aggressive phenotype (Hiscox *et al.*, 2004; Hiscox *et al.*, 2006). Furthermore, endocrine refractory disease in the clinic invariably results in rapid disease progression and is associated with a poor prognosis (Nicholson *et al.*, 2007), mirroring these *in vitro* observations.

Collectively, these findings illustrate the role of aberrant growth factor signalling in anti-hormone failure and concomitant enhanced metastatic potential and hence emphasize the urgent need for the delineation of the underlying molecular basis which will permit the development of successful combined targeting strategies to prevent, delay or overcome the resistant state and thereby significantly improve breast cancer survival.

1.5 Invasion and Metastasis

The ability of tumour cells to invade, colonise and impair the function of distant organs was first recognised by Jean-Claude Recamier in 1829. He termed this phenomenon 'metastasis'. This multi-step process involves a series of sequential, obligatory events termed the 'metastatic cascade' (**figure 1.6**).

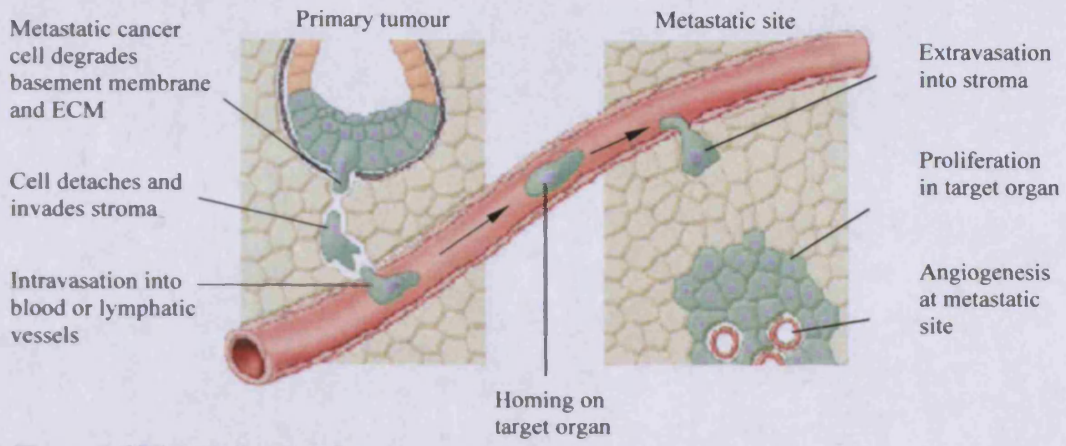


Figure 1.6 Metastatic cascade.
Diagram depicting the stages primary cancer cells must go through to successfully establish themselves at a metastatic site. (Adapted from Geho *et al.*, 2005).

First, cancer cells detach from neighbouring cells and penetrate the basement membrane and the interstitial stroma by way of enzymatic activity of strategically secreted proteases. They then invade both blood and lymphatic vessels, thereby gaining access to the circulatory system in a process known as 'intravasation'. Circulating tumour cells that successfully evade both anoikis and immunogenic surveillance attach to platelets and leukocytes forming emboli that are eventually arrested in the microcirculation of the target organ; furthermore they can also be arrested on the basis of their physical size or even directly interact with vessel walls. Finally, metastatic cells extravasate, i.e. they exit blood and lymph vessels where, after resuming proliferation, they can undergo local expansion in the parenchyma of distant organs (Geho *et al.*, 2005; Guo & Giancotti, 2004).

Thus, metastasis is an intricate interplay between altered cell adhesion, localised proteolysis at the invasive front, migration, lymph- and angiogenesis, evasion of immune surveillance and apoptosis, and homing on target organs involving profound changes in the adhesive, motile and invasive properties of primary tumour cells.

Breast carcinomas preferentially metastasise to bone, lung and liver (Weigelt *et al.*, 2005); and the presence of metastatic disease remains the primary cause of mortality in breast cancer patients.

In spite of the obvious clinical relevance of metastases, the inherently occult nature of these processes unfortunately means they are a challenging subject matter to study. Nonetheless, technological advances such as microarray expression profiling to identify new molecular markers and 'poor prognosis signatures' predictive of metastatic disease (Duffy, 2005; Minn *et al.*, 2005; van 't Veer *et al.*, 2003; van de Vijver *et al.*, 2002) and *in vivo* video microscopy to follow the fate of cancer cells inside the body (Chambers *et al.*, 2002) have furthered our knowledge. Whilst the traditional models of metastasis based on the work of Leighton (Leighton, 1965) and Fidler (Fidler, 1973) consider metastatic capacity to be a late, acquired event in tumourigenesis, new research challenges these long-held tenets suggesting that breast cancer is an inherently systemic disease with metastasis being an intrinsic capacity of all primary breast cancer cells (Bernards & Weinberg, 2002).

Regrettably, despite these findings, the prognosis for patients with advanced invasive and metastatic disease remains exceedingly poor, highlighting the pressing need for ongoing research and development of novel and effective anti-invasive treatments to overcome this impasse.

1.6 Matrix Metalloproteinases

The sheer complexity of proteolytic systems operating in human tissues is challenging, with human genome sequencing having revealed over five hundred protease-like proteins, many of which are associated with cancer dissemination (Puente & Lopez-Otin, 2004). However, among all the proteases linked to metastatic spread, matrix metalloproteinases (MMPs) are considered the most influential (Folgueras *et al.*, 2004). MMPs are a family of zinc- and calcium-dependent endopeptidases collectively capable of degrading essentially all components of the extracellular matrix (ECM) (Westermarck & Kahari, 1999)

and therefore associated with a variety of processes that involve matrix degradation and remodelling. They were first discovered when Gross and Lapiere described collagenolytic activity in tadpole tail resorption during metamorphosis (Gross & Lapiere, 1962). The structure, regulation and multifunctional roles of these proteases are delineated below, current inhibition strategies also being considered.

1.6.1 Domain Structure and Function

The availability of the complete human genome sequence and recent genomic studies have permitted identification and structural analysis of the entire set of 24 MMPs produced in human tissues. Based on their domain structure, MMPs can be divided into six groups: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT1-MMP to MT6-MMP) and other MMPs (**figure 1.7**) (Lemaitre & D'Armiento, 2006; Visse & Nagase, 2003). All family members with the exception of membrane-type MMPs contain an N-terminal signal peptide which directs the proenzyme for secretion. This predomain is followed by a prodomain which contains a conserved sequence in which a cysteine residue forms a covalent bond with a catalytic zinc ion located in the active site; this so-called cysteine switch maintains the latency of pro-MMPs. The catalytic domain contains the highly conserved zinc-binding site in which the zinc ion is coordinated by three histidine residues. A proline-rich hinge region links the catalytic domain to the hemopexin domain which mediates multiple protein interactions. It is involved in MMP activation and determines the substrate specificity (Pavlaki & Zucker, 2003).

Six distinct membrane-type MMPs have been identified; and localisation at the cell surface is achieved through a C-terminal type I transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or by a glycosylphsophatidylinositol anchor (MT4- and MT6-MMP) (Zucker *et al.*, 2003).

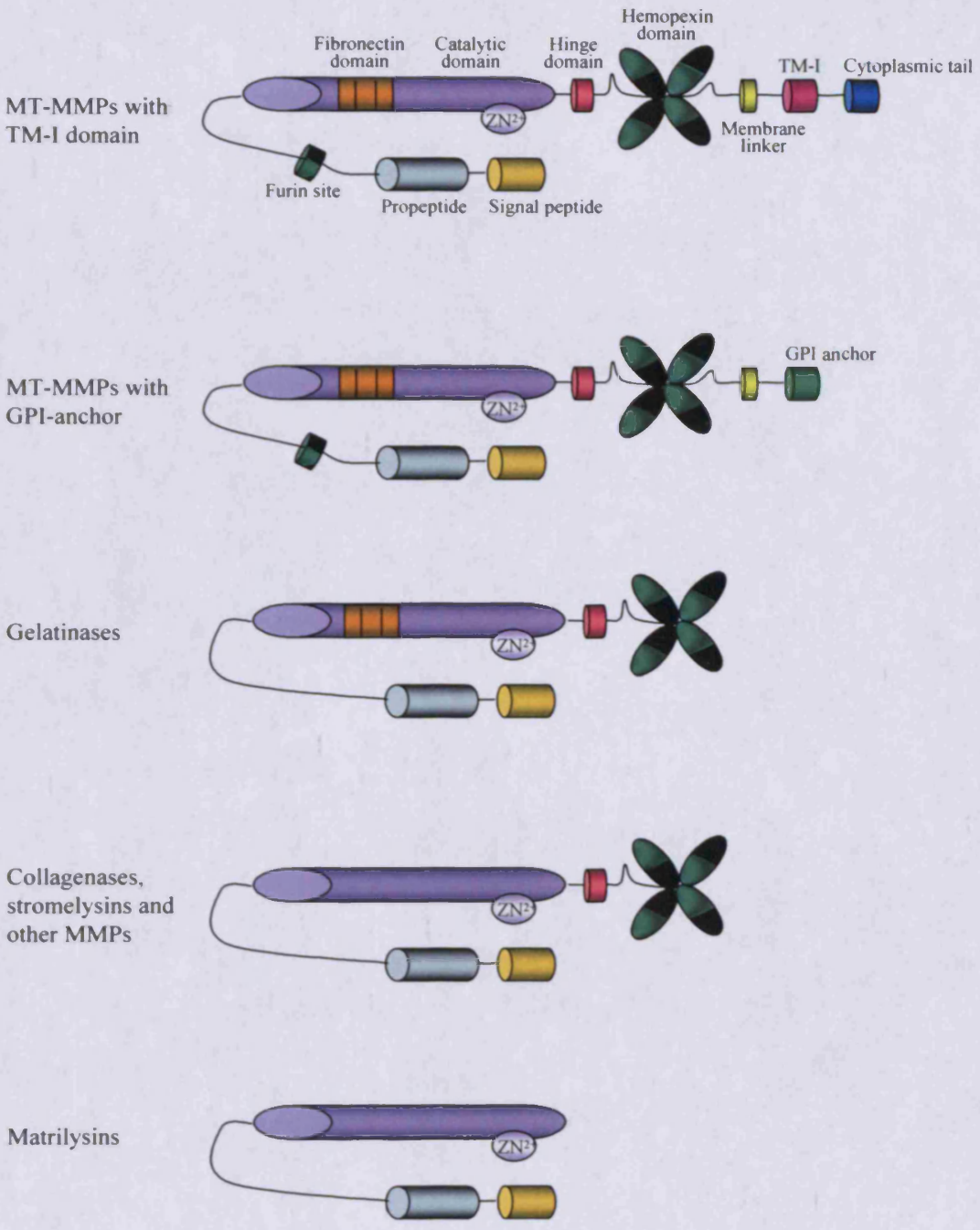


Figure 1.7 Schematic diagram of MMP domain structure.
Diagram showing domain structure of each of the 6 MMP groups, with complexity and size decreasing in descending order (adapted from Folgueras *et al.*, 2004). Zn^{2+}, catalytic zinc ion; TM-I, transmembrane-I; GPI, glycosylphosphatidyl.

1.6.2 Regulation of MMP Activity

Whilst MMPs are essential for physiological tissue turnover and homeostasis, uncontrolled proteolytic activity has clearly been shown to contribute to a plethora of pathologies such as arthritis, cardiovascular disease and cancer. Precise regulation of protease activity is therefore crucial in order to avoid the potentially devastating consequences of excessive tissue degradation. Not surprisingly, MMP expression and activity is tightly controlled at the level of gene transcription, proenzyme activation and enzyme inhibition (Clark *et al.*, 2008; Folgueras *et al.*, 2004; Overall & Lopez-Otin, 2002).

1.6.2.1 Gene transcription

In general, levels of MMP expression in unstimulated cells *in vitro* and intact tissue *in vivo* are low and only induced when ECM remodelling is required (Curran & Murray, 2000; Vihinen *et al.*, 2005). Expression of certain MMPs is induced by a wide variety of stimuli ranging from growth factors and cytokines to cell-cell and cell-matrix interactions (Benbow & Brinckerhoff, 1997; Westermarck & Kahari, 1999). The promoter regions of these inducible MMP genes (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12 and MMP-13) contain an activator protein-1 (AP-1) binding site (Benbow & Brinckerhoff, 1997; Pendas *et al.*, 1997). In addition, polyoma enhancer A binding protein-3 (PEA3) sites have been identified in the promoters of MMP-1, MMP-3 and MMP-9 (Crawford *et al.*, 2001; Westermarck & Kahari, 1999). The cytokines and growth factors that activate MMP expression typically act via the MAPK pathway which comprises the ERK1/2, JNK/SAPK1/2 and p38 proteins and include EGF (Epidermal Growth Factor), TGF-α (Transforming Growth Factor α) and HGF/SF (Hepatocyte growth Factor/Scatter Factor) (Bjorklund & Koivunen, 2005). In contrast, the promoters of the non-inducible, constitutively expressed MMP family members MMP-2, MMP-11 and MT-1-MMP do not contain AP-1 binding sites. More recently, further characterisation studies have revealed Sp1, Tcf/Lef-1, NFκB and RARE to be additional cis-acting elements

in MMP promoter regions (Clark *et al.*, 2008). Overall, control of MMP gene transcription is cell and tissue-specific, temporally variable and complex.

1.6.2.2 Proenzyme activation

MMPs are synthesised as inactive zymogens (pro-MMPs) (Harper *et al.*, 1971; Okada *et al.*, 1988). As outlined previously, latency is achieved through the interaction of the cysteine residue in the prodomain with the zinc ion bound to the catalytic site ('cysteine switch') (Windsor *et al.*, 1991); and activation requires proteolytic removal of the prodomain (Van Wart & Birkedal-Hansen, 1990). While the majority of MMPs *in vivo* are activated extracellularly or on the cell surface by other MMPs or serine proteases, intracellular activation by furin-like serine proteases has been described for the MT-MMPs, MMP-11, MMP-23 and MMP-28 (Pei & Weiss, 1995; Yana & Weiss, 2000). *In vitro,* several non-proteolytic agents such as thiol-modifying agents and SDS as well as heat and low pH result in zymogen activation through disturbance of the zinc-cysteine interaction of the cysteine switch (Van Wart & Birkedal-Hansen, 1990).

1.6.2.3 Enzyme inhibition

MMP activity is blocked by general inhibitors such as α2-macroglobulin as well as by specific endogenous inhibitors, tissue inhibitors of metalloproteases (TIMPs), which non-covalently bind to the zinc-binding site in the catalytic domain of active MMPs (Willenbrock & Murphy, 1994). Four homologous TIMPs (TIMP-1 to TIMP-4) have been identified (Brew *et al.*, 2000). With the exception of TIMP-3, which is anchored in the ECM, these cysteine-rich proteins are secreted. They share a conserved structure which consists of an N-terminal domain, a C-terminal domain and three disulfide bonds (Williamson *et al.*, 1990). The inhibitory activity of these proteins suggests that the net balance between the MMPs and their tissue-specific inhibitors is the primary determinant of the proteolytic potential of a tumour. This concept has been supported by several studies demonstrating that while TIMP overexpression results in reduced experimental metastasis (DeClerck & Imren, 1994), low TIMP levels correlate

with tumourigenesis (Khokha & Denhardt, 1989). However, more recent studies ascribing angiogenic and tumour-promoting functions to these proteins suggest more complex and even paradoxical roles for TIMPs in cancer (Jiang *et al.*, 2002)).

1.6.3 MMP Functions

While MMPs were initially characterised as matrix-degrading proteases, more recent identification of numerous novel substrates has forced us to revise our perhaps overly simplistic view of MMP proteolytic activity. By way of their ability to process additional substrates such as growth factors and their receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors MMPs are now recognised as signalling molecules rather than just ordinary 'weapons of mass destruction'. It is this substrate diversity which reflects the varied and sometimes even paradoxical functions that are now attributed to this protease family. Specific examples of the myriad of cellular processes controlled by MMPs are given below.

1.6.3.1 Apoptosis

Evasion of apoptosis permits cancer progression by allowing cell survival in the presence of low oxygen and nutrient levels, attack from the immune system and, most importantly, after detachment from the ECM - a prerequisite for invasion and metastasis (Reed, 1999). Due to the proteolytic activity MMPs have on various biologically active molecules, MMPs can positively or negatively regulate cell survival in a context-dependent manner (Hojilla *et al.*, 2003). MMP-3, MMP-7, MMP-9 and MMP-11 have been shown to regulate apoptosis (Egeblad & Werb, 2002). MMP-7, for example, releases membrane-bound Fas ligand which triggers apoptosis upon binding the Fas receptor (Powell *et al.*, 1999). Conversely, MMP-7 can also inhibit apoptosis by generation of mature heparin-binding epidermal growth factor (HB-EGF) which promotes survival by stimulating the ERBB4 tyrosine kinase receptor (Yu *et al.*, 2002).

1.6.3.2 Angiogenesis

Angiogenesis is a prerequisite for continued tumour growth as the newly formed blood vessels function as conduits for gas exchange and nutrient supply (Chang & Werb, 2001). MMPs are regarded as essential regulators of angiogenesis because of their ability to stimulate endothelial cell proliferation and migration through mobilisation of latent growth factors and receptor shedding. MMPs promote angiogenesis by increasing the bioavailability of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and transforming growth factor-β (TGF-β). However, MMPs also act anti-angiogenically through the cleavage of plasminogen and collagen XVIII which results in the generation of the angiogenesis inhibitors angiostatin and endostatin (Egeblad & Werb, 2002; Mott & Werb, 2004).

1.6.3.3 Escape from immune surveillance

While the immune system functions to recognise and eliminate tumour cells, the latter have developed several strategies to escape this immune surveillance by utilising MMPs. Sheu *et al.* (2001) showed that MMPs cleave the interleukin-2-receptor-α on T-lymphocytes thereby blocking T-cell proliferation while Gorelik *et al.* (2001) reported MMP-mediated TGF-β liberation, resulting in suppression of T-cell reactions against cancer cells. Cleavage of various members of the CC and CXC chemokine families blocks leukocyte attraction and thus assists the tumour cells in circumventing the host anti-tumour defense systems (McQuibban *et al.*, 2000).

1.6.3.4 Cell growth and proliferation

Cell proliferation is primarily controlled through the interaction of growth-promoting or growth-suppressing factors with their respective cell-surface receptors; and MMPs facilitate this interaction via several mechanisms. One such mechanism is the cleavage of matrix components bound to growth factors. Growth factors such as FGF preferentially bind to certain matrix components so

that cleavage of these matrix molecules leads to liberation of the associated growth factor. MMP-1 and MMP-3 - mediated cleavage of the proteoglycan perlecan, for example, releases FGF (Whitelock *et al.*, 1996). In addition to their proteolytic action on ECM components, MMPs also cleave non-matrix substrates which again results in the release of growth factors. Proteolysis of the Insulin Like Growth Factor-Binding Protein (IGF-BP), for example, results in the generation of active IGF ligand (Fowlkes *et al.*, 1994). Finally, MMPs also directly cleave and thereby activate growth factors; MMP-2 and MMP-9-mediated processing of TGF-β, for example, results in active ligand generation (Yu & Stamenkovic, 2000).

1.6.3.5 Cell migration

Coordinated regulation of cell-cell attachment, cell-matrix attachment and matrix remodelling is a prerequisite for cell migration. The diverse nature of their substrates has made the proteolytic action of MMPs seemingly indispensable to all aspects of the migration process (see **figure 1.8**).

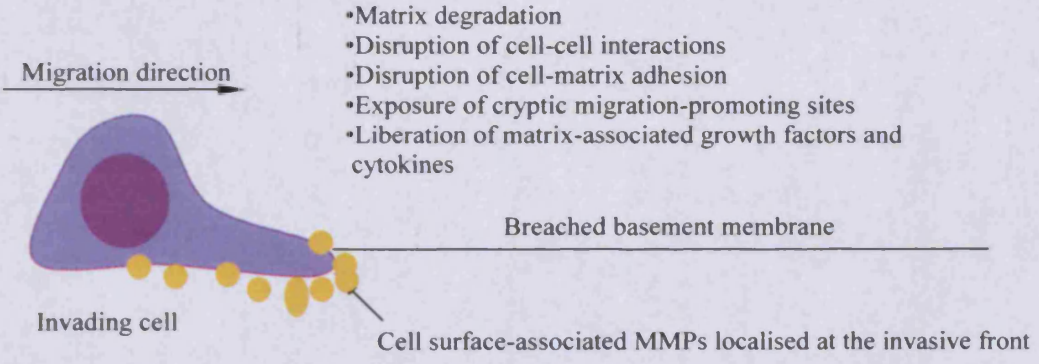


Figure 1.8 The functions of MMPs in cell migration.
(Adapted from Björklund and Koivunen, 2005).

For example, MMPs function in the direct modulation of cell-matrix adhesion by both removing adhesion sites and exposing binding sites. MMP-2-mediated cleavage of laminin-5 results in the release of the γ-chain of this basement component and the exposure of a cryptic promigratory site (Giannelli *et al.*, 1997). MMPs can furthermore modulate attachment and migration through direct cleavage of cell-cell or cell-matrix receptors; examples are MMP-7-

mediated cleavage of β4integrin (von Bredow *et al.*, 1997) and MMP-3 and MMP-7-mediated proteolysis of the cell adhesion molecule E-cadherin (Noe *et al.*, 2001).

1.6.4 Implication of MMPs in Invasion and Metastasis

As mentioned earlier, cancer cells must dissociate from their primary tumour and traverse several tissue barriers in order to metastasize. By way of their ability to process ECM and non-ECM substrates, to induce angiogenesis and cell migration and to promote evasion of apoptosis and host defense systems, MMPs are considered to be vital for tumour progression.

The concept that proteolytic activity could be critical for cell matrix degradation and the subsequent invasion of tumour cells into the surrounding stroma was pioneered several decades ago (Fisher, 1946). Individual proteases began to be identified in the 1970s, and in 1980 Liotta *et al.* demonstrated that the metastatic potential of various tumours correlated directly with the ability of cancer cells to degrade basement membrane collagen (Liotta *et al.*, 1980). Since then, countless *in vivo* and *in vitro* studies have suggested a role of MMPs in tumour cell dissemination. Importantly, many MMPs including MMP-9 and MMP-2 were originally cloned from cancer cell lines or tumours (Curran & Murray, 2000).

Enhanced MMP expression has been observed in many solid tumours, including cancers of the breast, and is usually accompanied by acquisition of an invasive phenotype, advanced stage metastatic disease and decreased overall survival (Fingleton, 2008; Mannello *et al.*, 2005).

Recent mouse model and patient transcriptome studies validated by *in vitro* data further highlight the pivotal roles of MMPs in metastasis. In a 'poor prognosis' signature of primary breast carcinomas, two of the 70 genes associated with short interval to distant metastasis were MMP-1 and MMP-9 (van 't Veer *et al.*, 2003; van de Vijver *et al.*, 2002) while in another 'poor prognosis' signature of 95 genes which mediate breast cancer metastasis to the lung the second most important gene was MMP-1 (Minn *et al.*, 2005).

Overall, the evidence for a role of MMPs in metastasis is compelling; and it is this prevailing view of proteases promoting tumour progression that prompted the development of synthetic MMP inhibitors.

1.6.5 MMPs as Therapeutic Targets

Currently, MMP inhibitors belong to five categories: peptidomimetics, non-peptidomimetics, chemically modified tetracyclines (CMTs), bisphosphonates and natural inhibitors (see **table 1.4**).

MMP Inhibitor	Company	Structure	Specificity
Batimastat (BB-94)	British Biotech	Peptidomimetic	Broad spectrum
Ilomastat (GM-6001)	Glycomed	Peptidomimetic	Broad spectrum
Marimastat BB-2516)	British Biotech; Schering-Plough	Peptidomimetic	Broad spectrum
Tanomastat (Bay-12-9566)	Bayer	Non-peptidomimetic	Higher specificity towards MMP-2, MMP-3 and MMP- 9
Prinomastat (AG-3340)	Agouron; Pfizer	Non-peptidomimetic	Broad spectrum
BMS-275291	Bristol-Myers Squibb; Celltech	Non-peptidomimetic	Broad spectrum
CGS27023A	Novartis	Non-peptidomimetic	Broad spectrum
Metastat (COL-3)	Collagenex	CMT	Broad spectrum; higher specificity towards MMP-2 and MMP-9
Neovastat	Æterna	Natural MMP inhibitor (shark cartilage extract)	Broad spectrum

Table 1.4 MMP inhibitors along with their structures and specificities.
Overview of the most important MMP inhibitors most of which have undergone clinical development which has been halted due to disappointing outcomes (Konstantinopoulos *et al.*, 2008; Overall and Kleifeld, 2006).

In retrospect, the development of MMP inhibitors was problematic from the very beginning. Administration of endogenous MMP inhibitors was the first attempt at therapeutic MMP inhibition after several studies had demonstrated the ability of TIMPs to inhibit tumour growth in transgenic mouse models (Kruger *et al.*, 1997; Martin *et al.*, 1999). However, difficulties with administration and poor pharmacokinetics further compounded by the paradoxical effects of these multifunctional proteins severely limited the feasibility of using TIMPs in cancer therapy (Folgueras *et al.*, 2004), thus highlighting the need for synthetic MMP inhibitors.

Over the past twenty-five years, the pharmaceutical industry has made an impressive effort to develop and establish specific, well-tolerated small molecule inhibitors as new anticancer agents. The first generation of MMP inhibitors to be developed were the peptidomimetics, pseudopeptide derivatives synthesised to mimic the structure of collagen at the site that binds to the catalytic domain of MMPs. Both Batimastat (BB-94) and its successor Marimastat (BB-2516) are examples of these early MMPIs that feature a hydroxamic acid zinc-chelating group which binds competitively and reversibly to the catalytic site of their target (Pavlaki & Zucker, 2003). Improved oral bioavailability as well as more acceptable pharmacokinetics were obtained through the subsequent design of non-peptidomimetic inhibitors such as Tanomastat (BAY12-9566) and Prinomastat (AG3340) which were synthesised on the basis of the 3-dimensional conformation of the MMP zinc-binding site (Mannello *et al.*, 2005; Vihinen *et al.*, 2005). However, despite extremely promising activity in a plethora of preclinical models, these compounds failed to demonstrate a statistically significant survival advantage in Phase III clinical trials for most human malignancies (Fingleton, 2003; Fingleton, 2008; Overall & Lopez-Otin, 2002), and a consequence, most MMP inhibitor development programs have been halted. So far, the only MMP inhibitor approved for clinical use is the collagenase inhibitor Periostat (AG-3340) in the treatment of adult periodontitis (Peterson, 2004).

In hindsight, the lack of efficacy of MMP inhibitors is not surprising. Both drugs and trials were designed at a time when knowledge of MMPs was still fragmentary, in terms of family members and their pleiotropic role in tumour progression. Since then, multiple etiologies have been proposed for the poor clinical performance of MMP inhibitors. Clearly, the utility of animal models to predict clinical outcome in humans was limited (Fingleton, 2008). Moreover, Phase III evaluation involved advanced stage cancers whereas patients with earlier stage disease might have derived more benefit (Bramshall *et al.*, 2002). Lack of a validated surrogate marker for effective inhibition meant that it was impossible to determine whether the optimal biologic dose had been achieved; and some trial results further revealed MMP inhibitors to have a very narrow therapeutic index as patients who derived the greatest benefit concomitantly developed musculoskeletal toxicities (King *et al.*, 2003). However, the most fundamental cause for the failure of Phase III trials was the inadvertent inhibition of beneficial protease activity which effectively counterbalanced the benefits of drug target inhibition. Indeed, at its inception, the idea of targeting proteolytic activity was inextricably linked to the basic assumption that all MMP activity was detrimental and that therefore indiscriminate inhibition would be desirable. However, more recent studies with mouse models of gain and loss of MMP function have shown certain MMPs to display host-protective functions (Balbin *et al.*, 2003; Hamano *et al.*, 2003; Pozzi *et al.*, 2002). As a consequence, MMP family members are currently being categorised into drug 'targets' and 'anti-targets' (Overall & Dean, 2006; Overall & Kleifeld, 2006) (see **figure 1.9**).

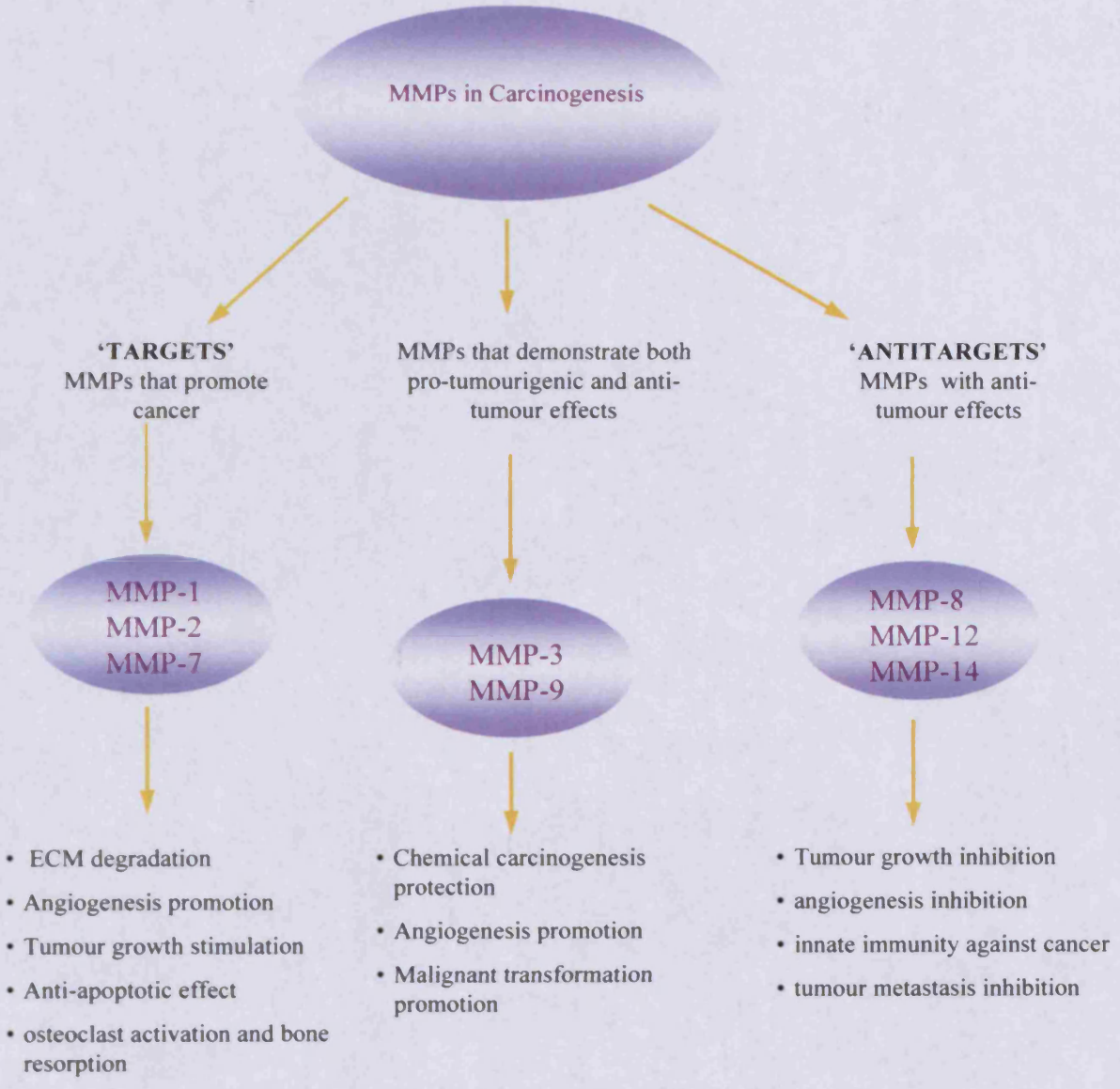


Figure 1.9 Target and Anti-target status of MMPs.
While some MMPs exert clear cancer-promoting or anti-cancer targets, some MMPs play a dual role in carcinogenesis (Konstantinopoulus *et al.*, 2007; Overall and Kleifeld, 2006).

MMP-1, MMP-2 and MMP-7 have been confirmed as valid drug targets whilst MMP-8, MMP-12 and MMP-14 have been designated anti-target status as they seem to exert only anticancer effects (Konstantinopoulos *et al.*, 2008). Even this classification is not without caveats, since studies have revealed MMPs to exert both positive and disease-promoting functions in the same disease, contributing to the pathology at early stages but being host-protective in advanced disease setting (Fingleton, 2008). Target and anti-target status is also likely to vary between individual cancer types and patients, further emphasizing the complexity of the MMP proteolytic system in cancer. What is becoming increasingly apparent with time is that precise identification of target MMPs for each disease setting and innovative chemistries that permit the synthesis of lead compounds that spare anti-targets over MMP targets are an absolute prerequisite if the third generation of MMP inhibitors are to form part of the drug armament against cancer in the future. However, despite these obstacles, the quest for specific and well-tolerated molecular therapeutics for MMP inhibition remains an exciting area of cancer research, and as recent reports on novel compounds in the chemical literature prove, there is a continued effort and sustained interest in meeting this goal.

1.7 HGF/SF and c-Met

Deregulated receptor tyrosine kinase (RTK) activity is a common feature of many human malignancies and is often associated with the acquisition of an invasive phenotype. While HGF/SF/c-Met-signalling-induced cellular responses are usually tightly regulated and required for many normal physiological processes such as wound healing and during embryogenesis, aberrant c-Met activation has been implicated in most types of solid tumours. Deregulated c-Met signalling is furthermore often associated with metastatic disease and has hence been recognised as a powerful expedient for cancer dissemination. The structure and function of HGF/SF and c-Met as well as current therapeutic strategies aimed at targeting this ligand-receptor complex are delineated below.

1.7.1 HGF/SF Structure and Regulation

HGF/SF is a pleiotropic cytokine so named due to its discovery both as a potent mitogen for hepatocytes (Nakamura *et al.*, 1984; Nakamura *et al.*, 1989) and as a fibroblast-derived factor which promotes cell motility or 'scattering' (Stoker & Perryman, 1985). The protein was subsequently purified as a disulfide-linked heterodimer consisting of a 69-kDa α-chain and a 34-kDa β-chain (Nakamura *et al.*, 1989) (**figure 1.10**).

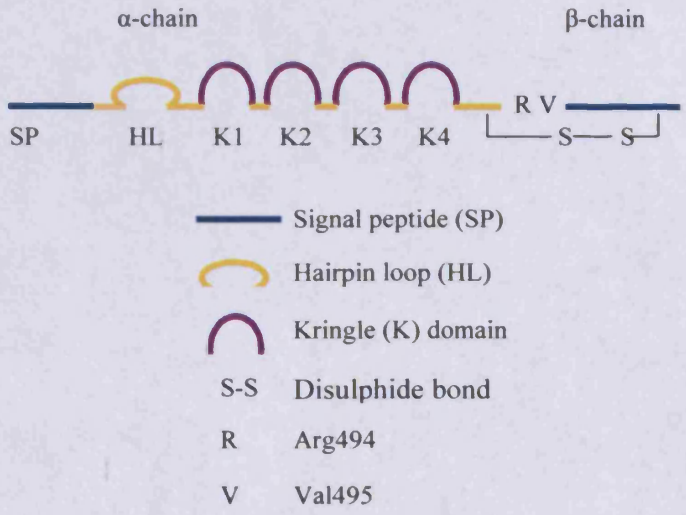


Figure 1.10 Schematic diagram of the structure of HGF/SF.

The α-chain consists of an N-terminal hairpin loop followed by four canonical kringle domains which are 80 amino acid double-looped structures stabilised by three internal disulfide bridges (Comoglio, 1993). The high-affinity binding site for its receptor, the receptor tyrosine kinase c-Met, is located in the first kringle domain, while the hairpin loop and the second kringle domain form a low-affinity binding site for membrane-associated heparin sulphate proteoglycans. The β-chain contains a serine proteinase homology domain but lacks enzymatic activity as a result of amino acid substitutions in the catalytic site (Maulik *et al.*, 2002). HGF/SF is synthesised and secreted by mesenchymal cells as a single-chain, inactive precursor (pro-HGF/SF) which is subsequently proteolytically converted into an active disulfide-linked α-β-chain heterodimer by serine

proteinases such as urokinase-type Plasminogen Activator (uPA) and tissue Plasminogen Activator (tPA), coagulation factors X, XI and XIII and HGF activator (HGFA) (Mars *et al.*, 1993; Parr *et al.*, 2004), (Naldini *et al.*, 1992). Factors that regulate the expression and secretion of HGF/SF include interleukins, injurin and glucocorticoids (Jiang *et al.*, 2005). The immediate HGF/SF regulatory system consists of its main activator HGFA and the two HGFA inhibitors, HAI-1 and HAI-2 (Parr *et al.*, 2004).

1.7.2 c-Met Structure and Function

The *Met* oncogene was originally isolated from a human osteogenic sarcoma cell line that had been chemically mutagenised *in vitro* (Cooper *et al.*, 1984). A translocated promoter region locus (*TPR*) from chromosome 1 was placed upstream of a portion of the *Met* gene on chromosome 7, and the resultant TPR-MET fusion protein displayed constitutively active Met kinase activity (Park *et al.*, 1986). Whilst HGF/SF is produced by surrounding mesenchymal cells, c-Met is predominantly expressed in epithelial cells.

The HGF/SF receptor is a transmembrane protein encoded by the MET proto-oncogene (Bottaro *et al.*, 1991). Its precursor protein is synthesized as single polypeptide chain which is then proteolytically processed into a mature, disulphide-linked 185kDa Met heterodimer (Migliore & Giordano, 2008). This heterodimer comprises an entirely extracellular α-subunit linked to a β-subunit which consists of a large extracellular region involved in ligand-binding, a transmembrane domain, a juxta-membrane domain, a tyrosine kinase domain and C-terminal docking site (Gao & Vande Woude, 2005; Maulik *et al.*, 2002). The extracellular segment of the c-Met receptor is subdivided into a Sema domain, a PSI domain (originally found in plexins, semaphorins and integrins) and four IPT domains (related to immunoglobulin-like domains present in plexins and transcription factors) (**figure 1.11**) (Comoglio *et al.*, 2008).

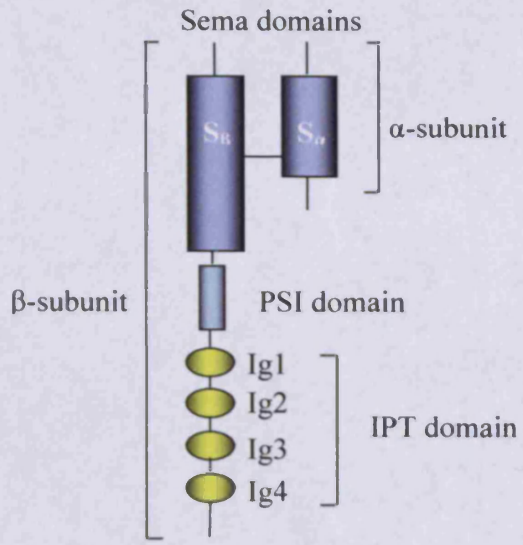


Figure 1.11 Schematic diagram of the modular structure of the extracellular domain of the c-Met receptor.
IG, immunoglobulin-like domain; PSI, plexin-semaphorin-integrin; IPT, immunoglobulin-like domain in plexins and transcription factors.

Upon activation by its ligand HGF/SF, c-Met undergoes autophosphorylation of specific tyrosine residues within its intracellular domain. Y1234 and Y1235 are located in the activation loop of the tyrosine kinase domain (**figure 1.11**) (Ferracini *et al.*, 1991). When inactive, this activation loop is in a 'closed' conformation and therefore inaccessible to substrates and ATP (Gentile & Comoglio, 2004). Upon ligand binding, the Met-Met dimers stabilise, and the activation loop is unlocked by trans-autophosphorylation of Y1234 and Y1235 (Longati *et al.*, 1994). Thus the phosphorylation of these tyrosine residues positively modulates the intrinsic kinase activity of the receptor (Naldini *et al.*, 1991a; Naldini *et al.*, 1991b) while negative regulation is achieved through phosphorylation of a serine residue (S985) in the juxta-membrane segment (Gandino *et al.*, 1994).

Once activated, the Met-Met dimers induce intramolecular phosphorylation of the tyrosine residues Y1349 and Y1356 located in the C-terminal (Gentile & Comoglio, 2004). These residues, along with the adjacent amino acids, constitute

the so-called multi-substrate docking site. Upon phosphorylation, this promiscuous motif mediates interactions with multiple SH2-domain-containing adaptors and signal transducers. Gab-1, an adaptor protein unique to c-Met, mediates most of the complex cellular responses to c-Met activation (Sachs *et al.*, 2000; Weidner *et al.*, 1996). Upon binding, Gab-1 is phosphorylated at several tyrosine residues which are required for recruiting downstream signalling molecules such as the non-receptor tyrosine kinase src (Ponzetto *et al.*, 1994), the lipid kinase PI3K (Ponzetto *et al.*, 1994) and the transcription factor STAT3 (Boccaccio *et al.*, 1998).

1.7.3 HGF/SF/c-Met Signalling in Invasion and Metastasis

As the name suggests, HGF/SF induces a potent scattering response in epithelial cells (Stoker & Perryman, 1985). Scattering involves cell spreading, cell-cell dissociation and cell migration. Attenuation or dissolution of cell-cell adhesions is achieved by HGF/SF-mediated β-catenin phosphorylation (Hiscox & Jiang, 1999a), cadherin-shedding (Davies *et al.*, 2001) and redistribution of E-cadherin (Hiscox & Jiang, 1999b). This, however, is just one of the pleiotropic responses c-Met activation evokes. The full complement of c-Met induced changes has been termed 'invasive growth' (Corso *et al.*, 2005); and the main signalling aspects of this genetic program are outlined below.

As briefly mentioned before, activation of c-Met results in the recruitment of scaffolding proteins such as Gab1 and Grb2, which in turn activate Shp2, Ras and ERK/MAPK (Birchmeier *et al.*, 2003). This alters gene expression of cell-cycle regulators such as pRB, Cdk6 and p27 which promotes cell cycle progression and proliferation, increases synthesis and secretion of extracellular matrix proteinases such as MMPs and uPA which facilitate invasion, and also modifies the cytoskeletal functions which results in enhanced proliferation, migration and invasion (**figure 1.12**) (Birchmeier *et al.*, 2003).

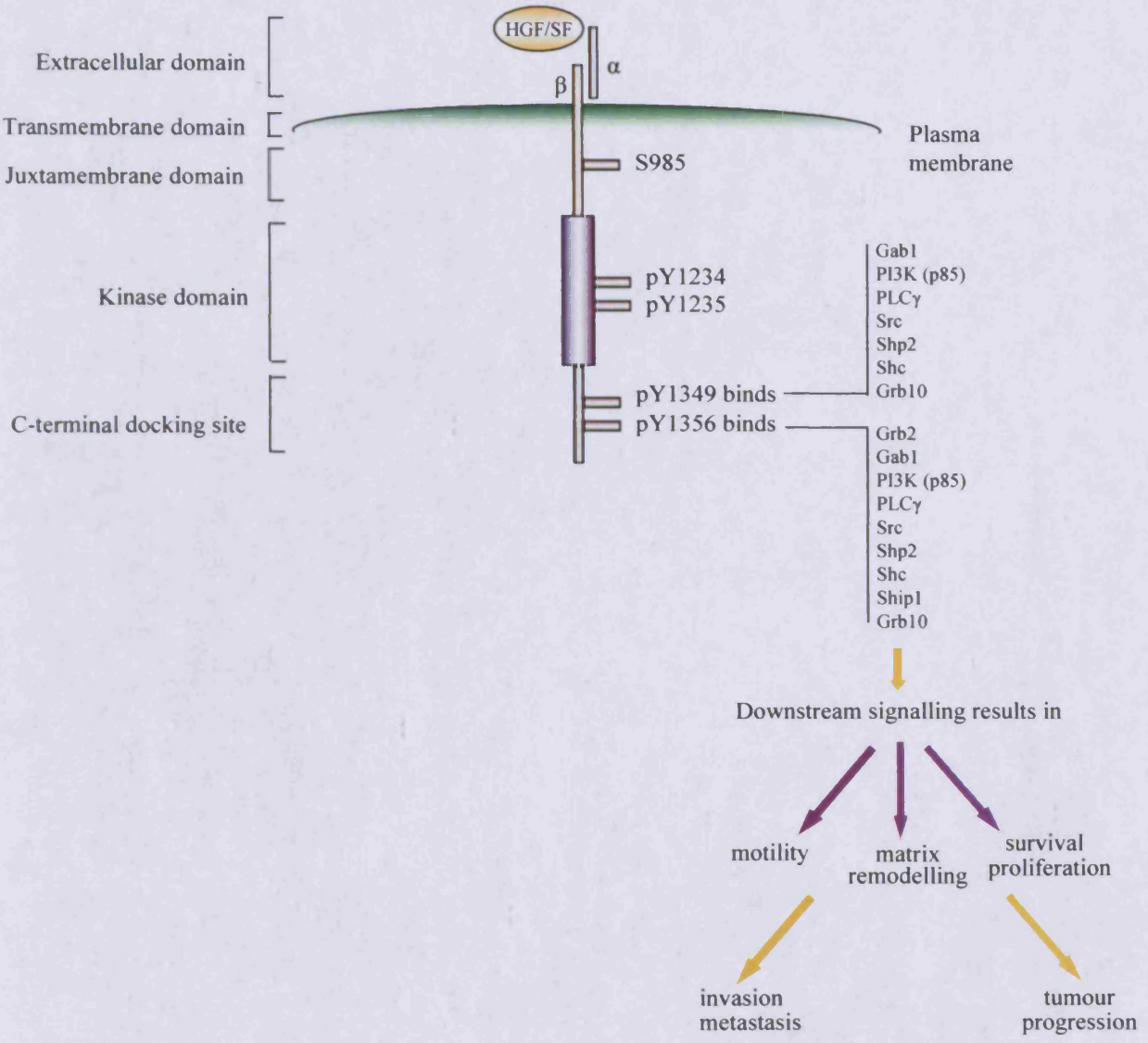


Figure 1.12 Diagram of c-Met phosphorylation and substrate-binding sites.
Diagram detailing the downstream signalling molecules bound by Y1349 and Y1356 and the pleiotropic cellular responses elicited. The negative regulatory site (Serine 985) and the tyrosine residues of the kinase domain, Y1234 and Y1235, are also shown. To the far right the 5 main domains of c-Met are indicated. (Modified from Birchmeier *et al.*, 2003)
Gab1, growth-factor-receptor-bound protein 2 (Grb2)-associated binder 1; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; Shc, Src-homology-2 (SH2)-domain-containing; Shp2, SH2-domain-containing protein tyrosine phosphatase 2; Grb2 and Grb10, growth-factor-receptor-bound protein 2 and 10; Ship, SH2-domain-containing inositol-5-phosphatase; p85 refers to the regulatory subunit of PI3K.

Moreover, Gab-1-mediated activation of PI3K-AKT/PKB promotes cell survival through inhibition of caspase-9 and Bad (Xiao *et al.*, 2001). Overall, the regulation of cell motility, cell dissociation, cell adhesion and invasion downstream of Gab-1 was shown to be dependent on both ERK 1/2 and PI3K pathways as demonstrated in experiments utilising specific pharmacological inhibitors (Potempa & Ridley, 1998). Finally, c-Met signalling has also been shown to evoke a powerful angiogenic response (Rosen *et al.*, 1993). In summary, the c-Met pathway activates a program of cell dissociation and motility coupled with increased protease secretion, promotion of angiogenesis and evasion of apoptosis which results in tumour invasion and metastatic progression.

Not surprisingly, c-Met and HGF/SF signalling activities have emerged as a crucial feature of virtually all human malignancies including breast cancer (Knudsen & Vande Woude, 2008; Peruzzi & Bottaro, 2006). Furthermore, aberrant c-Met activation, mostly due to transcriptional upregulation in the absence of gene amplification, is often linked to high tumour grade and poor prognosis (Jeffers *et al.*, 1996). By way of promoting proliferation, angiogenesis, survival and invasion inappropriate HGF/SF/c-Met signalling participates in all stages of malignant progression, and thus represents a promising drug target for molecular cancer therapy.

With the exception of c-Met receptor transactivation, ligand binding must occur before c-Met receptor dimerisation and autophosphorylation can take place. Therefore, activation of this signalling complex can be inhibited by interfering either with HGF/SF or c-Met directly, or both components simultaneously. Our present understanding of c-Met oncogenic signalling supports three main avenues for c-Met inhibition: (i) HGF/SF/c-Met competitors, (ii) anti-HGF or anti-c-Met antibodies and (iii) c-Met kinase inhibitors. Potent and selective drug candidates have been developed using these three strategies, and Phase I and II clinical trials for many of these compounds are now under way (**table 1.5**).

Compound	Stage of development	Additional information
HGF/c-Met biological antagonists		
NK4	Preclinical	Competitively inhibits HGF/SF binding to c-Met
DecoyMet	Preclinical	Inhibits HGF/SF binding and c-Met dimerisation
Antibodies against HGF/SF		
L2G7 (Galaxy Biotech)	Preclinical	Blocks binding of HGF/SF to c-Met
AMG102 (Amgen)	Phase I/II trial	–
Antibodies against c-Met		
OA-5D5 (Genentech)	Phase I trial	'one-armed' antibody
DN30 (Metheresis)	Preclinical	–
Small molecule c-Met inhibitors		
ARQ197 (ArQule)	Phase I/II trial	Non ATP competitive, c-Met-specific inhibitor
XL184 (Exelixis)	Phase I/II/III trials	ATP competitive, broad spectrum kinase inhibitor

Table 1.5 Typical examples of types of c-Met signal transduction inhibitors currently undergoing pre-clinical and clinical evaluation.
(Comoglio *et al.*, 2008; Eder *et al.*, 2009).

1.8 Aims

As mentioned previously in section 1.4, resistance to fulvestrant therapy presents a problem in the pharmacotherapeutic management of breast cancer (Buzdar, 2004; Cheung *et al.*, 2006; Robertson *et al.*, 2005), with most patients who progress on fulvestrant ultimately developing distant metastases, the primary cause of mortality in cancer.

As outlined before in section 1.4, cells resistant to endocrine therapy circumvent the need for oestrogen stimulation for growth by switching to growth factor signalling pathways to drive proliferation (Nicholson *et al.*, 1999). Preliminary in-house studies with our fulvestrant-resistant cell model have shown that development of resistance to the pure anti-oestrogen is accompanied by the acquisition of an *in vitro* aggressive cell phenotype, characterised by enhanced motile, migratory and invasive capabilities. Since proteolytic degradation of ECM barriers is a prerequisite for tumour invasion and metastasis, aberrant MMP/TIMP expression regulated by a growth factor signalling pathway significantly upregulated in FAS-R cells may be one of the factors contributing to their aggressive invasive phenotype. In order to test this hypothesis, the present study aims to:

- fully characterise the FAS-R cell phenotype with respect to its *in vitro* aggressive behaviour;
- establish the roles of MMP and TIMPs in this context;
- attempt to identify the dominant growth factor signalling pathway that promotes this aggressive behaviour.

Chapter Two

Materials and Methods

"If we knew what we were doing, it wouldn't be called research, would it?"

Albert Einstein (1879-1955)

2 Material and Methods

2.1 Materials

The materials used throughout this study along with their suppliers are detailed in **table 2.1.**

Table 2.1 List of materials and suppliers.

Material	Supplier
Acetic acid	Fisher Scientific UK Ltd., Loughborough, UK
Acrylamide/bis-acrylamide (30% solution (v/v), 29:1 ratio)	Sigma-Aldrich, Poole, Dorset, UK
Agarose	Bioline Ltd, London, UK
Alexa-488 fluorescent dye	Sigma-Aldrich, Poole, Dorset, UK
Ammonium persulphate (APS)	Sigma-Aldrich, Poole, Dorset, UK
Amphotericin B (Fungizone)	Invitrogen, Paisely, UK
Ampicillin	Sigma-Aldrich, Poole, Dorset, UK
Antibiotics	Invitrogen, Paisely, UK
Anti-mouse horseradish-peroxidase-linked IgG (source:sheep)	Amersham, Little Chalfont, UK
Anti-rabbit horseradish-peroxidase-linked IgG (source: donkey)	Amersham, Little Chalfont, UK
Aprotinin	Sigma-Aldrich, Poole, Dorset, UK
Bio-Rad D_C Protein Assaay	Bio-Rad Laboratories Ltd, HERTS, UK
Bovine serum albumin (BSA)	Sigma-Aldrich, Poole, Dorset, UK

Bromophenol blue (BPB)	BDH Chemicals Ltd, Poole, UK
Casein	Sigma-Aldrich, Poole, Dorset, UK
Cell culture medium : RPMI 1640 and Phenol-red-free RPMI 1640	Invitrogen, Paisely, UK
Cell culture medium: Phenol-red-free DCCM	Biological Industries, Ltd, Israel
Cell scrapers	Greiner Bio-One Ltd, Gloucestershire, UK
Chemiluminescent Supersignal® West HRP Substrate (Pico, Dura and Femto)	Pierce and Warrner Ltd, Cheshire, UK
Corning Standard Transwell® inserts (8μm pore size, 6.5mm diameter)	Fisher Scientific UK Ltd, Loughborough, UK
Coulter counter counting vials and lids	Sarstedt AG and Co., Nümbrecht, Germany
Crystal violet	Sigma-Aldrich, Poole, Dorset, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Poole, Dorset, UK
Disposable cuvettes	Fisher Scientific UK Ltd., Loughborough, UK
Di-thiothreitol (DTT)	Sigma-Aldrich, Poole, Dorset, UK
dNTPs (dGTP, dCTP, dATP, dTTP; 100mM)	Amersham, Little Chalfont, UK
Ethidium Bromide	Sigma-Aldrich, Poole, Dorset, UK
Ethylene glycol -bis(2-aminoethyl ether)-N,N,N',N' -tetraacetic acid (EGTA)	Sigma-Aldrich, Poole, Dorset, UK
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Faslodex (Fulvestrant)	AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK

Fibronectin (source: human plasma; 1mg/ml in 0.05M TBS: pH 7.5)	Sigma-Aldrich, Poole, Dorset, UK
Filter paper grade 3	Whatman, Maidstone, UK
Foetal Calf Serum (FCS)	Invitrogen, Paisley, UK
Gelatine	Sigma-Aldrich, Poole, Dorset, UK
General laboratory glass- and plasticware	Fisher Scientific UK Ltd., Loughborough, UK
Glacial Acetic Acid	Fisher Scientific UK Ltd., Loughborough, UK
Glass coverslips	Fisher Scientific UK Ltd., Loughborough, UK
Glass microscopy slides	Fisher Scientific UK Ltd., Loughborough, UK
Glycerol	Fisher Scientific UK Ltd., Loughborough, UK
Glycine	Sigma-Aldrich, Poole, Dorset, UK
Hepatocyte growth factor/ Scatter factor (HGF/SF)	R&D Systems Europe Ltd. Abingdon, UK
Hyperladder™ IV	Bioline Ltd, London, UK
Isoton® II azide-free balanced electrolyte solution	Beckmann Coulter Ltd, High Wycombe, UK
Kodak MXB Autoradiography film (blue sensitive, 18cm x 24cm)	Genetic Research Instrumentation (GRI), Rayne, UK
Leupeptin	Sigma-Aldrich, Poole, Dorset, UK
L-glutamine	Invitrogen, Paisley, UK
Lower buffer for SDS-PAGE gels (Tris 1.5M, pH 8.8)	Bio-Rad Laboratories Ltd, HERTS, UK
LY294002 (selective PI3K inhibitor)	Sigma-Aldrich, Poole, Dorset, UK
Magnesium Chloride ($MgCl_2$)	Sigma-Aldrich, Poole, Dorset, UK
Matrigel™ Basement Membrane Matrix	BD Biosciences, Oxford, UK

Micro-centrifuge tubes (0.5 and 1.5ml)	Elkay Laboratory Products, Basingstoke, UK
MMP2/9 Immunoblotting Standard (Biomol®)	Biomol® International, LP, Exeter, UK
MMP Inhibitor III (Calbiochem®)	Merck Chemicals Ltd., Nottingham, UK
Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase	Invitrogen, Paisely, UK
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Poole, Dorset, UK
Nitrocellulose transfer membrane (Protran® BA85; 0.45µm pore size)	Schleicher and Schuell, Dassell, Germany
PCR oligonucleotide primers	Invitrogen, Paisely, UK
PD98059 (specific MEK-inhibitor)	Alexis Corporation Ltd., Nottingham, UK
Phalloidin-594 fluorescent dye	Sigma-Aldrich, Poole, Dorset, UK
PhastBlue® tablet	GE Healthcare, Chalfont St. Giles, UK
pH buffer calibration tablets (ph 4, 7 and 10)	Fisher Scientific UK Ltd., Loughborough, UK
Phenylarsine oxide	Sigma-Aldrich, Poole, Dorset, UK
phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich, Poole, Dorset, UK
Pipette tips	Greiner Bio-One Ltd, Gloucestershire, UK
polyoxyethylene sorbitan monolaurate (Tween-20)	Sigma-Aldrich, Poole, Dorset, UK
Ponceau S solution (0.1% (w/v) in 5% acetic acid)	Sigma-Aldrich, Poole, Dorset, UK
Potassium Chloride (KCL)	Sigma-Aldrich, Poole, Dorset, UK
Precision Plus Protein™ All Blue Standards (10-250kDa)	Bio-Rad Laboratories Ltd, HERTS, UK
Random hexamers (RH)	Amersham, Little Chalfont, UK
RNase-free H_2O	Sigma-Aldrich, Poole, Dorset, UK
RNAsin™ ribonuclease inhibitor	Promega, Southampton, UK

Sodium azide	Sigma-Aldrich, Poole, Dorset, UK
Sodium chloride (NaCl)	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Poole, Dorset, UK
Sodium fluoride (NaFl)	Sigma-Aldrich, Poole, Dorset, UK
Sodium hydroxide (NaOH; 5M)	Fisher Scientific UK Ltd., Loughborough, UK
Sodium molybdate (Na_2MoO_4)	Sigma-Aldrich, Poole, Dorset, UK
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Poole, Dorset, UK
Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol)	Fisher Scientific UK Ltd., Loughborough, UK
Sterile bijou vials (5ml)	Bibby Sterilin Ltd, Stone, UK
Sterile cell culture plasticware (i.e. culture dishes, flasks, 12-and 24-well plates)	Nunc Int., Roskilde, Denmark
Sterile Falcon tubes (15 and 50ml)	Sarstedt AG and Co., Nümbrecht, Germany
Sterile phosphate buffered saline (PBS)	Invitrogen, Paisley, UK
Sterile syringe needles (BD MicrobalanceTM3; 25G x 5/8")	Becton Dickinson (BD) UK Ltd, Oxford, UK
Sterile syringes (BD Plastipak™; 1ml)	Becton Dickinson (BD) UK Ltd, Oxford, UK
Sterile universal containers (30ml)	Greiner Bio-One Ltd, Gloucestershire, UK
Sterile, disposable serological pipettes (1ml, 5ml, 10ml and 25ml)	Sarstedt AG and Co., Nümbrecht, Germany
Taq DNA polyemerase (BioTaq™; 5U/µl)	Bioline Ltd, London, UK
TRI® Reagent RNA Isolation Reagent	Sigma-Aldrich, Poole Dorset, UK
Tris HCl	Sigma-Aldrich, Poole, Dorset, UK
Triton X-100	Sigma-Aldrich, Poole, Dorset, UK

Trizma (Tris) base	Sigma-Aldrich, Poole, Dorset, UK
Trypsin/EDTA 10x solution	Invitrogen, Paisely, UK
Upper buffer for SDS-PAGE gels (Tris 0.5M, pH 6.8)	Bio-Rad Laboratories Ltd, HERTS, UK
VectorShield® hard-set mounting medium containing DAPI nuclear stain	Vector Laboratories, Inc., Peterborough, UK
Western Blocking Reagent	Roche Diagnostics, Mannheim, Germany
X-ray film developer solution (X-O-dev)	X-O-graph Imaging Sytem, Tetbury, UK
X-ray film fixative solution (X-O-fix)	X-O-graph Imaging Sytem, Tetbury, UK

2.2 Cell Culture

2.2.1 Routine Cell Culture

The hormone-sensitive human mammary carcinoma wild-type cell line MCF-7 (WTMCF-7) was kindly donated to TCCR by AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK) and had originally been obtained from the American Type Culture Collection (ATCC® Number HTB-22™). WTMCF-7 cells were removed from liquid nitrogen storage and routinely cultured in RPMI 1640 medium containing the pH indicator phenol-red (rRPMI) supplemented with 5% foetal calf serum (FCS), antibiotics (penicillin 100units/ml and streptomycin 100μg/ml) and an antifungal agent (amphotericin B; 2.5μg/ml).

Cultures were maintained as monolayers in 75 cm^2 flasks (T-75) at 37°C in a humidified 5% CO_2 atmosphere in a Sanyo MCO-17AIC incubator (Sanyo E&E Europe BV, Loughborough, UK); culture medium was replaced every 3-4 days. Cell confluency and viability were monitored by phase-contrast microscopy (Nikon Eclipse TE200 (Nikon UK Ltd, Kingston-upon-Thames, UK)). Cells were routinely passaged upon reaching 90% confluency and seeded in a 1:10 dilution (see section 2.2.3 for details of procedure). All cell culture was performed in MDH Class II laminar-flow safety cabinets (BIOQUELL UK Ltd, Andover, UK) using sterile technique and sterile plastic ware. Routine cell culture equipment was autoclaved at 119°C in a Denley BA852 autoclave (Thermoquest Ltd, Basingstoke, UK).

2.2.2 Experimental Cell Culture

24 hours prior to experiments, WTMCF-7 cells were washed 3 times with Dulbecco's phosphate- buffered saline (PBS) and transferred to phenol-red-free RPMI 1640 (wRPMI) supplemented with 5% charcoal-stripped foetal calf serum (SFCS), L-glutamine (200mM) and antibiotics and antifungal as detailed above; thereby eliminating any oestrogenic effects arising from the ph indicator

or the steroid hormones of full serum. This experimental cell culture medium will be referred to as 'EM'.

2.2.3 Cell Passaging

First, cultured medium was replaced with 10ml trypsin (0.05%)/EDTA (0.02%) in PBS and the flasks were returned to the incubator for approximately 5 minutes until the cells had detached. Then, 10ml of serum-containing medium was added to the trypsinised cells to neutralise trypsin/EDTA action and the resultant cell suspension centrifuged (Jouan C312 (Thermo Fisher Scientific Inc., MA, USA); 1000rpm, 5 min). The supernatant was discarded and the cell pellets carefully resuspended in 1ml culture medium until a uniform suspension was obtained. 1/10 of this cell suspension was then added to 15ml medium in a T-75 which was then cultured as described above until required.

Upon reaching the maximum number of 25 cell passages, cryopreserved original stocks were resuscitated thereby eliminating the cell line character changes that are induced by high passage numbers.

2.2.4 Cell Number Determination Prior to Experimental Analysis

To ensure seeding of cells at specific densities and equivalent cell numbers in duplicate flasks cell number determination using Coulter counter analysis (Coulter^{TM} Multisizer II (Beckmann Coulter UK Ltd, High Wycombe, UK)) was carried out. Cells were harvested by trypsinisation as described above and the resultant cell suspension passed through a 25G syringe three times to obtain a single-cell suspension. 100µl of this cell suspension were then added to 10ml of Isoton® II solution and cell counts performed in triplicate. In order to obtain the total number of cells present in the flask the average of the above cell counts was multiplied by a factor of 200. An appropriate volume of cell culture medium was then added to the remaining cell suspension and cells seeded onto new flasks at the desired density; the latter being governed by the cell line used and experimental design.

2.2.5 Establishment of a Faslodex (ICI 182,780) - resistant cell line (FAS-R)

The faslodex-resistant MCF-7 cell line (FAS-R) was established in-house by McClelland *et al.* (2001). WTMCF-7 cells were removed from liquid nitrogen storage and maintained and subcultured in EM as described in sections 2.2.1 and 2.2.3 for 2 weeks. After this period of routine maintenance, WTMCF-7 monolayers were washed with PBS and transferred to EM supplemented with faslodex (10^{-7}M final concentration); and from this point onwards, the cells were continuously exposed to the anti-oestrogen. In the initial phase, cell growth was drastically reduced by the drug rendering passaging unnecessary while the culture medium was still being replaced every 3-4 days. After 3 months of culture in the continuous presence of faslodex, cell growth rates began to increase again and stabilise, suggesting the cells had developed resistance to the growth-inhibitory properties of the drug; and the resultant cell line was therefore designated FAS-R. The resistant cells were cultured for an additional 4 months before the commencement of characterisation studies. FAS-R cells were routinely maintained in EM supplemented with faslodex (10^{-7}M).

2.2.6 Treatments

Many experiments in this study required treatment of cells with anti-oestrogens, growth factors and signal transduction inhibitors (see **table 2.2**). 24h prior to all treatments, cells were serum-starved in phenol-red-free DCCM supplemented with L-glutamine and antibiotic/antifungal agents (as above). Detailed information on compound concentration and treatment duration can be found in the relevant sections of the results chapters.

Table 2.2 Treatments used in cell culture experiments.

Treatments	Classification	Target Protein	Final concentration used	Supplier
4-hydroxytamoxifen	anti-hormone	ER	100nM	Sigma-Aldrich, Poole, Dorset, UK
Faslodex™	anti-hormone	ER	100nM	AstraZeneca Pharmaceuticals, Cheshire, UK
TGF-α	growth factor	EGFR	10ng/ml	Sigma-Aldrich, Poole, Dorset, UK
EGF	growth factor	EGFR	10ng/ml	Sigma-Aldrich, Poole, Dorset, UK
IGF-II	growth factor	IGF-1R	10ng/ml	Sigma-Aldrich, Poole, Dorset, UK
TPA	growth factor	PKC	100nM	Sigma-Aldrich, Poole, Dorset, UK
HGF/SF	growth factor	c-Met	20ng/ml	R&D Systems Europe Ltd., Abingdon, UK
MMP inhibitor III (Calbiochem®)	inhibitor	MMPs	40μM	Merck Chemicals Ltd., Nottingham, UK
PD98059	inhibitor	ERK1/2	50μM	Alexis Corporation Ltd., Nottingham, UK
LY294002	inhibitor	PI3K	10μM	Sigma-Aldrich, Poole, Dorset, UK

2.3 Cell Counting Assay

Cell growth was assessed by cell counting experiments using Coulter counter analysis. Cells were harvested by trypsinisation as described previously and seeded into a 24-well-plate in EM ± Faslodex (10^{-7}M) at a density of $4x10^4$cells/well. Cells were allowed to attach for 24h prior to the addition of treatments. Following treatment, the plate was incubated at 37°C in a humidified 5% CO_2 atmosphere for 15 days; medium changes took place every 3-4 days. On day 15, medium was replaced with trypsin/EDTA (1ml/well) and the plate returned to the incubator for approximately 5 minutes until the cells had detached. The resultant cell suspension was passed through a 25G syringe three times to obtain a single-cell suspension and then drawn into the syringe. Wells were subsequently rinsed with fresh Isoton II solution (1ml/well) three times and this volume was also drawn into the syringe each time to give a final volume of 4ml in the syringe. These 4ml of suspension were then added to a counting cup containing 6 ml of Isoton II solution to give a total volume of 10ml in the cup and cell counts performed in triplicate. In order to obtain the total number of cells present in each well the average of the above cell counts was multiplied by a factor of 20.

2.4 Cell Morphology Assessment

Representative images of live cells were captured by differential interference microscopy using a Leica DM-IRE2 inverted microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) connected to a Hamamatsu C4742-96 digital camera (Hamamatsu Photonics UK Ltd, HERTS, UK) and a PowerMAC G5 computer (Apple Computer Inc., CA, USA) running Improvision® OpenLab V4.04 software (Improvision®, Coventry, UK).

2.5 Gene Expression Analysis

Gene expression in WTMCF-7 and FAS-R cell lines was analysed using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

(Saiki *et al.*, 1985, Mullis *et al.*, 1986, Mullis and Faloona, 1987), a technique for enzymatic DNA amplification. RT-PCR was first employed to establish basal gene expression levels and subsequently to determine the effect of growth factor and inhibitor treatment on the expression levels of some of the above genes. All reactions were set up in a Labonco Purifier PCR Enclosure (GRI, Rayne, UK) using sterile pipette tips and microcentrifuge tubes. Equipment and work surfaces were cleaned with 70% ethanol to remove contaminating RNAse and DNAse enzymes.

2.5.1 Total RNA isolation

Cells were seeded into 60mm culture dishes at a density of $5x10^5$cells/dish and cultured until log-phase growth; at this point treatments were added for the desired duration where appropriate. Total RNA was extracted from cell monolayers using TRI® Reagent RNA Isolation Reagent (Chomczynski, 1993).

Culture medium was aspirated and monolayers washed twice with PBS prior to the addition of TRI® Reagent (1ml per $10cm^2$ area of culture dish at RT). After 5 minutes lysates were collected using a cell scraper, homogenised by pipetting and transferred to sterile 1.5ml microcentrifuge tubes. Phase separation into aqueous (RNA), interphase (DNA) and organic phase (protein) was achieved by adding chloroform (0.2ml chloroform per 1ml TRI reagent) to the lysates. Samples were gently shaken for 15 seconds and incubated for 15 minutes at RT. Following centrifugation (IEC Micromax RF microcentrifuge (Thermo Electron Corporation, Hampshire, UK); 11,3000rpm, 15 minutes, 4°C), the aqueous upper phase was transferred to fresh 1.5ml microcentrifuge tubes. After the addition of isopropanol (0.5ml isopropanol per 1ml TRI® Reagent) samples were gently shaken and incubated for 10 minutes at RT and subsequently centrifuged (11,300rpm, 10 minutes, 4°C). The resulting RNA pellets were washed with ethanol (75% in H_2O, 1ml per pellet), vortexed and centrifuged (9000rpm, 5 minutes, 4°C). Following a second ethanol wash, ethanol was removed and RNA pellets allowed to dry briefly before

resuspension in RNAse-free sterile H_2O (20µl per pellet). RNA samples were stored at -80°C until required.

2.5.2 Total RNA Quantitation

RNA concentration (µg/ml) was determined by diluting 1µl RNA in 499µl RNAse-free H_2O and measuring the absorbance at 260nm (A_{260}) on a spectrophotometer (Cecil CE2041 spectrophotometer (Cecil Instruments, Cambridge, UK)) based on the following formula:

$$(RNA) = A_{260} \times 40 \times \text{Dilution Factor}$$

2.5.3 Total RNA Qualitation and Agarose Gel Electrophoresis

RNA qualitation was carried out by measuring the absorbance at 280nm (A_{280}) and calculating the A_{260}/A_{280} ratio (a ratio of 1.8–2.0 being indicative of good-quality RNA) and by electrophoresing RNA.

RNA samples were resolved on a 2% (w/v) agarose gel in Tris-Acetate-EDTA buffer (TAE, pH 8.3; see buffer appendix) containing ethidium bromide (1µl of a 10mg/ml solution per 100ml agarose gel). Gels were cast and electrophoresed using the Sub-cell® Agarose Electrophoresis System linked to a Powerpac 1000 power pack (both Bio-Rad Laboratories Ltd, HERTS, UK) following manufacturer's instructions. 500ng RNA per sample were combined with sample loading buffer (5µl; see buffer appendix) and loaded into the wells of the gel. TAE buffer was added to the electrophoresis tank and the gel electrophoresed at 80V constant voltage for 1 hour. Gels were subsequently visualised under UV light using a FOTODYNE 3-3002 UV trans-illuminator and photographed using a Polaroid GelCam camera (both GRI, Rayne, UK). Strong 28S and 18S ribosomal bands with the 28S band appearing twice as strong as the 18S band were indicative of good-quality RNA.

2.5.4 Reverse Transcription (RT)

Complementary DNA (cDNA) was generated by reverse transcribing RNA using the Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase enzyme.

1µg of total RNA (made up to a total volume of 6µl with RNAse-free H_2O) was added to 6µl of master mix comprising 2µl random hexamer oligonucleotides (100µM) and 4µl dNTP mix (0.625mM of dGTP, dCTP, dATP and dTTP) to make up a final volume 12µl. Samples were then placed in a PTC-100 thermocycler (MJ Research Ltd, Massachusetts, USA), denatured at 65°C for 5 minutes and cooled on ice. 8µl of RT master mix comprising 4µl First Strand Buffer (5x), 2µl dithiothreitol (DTT, 0.1M), 1µl RNasin™ RNase inhibitor (40U/µl) and 1µl MMLV-reverse transcriptase (200U/µl) were added to each denatured sample. Samples were incubated at 37C° for 50 minutes, inactivated by heating to 70C° for 15 minutes and then cooled at 4C°. The resulting cDNA samples were aliquotted and stored at -20C° until required.

2.5.5 Primer design

The oligonucleotide primers employed were either based on published sequences or designed using the software program 'Primer3' (http://frodo.wi.mit.edu/), using gene sequences that had been obtained from the NCBI online database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar) (see **table 2.3** for primer sequences).

Table 2.4 General guidelines used for PCR primer design.

Parameter	Optimum
Length	18-22 nucleotides
G/C content	40-60%
Melting temperature	Forward and reverse primers should have similar melting temperatures. T_m = (number of A+T) x 2° C + (number of G+C) x 4°C (Suggs *et al.*, 1981)
Sequence	Primer sequence should span a region towards middle of gene sequence to avoid potential partial RNA degradation Avoid complementarity at 3' ends to avoid primer dimer formation Avoid 3 G or C nucleotides in a row near the 3' end Aim for high G/C content at 3' end as this is where RNA polymerase enzyme binds

Table 2.5 Expected product sizes of PCR primers.

Primer pair	Expected product size (bp)
MMP-1	1030
MMP-2	440
MMP-7	373
MMP-9	1100
MMP-10	408
MMP-11	700
MMP-13	359
TIMP-1	655
TIMP-2	335
TIMP-3	201
TIMP-4	226
c-Met	320
ER-α	432
β-actin	204

Table 2.3 Primer sequences and reaction conditions used for semi-quantitative RT-PCR alongside expected product sizes.

Target gene	Primer Sequence	Conditions (annealing temperate - cycle number)
MMP-1F	5'-GTC ACA CCT CTG ACA TTC AC-3'	56°C 33 cycles
MMP-1R	5'-CAC CTT CTT TGG ACT CAC AC-3'	
MMP-2F	5'-CTC GAA TCC ATG ATG GAG AG-3'	58°C 34 cycles
MMP-2R	5'-CAG CTG TCA TAG GAT GTG CC-3'	
MMP-7F	5'-ACA GGC TCA GGA CTA TCT CA-3'	55°C 30 cycles
MMP-7R	5'-AGT TCC CCA TAC AAC TTT CC-3'	
MMP-9F	5'-AGA CCT GAG AAC CAA TCT CAC-3'	56°C 33 cycles
MMP-9R	5'-GGC ACT GAG GAA TGA TCT AA-3'	
TAG-MMP-10F	5'-CAC TCT ACA ACT CAT TCA CAG AGC T-3'	56°C 33 cycles
TAG-MMP-10R	5'-CTT GGA TAA CCT GCT TGT ACC TCA T-3'	
MMP-11F	5'-CCG CCT TCT ACA CCT TTC GC-3'	56°C 33 cycles
MMP-11R	5'-AGC CTT CCA GAG CCT TCA CC-3'	
MMP-13F	5'-GAT GAT ACT TCC CAC ACC AC-3'	56°C 33 cycles
MMP-13R	5'-CTG CTC TTT TGT CTC CTG TC-3'	
TIMP1F	5'-TTG TTG CTG TGG CTG ATA G-3'	55°C 23 cycles
TIMP1R	5'-CGG AAG AAA GAT GGG AGT G-3'	
TIMP2F	5'-ATC AGG GCC AAA GCG GTC AGT GAG-3'	61°C 23 cycles
TIMP2R	5'-ATC TTG CAC TCG CAG CCC ATC TGG-3'	
TIMP3F	5'-TTC CCA TTG TGG TCA CGC CAT TTG-3'	55°C 30 cycles
TIMP3R	5'-GGG AAC TTG TGT AGG TTG GTC AGG-3'	
TIMP4F	5'-GTG CCG TCA ACA TGC TTC ATA CAG-3'	58°C 30 cycles
TIMP4R	5'-ATC TGT GCA ACT ACA TCG AGC CCT-3'	
ER-αF	5'-GGA GAC ATG AGA GCT GCC AAC-3'	55°C 27 cycles
ER-αR	5'-CCA GCA GCA TGT CGA AGA TC-5'	
c-MetF	5'-AAG CCA GAT TCT GCC GAA CCA ATG G-3'	55°C 27 cycles
c-MetR	5'-CCA AGA TTA GCT ATG GTG AGG TCT CC-3'	
β-actinF	5'-GGA GCA ATG ATC TTG ATC TT-3'	55°C 27 cycles
β-actinR	5'-CCT TCC TGG GCA TGG AGT CCT-3'	

Primers were designed according to the general guidelines outlined in **table 2.4** (Taylor *et al.*, 1991); expected product sizes are detailed in **table 2.5.**

All primer sequences were cross-checked using an online database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure sequence specificity (Altschul *et al.*, 1997). All PCR primers were synthesized by Invitrogen. Primer stock solutions (20μM) in RNAse-free H_2O were prepared and stored at -20°C until required.

2.5.6 Polymerase Chain Reaction (PCR)

2.5.6.1 Reaction Mixtures and Thermocycling Conditions

PCR reactions were performed in a total volume of 25 μl (i.e. 0.5 μl cDNA and 24.5 μl of master mix solution). The master mix contained 2μl dNTP mix (0.625mM of dGTP, dCTP, dATP and dTTP), 2.5μl PCR buffer (10x; see buffer appendix), 0.625μl forward primer (20μM), 0.625μl reverse primer (20μM), 0.2 μl Taq polymerase enzyme (5U/μl) and 18.6μl sterile RNAse free H_20 to check for contamination. Each PCR reaction included a negative control in which cDNA was replaced by the same amount of RNAse-free H_2O to check for contamination. The reaction mixture was briefly vortexed and pulsed in a microcentrifuge before being placed in a PTC100-thermal cycler with the heated lid programmed at 100°C. The PCR cycling parameters employed are outlined in **table 2.6**; annealing temperature and cycle number varied for each target gene. Amplification of the house-keeping gene β-actin was performed in parallel on the same set of cDNA samples used for target gene amplification and used as an equal loading control.

Table 2.6 Thermocycler program conditions for PCR amplification.

	Step	Temperature	Duration	Cycle number
1	Denaturing Annealing Extension	95°C dependent on target gene 72°C	2 minutes 1 minute 2 minutes	1x
2	Denaturing Annealing Extension	94°C dependent on target gene 72°C	1 minute 30 seconds 1 minute	dependent on target gene
3	Denaturation	94°C	1 minute	1x
4	Final Extension	60°C	7 minutes	1x
5	Short-term storage	12°C	∞	

2.5.6.2 Gel electrophoresis and Visualisation of cDNA PCR products

PCR cDNA products were essentially electrophoresed as described before (section 2.5.3). 15µl PCR product were combined with sample loading buffer (5µl; see buffer appendix) and resolved alongside a DNA molecular weight marker (Hyperladder^{TM} IV 100-1000bp; 5µl). cDNA products were visualised and photographed as before, and images scanned using a Bio-Rad GS-690 Imaging Densitometer (Bio-Rad Laboratories Ltd, HERTS, UK) connected to a computer running Molecular Analyst Version 1.5 (Bio-Rad). Densitometry was measured as 'adjusted volume Optical Density x mm^2' and raw data was subsequently normalised using β-actin.

2.6 Analysis of Protein Expression and Activation

SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and Western Blotting (Towbin *et al.*, 1979) were used to both identify and quantify the expression of specific proteins and to determine their activity levels where appropriate. Protein expression and activation analysis was performed on WTMCF-7 and FAS-R cell lines under basal conditions and following growth factor and inhibitor treatment.

2.6.1 Cell Lysis

Cells were seeded into 60mm culture dishes at a density of $5x10^5$cells/dish and cultured until log-phase growth; at this point treatments were added for the desired duration where appropriate.

Cell monolayers were gently washed twice with ice-cold PBS and then lysed by the addition of ice-cold Triton-X100 lysis buffer containing protease and phosphatase inhibitors (see buffer appendix). 150-200µl lysis buffer were added per dish, the volume depending on dish size and cell confluency. Lysates were collected using a cell scraper and transferred to 1.5ml-microcentrifuge tubes; these were then incubated on ice for 20 minutes. Cell lysates were subsequently centrifuged (13,000rpm, 15 min, 4°C). The resultant supernatants were aliquotted and stored at -20°C until required.

2.6.2 Cell Supernatant Collection

In an attempt to detect MMPs potentially secreted into the culture medium, protein expression analysis was also carried out on cell supernatants; this required collection and concentration of the cell culture medium. As above, cells were seeded into 60mm dishes and treated where appropriate. Cell supernatants were then removed from the dishes, transferred to 15ml-centrifuge tubes and immediately centrifuged (Jouan C312 (Thermo Fisher Scientific Inc., MA, USA); 4000rpm, 5 min) to pellet debris and clarify the medium. As above, the resultant supernatants were aliquotted and stored at -20°C until required.

2.6.3 Protein Concentration Assay

Determination of the total soluble protein concentration of cell lysates was carried out using the detergent-compatible Bio-Rad D_C Protein Assay Kit (Bio-Rad Laboratories Ltd, UK), a modified version of the assay described by Lowry *et al.* (1951).

First, BSA protein concentration standards ranging from 0 to 1.45mg/ml were prepared (see appendix), their absorbance analysed at 750nm using a spectrophotometer (see section 2.5.2) and a BSA standard curve generated. Then, lysates were diluted with Triton-X100 lysis buffer (1:5; final volume 50µl). Test samples were prepared by adding 0.25ml Reagent A (containing 5µl Reagent S) and 2ml Reagent B to each diluted lysate in a spectrophotometer cuvette. Test samples were gently vortexed, incubated at RT for 15 minutes for full colour development and absorbances analysed as before. Based on the BSA standard curve and the absorbance values the total soluble protein content of the lysates was calculated.

2.6.4 SDS-PAGE Analysis

Whole cell extracts were separated by size using the discontinuous polacrylamide gel electrophoresis method by Laemmli (Laemmli, 1970). This comprised a 7-8% (w/v) acrylamide/bis-acrylamide resolving gel (pH 8.8) and a 4% (w/v) acrylamide/bis-acrylamide stacking gel (pH 6.8), the acrylamide percentage of the resolving gel varying depending on the molecular weight of the protein of interest (higher acrylamide percentage being used to separate lower molecular weight proteins). All SDS-PAGE was performed using the Bio-Rad Mini-Protean® III apparatus powered by a Powerpac Basic™ power pack (both Bio-Rad Laboratories Ltd, HERTS, UK) following manufacturer's instructions. Acrylamide gel solutions were prepared according to the recipes in **tables 2.7** and **2.8** and carefully dispensed between the glass plates of the Bio-Rad gel casting apparatus. Immediately after pouring the resolving gel it was overlaid with 0.05% (w/v) SDS in H_2O to level the interface between resolving and stacking gel. The gel was then allowed to set for 30 minutes at RT. Once set, the SDS solution was discarded and the stacking gel rinsed with distilled H_2O before being overlaid with the resolving gel. A 10-well comb (1.5mm width) was inserted and the resolving gel allowed to set for 30 minutes at RT. The gels were then positioned in the electrophoresis tank and SDS-PAGE running buffer (1x, see buffer appendix) poured into the inner and outer

reservoirs of the tank. Once immersed, the comb was gently removed from the gels and the resultant wells rinsed out with SDS-PAGE running buffer.

Cell lysates containing 40µg total soluble protein per sample were diluted with 3x Laemmli sample loading buffer (see buffer appendix), denatured/reduced at 100°C in a dry heating block (Techne DB-2A Dri-Block) for 5-10 minutes (depending on the sample volume) and pulsed before being loaded into the wells of the stacking gel. Samples were run alongside a protein molecular weight marker (Precision Plus Protein™ All Blue Standards 1-250kDa; 5µl) and, where appropriate, a MMP-2/-9 Immunoblotting Standard (Biomol®) International, LP, Exeter, UK). Gels were electrophoresed at 150V constant voltage until the Laemmli sample loading buffer had migrated the desired distance down the gel (approximately 1-1.5 hours).

Table 2.7 Recipe for stacking gel.

	5% (w/v) For 10ml:	**Final concentration in gel**
Acrylamide/bis-acrylamide 30% solution (ratio 29:1)	1.65ml	5% (w/v)
H_20	5.7ml	-
Tris (0.5M, pH 6.8)	2.5ml	125mM
SDS (10% solution in H_20)	100µl	0.1% (w/v)
APS (10% solution in H_20)	50µl	0.5% (w/v)
TEMED	20µl	0.2% (v/v)

Table 2.8 Recipe for resolving gel.

	8% (w/v) gel (70-200kDa) For 10ml:	**10% (w/v) gel (20-100kDa) For 10ml:**	**Final concentration in gel**
Acrylamide/bis-acrylamide 30% solution (ratio 29:1)	2.7ml	3.3ml	8 or 10% (w/v)
H_20	4.6ml	4ml	-
Tris (1.5M, pH 8.8)	2.5ml	2.5ml	375mM
SDS (10% solution in H_20)	100µl	100µl	0.1% (w/v)
APS (10% solution in H_20)	100µl	100µl	0.1% (w/v)
TEMED	40µl	40µl	0.4% (v/v)

2.6.5 Western Blotting

Western Blotting involves the electrophoretic transfer of size-fractionated proteins from a gel matrix to a solid membrane support. Proteins were transferred from SDS-PAGE gels to a nitrocellulose membrane using the equipment described above.

Following SDS-PAGE gel plates were separated, the stacking gel discarded and the resolving gel rinsed in Western Blot transfer buffer (see buffer appendix) to remove excess SDS. Two sheets of grade 3 filter paper and one sheet of Protran B85 nitrocellulose membrane (pore size 0.45µm) per gel were cut to size and, together with two fibre pads, pre-soaked in Western Blot transfer buffer for 30 minutes. A Western Blot cassette was then assembled as outlined in **figure 2.1** and a universal plastic tube gently rolled over the surface of the 'gel-sandwich' to expel trapped air bubbles which might interfere with the protein transfer.

subsequently removed by washes in TBS-Tween (0.05% (v/v) Tween-20 in 1xTBS, see appendix) (3 x 5 minutes) and membranes incubated with blocking buffer (5% Western Blocking Reagent (v/v) in 1xTBS-Tween) on a rotor-shaker for 1 hour at RT to prevent non-specific binding of the primary antibody (1°AB).

Following blocking, membranes were washed in TBS-Tween and incubated with a 1°AB specific to the protein of interest. 1°ABs were diluted in TBS-Tween containing 5% (v/v) Western Blocking Reagent and 0.05 (w/v) sodium azide. 1°AB concentration and incubation conditions varied depending on the protein of interest and were optimised individually, see **table 2.9** for details.

After exposure to the 1°AB, membranes were washed in TBS-Tween at RT (3 x 10 minutes) and incubated with Horse Radish Peroxidase (HRP)-conjugated anti-rabbit-or anti-mouse-antibody (1:20,000 dilution for β-actin; 1:10,000 or 1:5,000 dilution for all other antibodies) for 1 hour at RT. Following repeated TBS-Tween washes (3 x 10 minutes) chemiluminescence was performed to visualise the presence and, where appropriate, phosphorylation status of the protein of interest in each sample (Kricka *et al.*, 1991). Chemiluminescence was detected using a commercial kit containing the luminol/peroxide-based enhanced chemiluminescence (ECL) reagents Supersignal™ West Pico, Dura or Femto (Mattson and Bellehumeur, 1996); the choice of reagent (in descending order) being dictated by signal strength.

Membranes were then transferred to an autoradiography cassette and incubated with the appropriate ECL reagent (500µl per membrane) for 5 minutes at RT. HRP-mediated oxidisation of the substrate luminol in the presence of peroxide generates an excited state molecule. The subsequent decay to a lower energy state molecule is accompanied by the emission of light; this light is detected using x-ray film. Exposure times vary from several seconds to overnight, depending on signal strength. Following detection of the protein of interest membranes were washed in TBS-Tween (3 x 10 minutes) and re-immunoprobed for β-actin and the detection procedures described above were repeated.

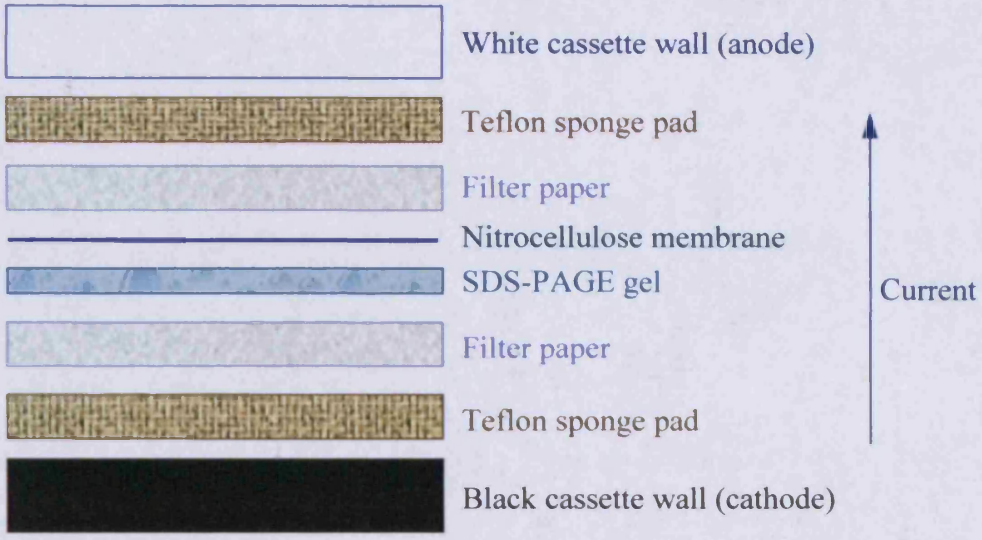


Figure 2.1 Schematic diagram of the electrophoretic transfer sandwich.
The individual components of the electrophoretic transfer sandwich were assembled in the above order and the Western Blot cassette closed and inserted into the transfer apparatus.

Following its assembly the cassette was inserted into the transfer apparatus with the nitrocellulose membrane nearest the anode to ensure deposition of the negatively charged SDS-coated proteins from the gel matrix onto the membrane. Both the transfer apparatus and an ice-pack were then placed in the transfer tank filled with ice-cold Western Blot transfer buffer, the tank placed onto a magnetic stirrer plate and the transfer allowed to proceed at 100V constant voltage for 1 hour under gentle stirring.

2.6.6 Immunoprobing of Western Blots

Following transfer, cassettes were dismantled, gels discarded and membranes washed in 1xTris buffered saline (TBS, see buffer appendix) (3 x 5 minutes). Membranes were briefly stained with Ponceau S solution (0.1% (w/v) in 5% acetic acid) to ascertain equal protein transfer to the membrane, the stain

X-ray films were developed using an X-O-graph Compact X2- x-ray developer (X-O-graph Imaging System, Tetbury, UK) and the resultant signals scanned and analysed using a Bio-Rad GS-690 Imaging Densitometer as described in section 2.5.6.2.

Variation in sample protein loading was corrected by normalising all signal values using the signal value of β-actin which does not vary significantly and is thus used as an internal loading control (Liao *et al.*, 2000).

Table 2.9 Details of antibodies used for immunoprobing.

Target	Source	Dilution	Incubation conditions	Supplier	Catalogue number
MMP-9	Rabbit	1:2000	O/N 4°C + 2h RT	Biomol	#SA-106
MMP-9	Mouse	1:2000	O/N 4°C + 2h RT	Labvision	#MS-569-P0
MMP-9	Rabbit	1:2000	O/N 4°C + 2h RT	Stratech Scientific	#RB-1539-P0
MMP-2	Mouse	1:1000	O/N 4°C + 2h RT	Labvision	#MS-804-P0
MMP-2	Mouse	1:2000	O/N 4°C + 2h RT	Stratech Scientific	#MS-567-P0
MMP-2	Rabbit	1:2000	O/N 4°C + 2h RT	Santa Cruz	SC-10736
MMP-1	Mouse	1:400	O/N 4°C + 2h RT	Stratech Scientific	#MS-801-P0
MMP-1	Rabbit	1:2000	O/N 4°C + 2h RT	Biomol	#SA-102
CD44s	Mouse	1:2000	O/N 4°C + 2h RT	Stratech Scientific	#MS-668-P0
ERK 1/2 Y202/Y204	Rabbit	1:1000	O/N 4°C	Cell Signalling	#9101
T-ERK 1/2	Rabbit	1:1000	O/N 4°C	Cell Signalling	#9102
AKT S473	Rabbit	1:1000	O/N 4°C	Cell Signalling	#9271
T-AKT	Rabbit	1:1000	O/N 4°C	Cell Signalling	#9272
T-c-MET	Mouse	1:2000	O/N 4°C	Zymed	#18-7366
c-Met Y1349	Rabbit	1:1000	O/N 4°C	Cell Signalling	#3121
c-Met Y1234	Rabbit	1:1500	O/N 4°C	Upstate	#07-211
β-actin	Mouse	1:20,000	2h RT	Sigma	A2228

T = Total

O/N = Overnight

Y = Phosphorylation on tyrosine

S = Phosphorylation on serine

2.7.1 Cell-conditioned Medium Collection

As described before cells were seeded into 60mm culture dishes at a density of $5x10^5$cells/dish and cultured until log-phase growth; at this point treatments were added for the desired duration where appropriate. However, when growing cultures for zymographic analysis of MMP expression cells were cultured in the lowest possible culture medium volume (1ml per dish) to increase the MMP concentration in the supernatant. Also, cells were cultured in Defined Cell Culture Medium (DCCM) to eliminate contamination with exogenous constitutively expressed MMPs contained in the SFCS of routine culture medium. Cell-conditioned medium was collected, processed and stored as described in section 2.6.2.

2.7.2 Substrate Gel Electrophoresis

Clarified supernatants were separated according to their specific molecular weight and electrical charge using a modified version of the discontinuous polyacrylamide gel electrophoresis method described in 2.6.4 (Laemmli, 1970); and zymography was essentially performed according to Hauck (2001). While the stacking gel was prepared as detailed in section 2.6.4, a substrate, either gelatin (1% gelatin in resolving gel solution) or casein (1% casein in resolving gel solution), was incorporated into the resolving gel (see **table 2.10**).

Prior to loading samples were combined with an appropriate amount of non-reducing sample loading buffer (1x; see buffer appendix), vortexed, pulsed and incubated for 30 minutes at RT to allow coating of the proteins with SDS. Following incubation, samples were applied to the gel as described in 2.6.4 alongside the protein molecular weight marker and the MMP-2/-9 Immunoblotting Standard detailed above. A standard volume of 40µl was loaded for each sample and densitometric values were subsequently corrected based on protein concentration values obtained for these samples. Gels were electrophoresed at 70V for 4 hours at 4°C to prevent spontaneous enzyme activation.

2.7 Zymography

Numerous methods have been developed to measure the expression and activity levels of proteolytic enzymes in tissues and biological fluids (Cheng *et al.*, 2008). Zymography, a technique now routinely used for the detection of MMPs in complex biological samples (Snoek-van Beurden and Von den Hoff, 2005), was first pioneered by Granelli-Piperno and Reich (1978), and subsequently described by Heussen and Dowdle (1980) as a method for detecting plasminogen activators in gelatine substrate slab gels. Zymographic analysis involves the electrophoretic separation of proteins under denaturing but non-reducing conditions through an SDS-polyacrylamide gel containing a co-polymerised protease substrate such as gelatin (Lombard *et al.*, 2005, Hattori *et al.*, 2002). Upon electrophoresis, resolved proteins are renatured in non-ionic detergent which removes the SDS, and susbsequently incubated in a buffer system which supplies the chloride and zinc ions the enzymes require to be active. It is thought that zymogen forms of MMPs are activated through this process of gentle denaturation and renaturation; and they can therefore be detected in zymograms along with the active forms based on their different molecular weights (Kleiner and Stetler-Stevenson, 1994). Following Coomassie Blue staining, MMPs are visualised as clear zones in which proteolytic activity resulted in degradation of the gelatin substrate. Zymography has been described as an extremely sensitive technique, and Leber and Balkwill (1997) reported the detection of less than 10 pg of MMP-2 on gelatine zymograms which compares favourably with Enzyme-Linked ImmunoSorbent Assays (Kleiner and Stetler-Stevenson, 1994). Moreover, zymography has been used extensively in the qualitative evaluation of proteases present in tumours and cell culture media (Kleiner and Stetler-Stevenson, 1994). In this study, gelatin zymography was employed which is routinely used for the detection of the gelatinases MMP-2 and MMP-9. Casein zymography for the detection of MMP-1 was also performed.

Table 2.10 Recipe for resolving gel for zymography.

	8% (w/v) gel For 10ml:	**Final concentration in gel**
Acrylamide/bis-acrylamide 30% solution (ratio 29:1)	2.7ml	8 % (w/v)
H_20	3.6ml	-
1% gelatin stock solution	1ml	0.1%
Tris (1.5M, pH 8.8)	2.5ml	375mM
SDS (10% solution in H_20)	100µl	0.1% (w/v)
APS (10% solution in H_20)	100µl	0.1% (w/v)
TEMED	40µl	0.4% (v/v)

2.7.3 Staining and Destaining

Following electrophoresis, gels were washed in the detergent Triton X-100 (2.5% in H_2O) three times for a total of 1 hour to allow renaturation of the MMP proteins contained in the gel matrix before overnight incubation in activation buffer (see buffer appendix) at 37°C to allow digestion of the gelatine substrate by the enzymes. Upon overnight incubation, gels were briefly rinsed in distilled H_2O. A stain stock solution (0.2%) was prepared by dissolving 1 PhastBlue® tablet (GE Healthcare, UK) in 80ml of distilled H_2O, adding 120ml methanol, and stirring until all dye was dissolved. The solution was then filtered through Whatman filter paper to remove undissolved stain particles. A stain working solution was prepared by adding a certain volume of stain stock solution to an equal amount of glacial acetic acid (0.2% in H_2O). Gels were incubated in stain working solution (200ml per gel) on a rotor-shaker for 6 hours and then washed in destain solution (see buffer appendix) until clear bands against a dark blue background appeared. Gels were placed on a light-box and photographed using a Polaroid GelCam camera (GRI, Rayne, UK). Images were subsequently scanned in and analysed using a Bio-Rad GS-

690 Imaging Densitometer (Bio-Rad Laboratories Ltd, HERTS, UK) connected to a computer running Molecular Analyst Version 1.5 (Bio-Rad). Densitometry was measured as 'adjusted volume Optical Density x mm^2'.

2.8 Cell Invasion Assay

A modified version of the Boyden chamber assay (Albini *et al.*, 1987) was used to determine the ability of WTMCF-7 and FAS-R to invade an artificial matrix under both basal conditions and following growth factor and inhibitor treatment.

Briefly, the assay consists of a hollow plastic chamber with a porous membrane at the lower end. This invasive chamber, coated with a gel matrix designed to mimic the ECM, resides in another chamber filled with medium. Cells seeded into the insert invade through the pores to the other side of the membrane; and at the assay endpoint, the number of invading cells is quantified.

Assays were performed using Corning Standard Transwell® inserts (8μm pore size; 6.5mm diameter (**figure 2.2**)).

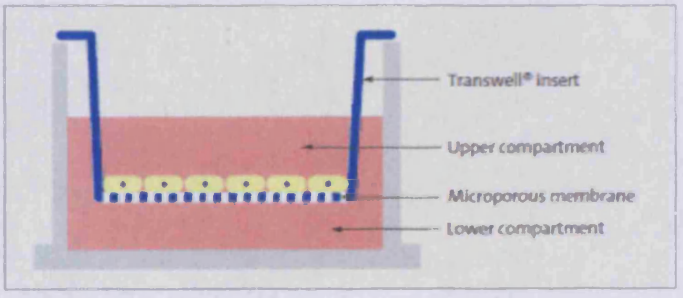


Figure 2.2 Schematic diagram of a Corning Standard Transwell® insert.
Diagram of a Corning Standard Transwell® insert resting in a well of a 24-well-plate. During migration and invasion assays the cells that are here depicted as resting on the upper side of the microporous membrane, traverse through the pores and attach to the bottom of the insert. Figure acquired from
http://catalog2.corning.com/Lifesciences/media/pdf/transwell_guide.pdf

The inner membrane of the inserts was coated using BD Matrigel™ Basement Membrane Matrix, a solubilised biologically active basement membrane prepared from the Engelbreth-Holm-Swarm mouse sarcoma containing laminin and collagen IV as its major components. 50µl of a 1 in 3 dilution of BD Matrigel™ Basement Membrane Matrix in ice-cold wRPMI (without supplements) was pipetted unto the porous membrane of each insert, the inserts placed in a 24-well plate and the plate incubated at 37°C for 2 hours until the gel matrix had set.

Cells were harvested by trypsinisation as described previously and then seeded into the inserts at a density of $5x10^5$ cells/ml in EM ± Faslodex (10^{-7}M) ± treatments (200µl). All conditions were performed in duplicate inserts. EM ± Faslodex (10^{-7}M) ± treatments (650µl) was pipetted into the wells of the plate containing the respective inserts and the plate incubated at 37°C in a 5% CO_2 atmosphere for 48 hours.

Following incubation, the supernatants of the inserts were discarded; and non-invasive cells and gel matrix removed by gently wiping the inside of the insert membrane with a cotton swab. Those cells that had invaded the gel matrix and the pores of the membrane and adhered to the outside of the insert were fixed in 3.7% formaldehyde in PBS for 10 minutes, washed with PBS and left to dry for 5 minutes. The membranes were then excised from the inserts using a scalpel blade and mounted (cells pointing upwards) onto microscope slides in duplicate using VectorShield® hard-set mounting medium which contains the nuclear stain DAPI. Microscope slides were stored in the dark at 4°C. To determine the number of invading cells the number of DAPI-stained nuclei of each membrane in 5 non-overlapping fields of view at 20 x magnification was recorded using a Leica DM-IRE2 fluorescent microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) connected to a Hamamatsu C4742-96 digital camera (Hamamatsu Photonics UK Ltd, HERTS, UK) and a PowerMAC G5 computer (Apple Computer Inc., CA, USA) running Improvision® OpenLab V4.04 software (Improvision®, Coventry, UK). Data

was presented as mean cell count/membrane. Also, general observations were made, recording the appearance of the nuclei following different treatments.

2.9 Cell Migration Assay

Analysis of migratory capacity of WTMCF-7 and FAS-R cells under basal conditions and following growth factor and inhibitor treatment was carried out using the Transwell® inserts described for the invasion assay (**figure 2.2**); however, for this assay the outer side of the membrane of the inserts was coated with the chemo-attractant fibronectin. The lower sides of the inserts were coated by pipetting 250µl fibronectin solution (10µg/ml in wRPMI without supplements) into the wells of a 24-well-plate and placing the Transwell® inserts into the wells such that the membrane undersides were submerged in the solution. The plate was then incubated at 37°C; 2 hours later the inserts were rinsed with PBS and left to dry.

Cells were harvested and seeded into wells as described in section 2.8 and cultured for 24 hours.

Following incubation, the supernatants of the inserts were discarded; and non-migratory cells removed by gently wiping the inside of the insert membrane with a cotton swab. Those cells that had migrated through the pores of the membrane and adhered to the outside of the insert were fixed in 3.7% formaldehyde in PBS for 15 minutes, washed with PBS and then stained with crystal violet (0.5% in H_2O) for 20 minutes. Following several sequential washes in PBS to remove excess crystal violet dye, inserts were left to dry before being photographed and analysed using an Olympus BH-2 phase contrast microscope attached to an Olympus DP-12 digital camera system (both Olympus, Oxford, UK). For each membrane, the number of cells in 5 non-overlapping fields of view at 10 x magnification was recorded; and data was presented as mean cell count/field.

2.10 Scatter Assay

The extent to which HGF/SF induced dispersion of compact WTMCF-7 and FAS-R cell colonies and the effect of inhibitors on the scatter response was determined using scatter assays.

Cells were harvested by trypsinisation as described previously and seeded into the wells of a 12-well plate at a density of $0.2x10^5$ cells/ml in EM ± Faslodex (10^{-7}M) (1ml/well; seeding density had been optimised empirically). The plate was then incubated at 37°C in a 5% CO_2 atmosphere until cells had attached and formed discrete colonies (approximately 3 days, depending on cell type). Upon colony formation, medium was replaced and HGF/SF (20ng/ml) and inhibitors added where appropriate. 24 hours later, medium was aspirated and cells fixed by the addition of 3.7% formaldehyde in PBS (1ml/well). Scatter response was assessed visually and selected representative colonies were photographed using a Leica DM-IRE2 inverted microscope (see section 2.4).

2.11 Immunofluorescence

Expression and localisation of MMP-1, MMP-2 and MMP-9 was investigated using immunofluorescence microscopy. As with Western blotting, the choice of MMP family members to be further studied at protein level was dictated by both which family members had primarily been linked to invasive behaviour in the literature and also by which MMPs had previously been successfully detected using immunofluorescent analysis by other investigators.

Since the location of MMPs can be both intracellular and extracellular, i.e. on the cell membrane; both permeabilised and non-permeabilised cells were set up for immunofluorescence analysis.

WTMCF-7 and FAS-R cells were cultured on thin coverslips until log-phase growth was reached. After the removal of cell culture medium, cells were first washed gently with PBS, then fixed with 3.7% formaldehyde in PBS (100µl/coverslip) for 15 minutes at room temperature, followed by a further washing with PBS. Permeabilisation buffer (see buffer appendix) was added to

one set of coverslips (100µl/coverslip) for 10 minutes before cells were washed and blocked by the addition of 10% normal goat serum in immunofluorescence buffer (see buffer appendix) (100µl/coverslip) at room temperature for 15 minutes. The other set of coverslips containing the non-permeabilised cells was blocked immediately following formaldehyde fixation. After blocking, cells were washed with PBS and incubated with the appropriate primary antibody (MMP-1, MMP-2 or MMP-9 primary antibody (**table 2.9**)) in a 1:50 dilution in immunofluorescence buffer (see buffer appendix) (100µl/coverslip) for 1 hour at room temperature in a humidified chamber. After 3 consecutive PBS washes, cells were then incubated with the appropriate secondary Alexa-488-conjugated antibody (1:1000 dilution in immunofluorescence buffer; 100µl/coverslip) for 1 hour in a dark chamber. Cells were subsequently washed with PBS and incubated with Phalloidin-594 (100µl/coverslip) to stain actin filaments for 15 minutes. Cells were then mounted onto microscope slides using VectorShield® soft-set mounting medium containing the nuclear stain DAPI; and nail varnish was used to seal the edges of the coverslips. Slides were kept in the dark until they were analysed on a Leica fluorescence microscope (section 2.8) using an oil immersion lens at 63x magnification.
Relevant controls (secondary antibody only; a secondary antibody from a different species) were used to avoid any chances of cross staining and false positive results.

2.12 Statistical Analysis

Where possible, statistical significance of data generated from comparisons between cell lines or from comparisons between treated and control cells was analysed using independent two-tailed Student's t-test and ANOVA (ANalysis Of VAriance) with post-hoc tests (Dunnett's t-test and Tamhane's T2 test). These analyses were performed using the statistical analysis program SPSS v12.0.2 (SPSS Inc., Illinois, USA).

Chapter Three

Results

Characterisation of the Adverse Phenotype of FAS-R Cells

"I have had my results for a long time but I do not yet know how I am to arrive at them."

Karl Friedrich Gauss (1777-1855)

3 Characterisation of the Adverse Phenotype of FAS-R Cells

Introduction and Aims

As is the case with tamoxifen, resistance can also develop to long-term faslodex treatment resulting in disease relapse and increased patient mortality (Cheung *et al.*, 2006, Robertson *et al.*, 2005). Clarification of the molecular changes underlying development of resistant growth is therefore urgently needed in order to inhibit, or at least delay, the emergence of such a state. To this end, a faslodex-resistant xenograft model (Massarweh *et al.*, 2006) as well as several faslodex-resistant cell lines (Frogne *et al.*, 2009, Shaw *et al.*, 2006, Liu *et al.*, 2006, Sommer *et al.*, 2003, Brunner *et al.*, 1997, Lykkesfeldt *et al.*, 1995) including our in-house cell model (Nicholson and Johnston, 2005) have been established. The majority of studies using the above models have primarily focused on the cellular growth component of faslodex resistance, investigating the changes in the cell signalling system that enable resistant cells to circumvent the growth-inhibitory effect of faslodex treatment.

In contrast, one of the aims of this study was to determine whether, and how, acquisition of faslodex resistance *in vitro* affects cellular phenotype with respect to aggressive behaviour. Previous investigations using models of acquired tamoxifen resistance have revealed that acquisition of an endocrine-insensitive state is accompanied not only by oestrogen-independent growth but also by altered cell-cell and cell-matrix adhesive interactions which promote an aggressive *in vitro* phenotype as demonstrated by migration and invasion assays (Hiscox *et al.*, 2006, Hiscox *et al.*, 2004). In order to determine whether acquisition of faslodex resistance gives rise to a similar phenomenon, FAS-R cells were further characterised with particular emphasis on their *in vitro* aggressive behaviour.

3.1 Results

3.2.1 FAS-R Cells Display an Aggressive Invasive *In Vitro* Phenotype compared to WTMCF-7 Cells

Cell counting experiments were performed to compare the growth rates of WTMCF-7 and FAS-R cells. As **figure 3.1A** demonstrates, FAS-R cells displayed comparable growth rates until approximately day 9 under control conditions, i.e. in the absence of faslodex. After this point, however, FAS-R cell growth rates increased until they were noticeably higher compared to WTMCF-7 growth rates at day 11. Furthermore, WTMCF-7 cell growth rates were significantly inhibited in the presence of faslodex whilst FAS-R cell growth was unaffected. The effect of E_2 and tamoxifen on WTMCF-7 and FAS-R cell growth was also examined. In the wildtype, growth rates were virtually unchanged only until day 4; with the growth-promoting effect of E_2 and the inhibitory action of tamoxifen becoming obvious by day 6 and peaking at day 11 (**figure 3.1B**). In FAS-R cells, however, E_2 and tamoxifen had no effect on growth rates whatsoever (**figure 3.1C**).

Development of tamoxifen resistance was accompanied by morphological changes; hence WTMCF-7 and FAS-R cell morphology was studied using differential interference contrast microscopy in order to see whether development of faslodex resistance changed the appearance of FAS-R cells. A Leica DM-IRE2 inverted microscope with a Hoffman condenser was used to photograph live cells; representative images are shown in **figure 3.2**. While WTMCF-7 cells grew in distinct, tightly packed colonies FAS-R cells were spiky and smaller in size, lacked cell-cell contacts, and thus had a predilection for growth in a looser, disorganised fashion, overall displaying a more de-differentiated, angular morphology reflective of EMT (Kang and Massague, 2004, Thiery, 2003). Closer inspection of individual cells suggested membrane ruffling and potential filopodia and lamellipodia formation.

A

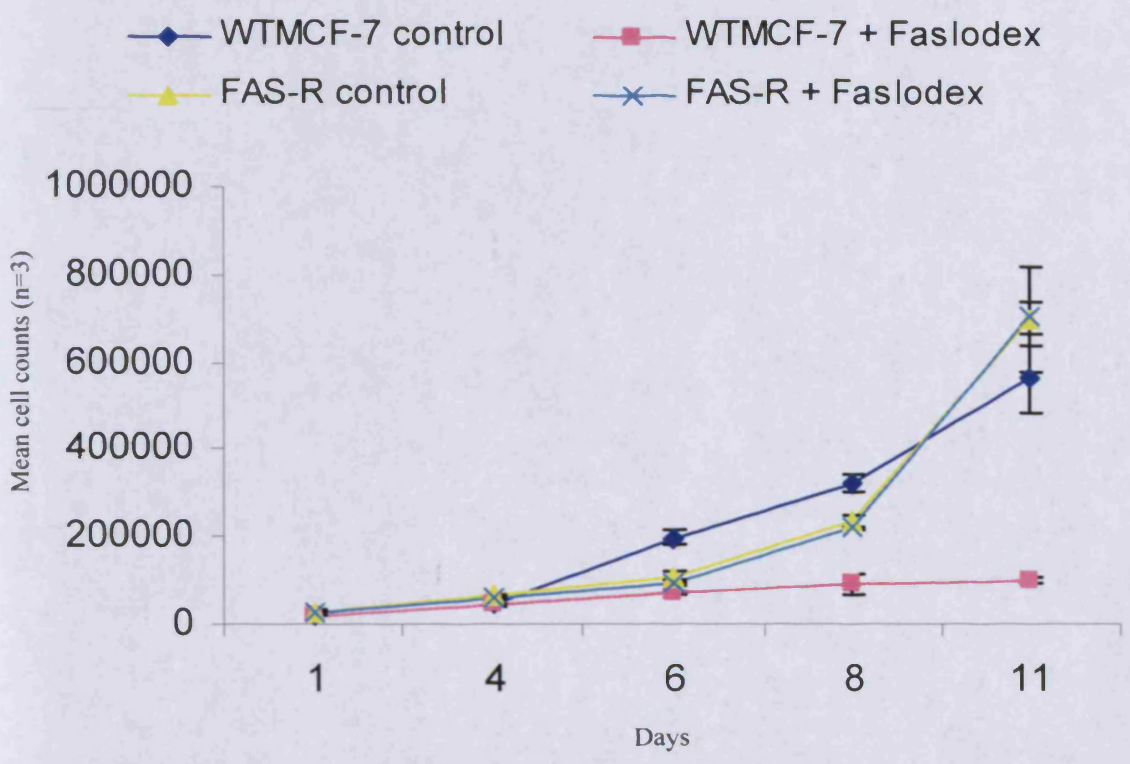


Figure 3.1 Growth of WTMCF-7 and FAS-R cells.
WTMCF-7 and FAS-R cells were seeded in EM ± Faslodex (10^{-7}M) at a density of 30,000 cells/well and cell counts were performed on days 1, 4, 6, 8 and 11 using a Beckman Coulter™ Multisizer II. Cell numbers at each time point were determined in triplicate and the experiment repeated 3 times; a representative graph displaying mean cell counts ± S.D. is shown above (A).
WTMCF-7 (B) and FAS-R (C) cells were also seeded in EM ± tamoxifen (10^{-7}M) and EM ± E_2(10^{-7}M). Cell counts were performed on days 1, 4, 6, 8 and 11 as before; representative graphs displaying mean cell counts ± S.D. are shown on the following page (B and C).

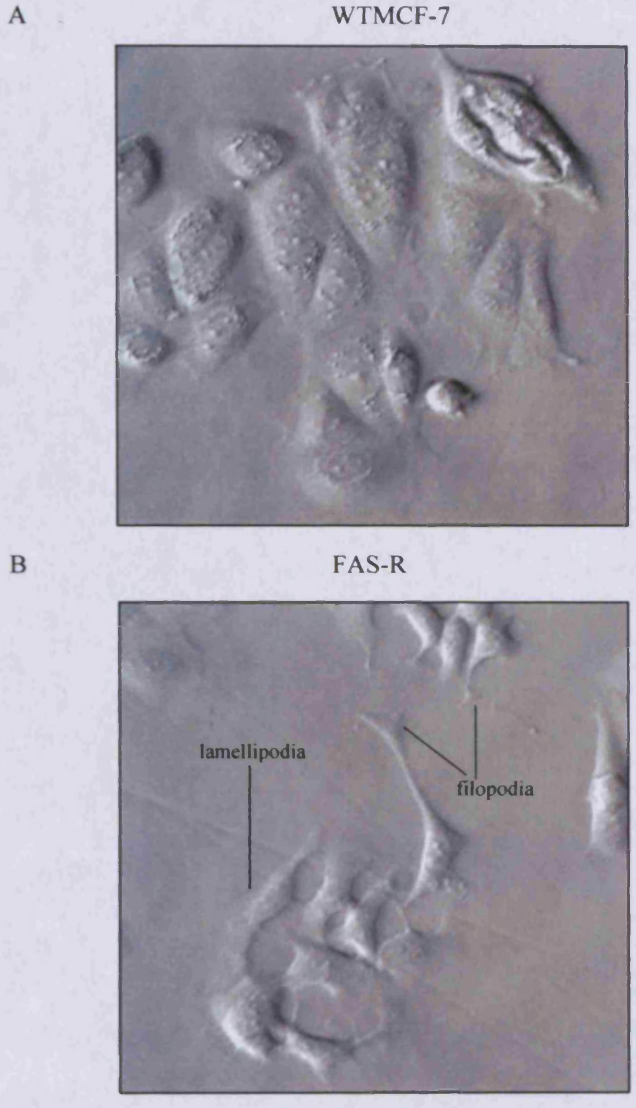
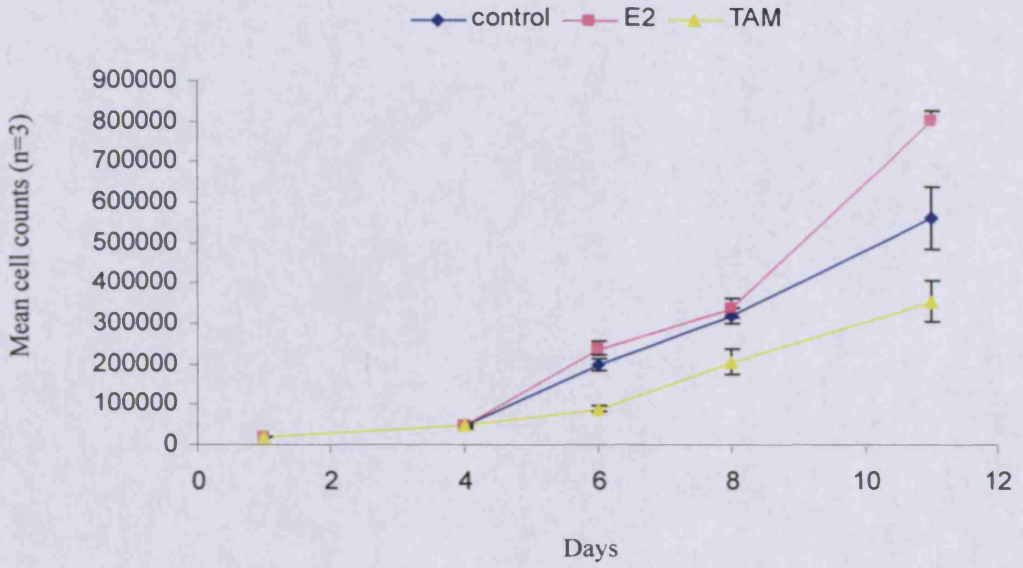


Figure 3.2 Basal morphology of WTMCF-7 and FAS-R cells.
WTMCF-7 (A) and FAS-R (B) cells were cultured in EM ± Faslodex (10^{-7}M) until log-phase growth was reached. Representative images of live cells were captured at 20x magnification using a Hamamatsu C4742-96 digital camera attached to a Leica DM-IRE2 inverted microscope with a Hoffman condenser.

B WTMCF-7



C FAS-R

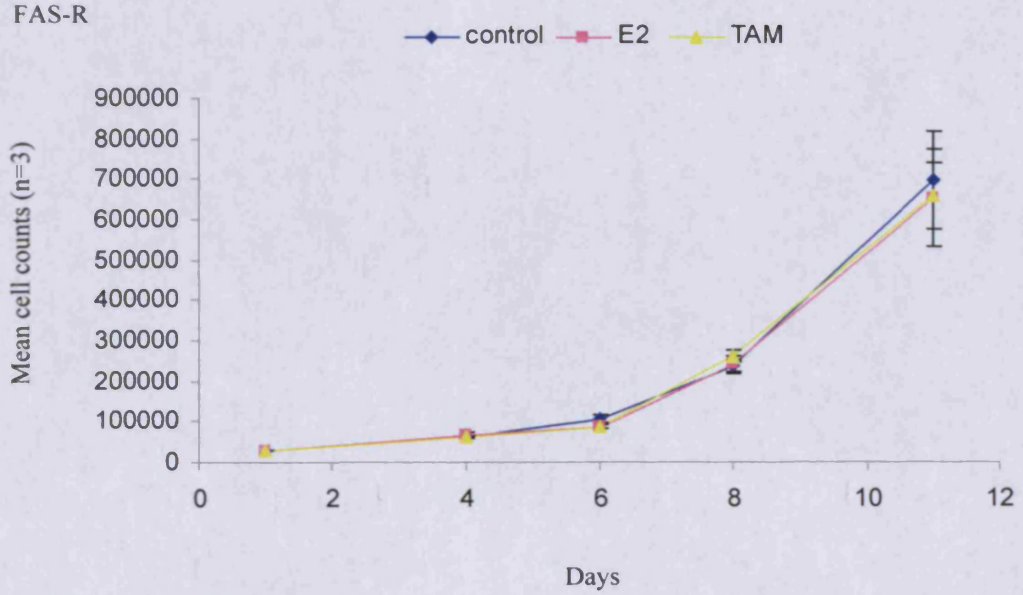


Figure 3.1 continued.

To elucidate the mechanisms underlying endocrine insensitivity Tenovus Centre for Cancer Research had previously established a tamoxifen-resistant breast cancer cell model (Knowlden *et al.*, 2003). Studies with this in-house model revealed that acquisition of an endocrine-resistant state in these cells was accompanied by transition to a more aggressive phenotype displaying significantly increased motile and invasive behaviour *in vitro* (Hiscox *et al.*, 2004). In order to establish whether acquisition of faslodex resistance had resulted in a similar augmentation of metastatic potential, the intrinsic migratory and invasive capabilities of FAS-R cells were assessed using *in vitro* migration and invasion assays respectively.

As representative images of Transwell® insert membranes in **figure 3.3A** illustrate, an increased number of cells (stained purple) traversed the pores (smaller clear circles) of the fibronectin-coated membrane in the FAS-R cell population as compared to WTMCF-7 cells. This increase in migratory capacity compared to WTMCF-7 cells was confirmed by quantitation of the assay. Data obtained from three independent experiments revealed a 1.6-fold increase in the migration rate of basal FAS-R cells as compared to that of basal WTMCF-7 cells (**figure 3.3B**).

A similar trend was observed for the invasive capacity of FAS-R cells. As representative images of DAPI-stained cell nuclei of the insert membranes in **figure 3.4A** show, an increased number of cells invaded the matrigel-coated membrane in the FAS-R cell population as compared to the WTMCF-7 cell population. Quantitation of the assay confirmed the increase in invasive behaviour in FAS-R cells as compared to their wild-type counterpart. Results of three independent experiments showed that basal FAS-R cell invasion was 8.2-fold greater than that of basal WTMCF-7 cells (**figure 3.4B**).

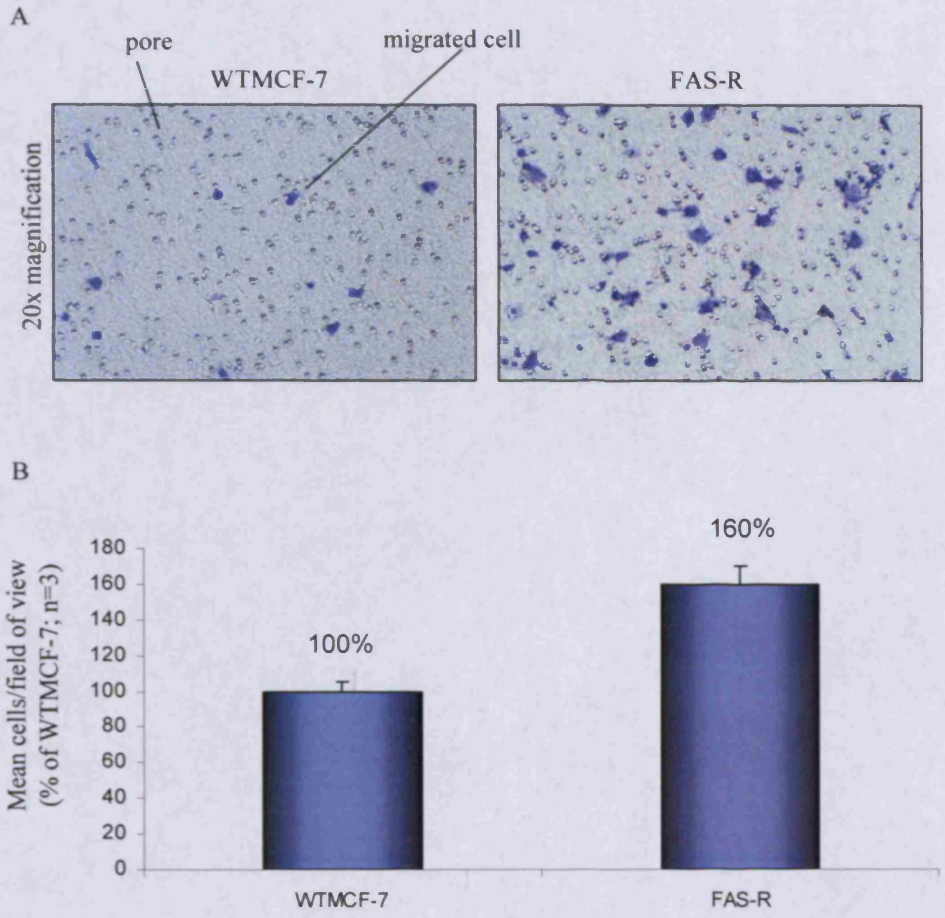


Figure 3.3 Basal migration of WTMCF-7 and FAS-R cells.
The migratory capacities of WTMCF-7 and FAS-R cells were determined using *in vitro* cell migration assays as described in section 2.9. Crystal-violet-stained cells were photographed at 20x magnification using an Olympus BH-2 phase contrast microscope; representative images are shown above (A). Quantification was by counting the number of migratory cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of WTMCF-7 ± S.D. (B). As can be seen, FAS-R cells display an increased migration rate compared to WTMCF-7 cells.

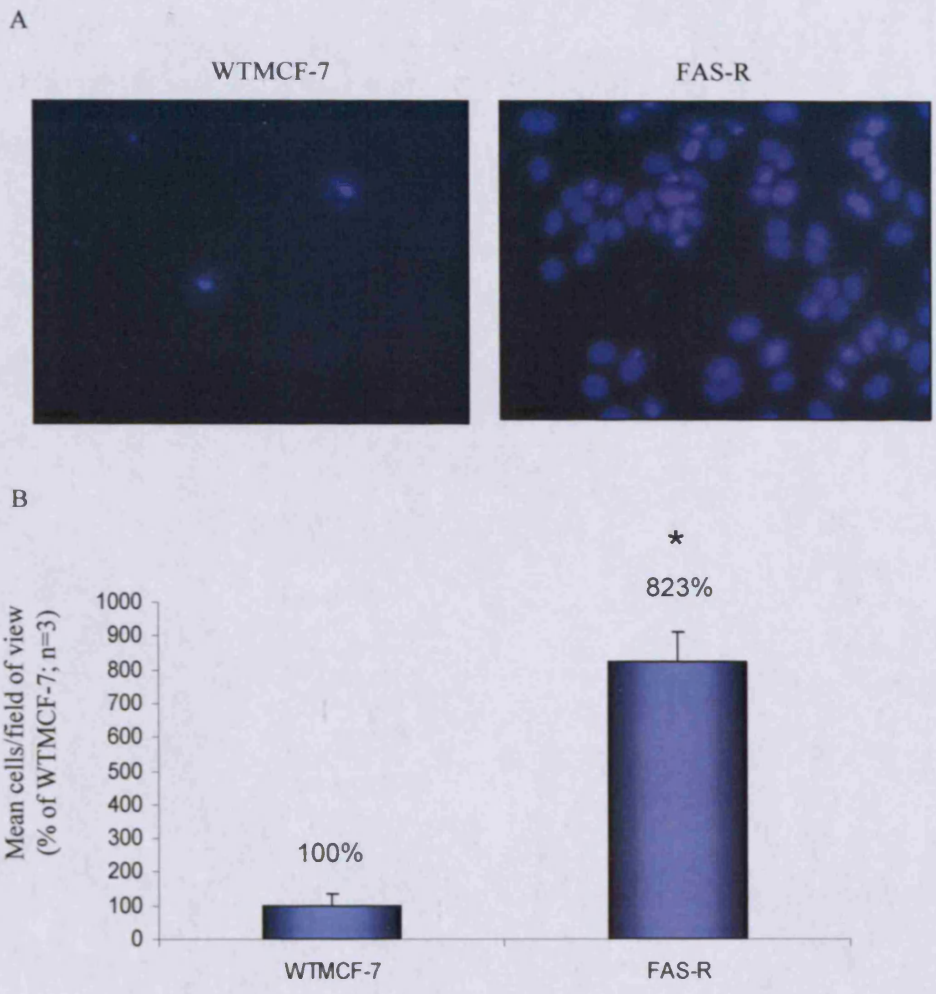


Figure 3.4 Basal invasion of WTMCF-7 and FAS-R cells.
The invasive capacities of WTMCF-7 and FAS-R cells were determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera; representative images are shown above (A). Quantification was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of WTMCF-7 ± S.D. (* $p<0.001$ vs. WTMCF-7) (B). As can be seen FAS-R cells display a significantly increased invasion rate compared to WTMCF-7 cells.

ER positivity and oestrogen signalling through the ER have been reported to suppress acquisition of a more motile and invasive phenotype (Hiscox *et al.*, 2007, Platet *et al.*, 2004, Rochefort *et al.*, 1998). Conversely ER negativity, an important clinical feature of 30% of breast cancer patients on presentation and also apparent in some ER-positive carcinomas at the time of tamoxifen relapse (Gee *et al.*, 2005), heralds a more proliferative and aggressive phenotype and consequently, poor prognosis (Nicholson *et al.*, 1993). In order to establish whether the aggressive phenotype of FAS-R cells was accompanied by ER negativity, ERα gene and protein expression studies were performed. ERα gene expression profiles of FAS-R and WTMCF-7 cells generated using Affymetrix cDNA array technology clearly show markedly lower ERα mRNA levels in FAS-R cells as compared to in WTMCF-7 cells (**figure 3.5**; [data provided courtesy of J. Gee and R. McClelland]). This finding was corroborated by semi-quantitative PCR (**figure 3.6A**).

ERα protein levels were measured using Western Blotting and showed FAS-R cells to be ERα-negative as opposed to their ERα-positive wild-type counterparts (**figure 3.6B**). Immunocytochemical analysis further confirmed the ERα-negative status of FAS-R cells; as can be seen in **figure 3.6C**, intense nuclear staining was observed in WTMCF-7 cells whereas virtually no staining could be detected in FAS-R cells.

Collectively the above results demonstrate that loss of ERα in FAS-R cells results in the development of an adverse phenotype characterised by increased growth rate, altered morphology and augmented intrinsic *in vitro* aggressive behaviour.

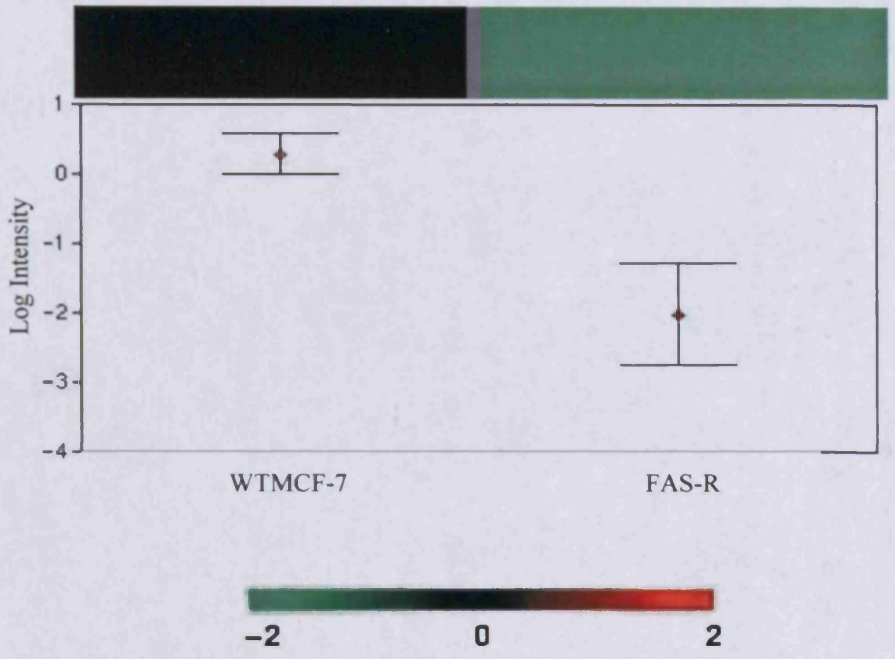


Figure 3.5 ERα gene expression profiles of WTMCF-7 and FAS-R cells generated using Affymetrix cDNA array technology.
WTMCF-7 and FAS-R cells were cultured in EM ± Faslodex (10^{-7}M) until log-phase growth was reached. Total RNA was extracted as described in section 2.5.1. Total RNA samples were DNase1-treated to eliminate any genomic DNA contamination and subsequently purified using the QIAGEN RNeasy mini kit. Following RNA quantification and qualitation by denaturing agarose gel electrophoresis, samples were processed and hybridised to Affymetrix GeneChip® Human Genome U133A arrays (Central Biotechnology Services, Cardiff University, Cardiff, UK). Hybridised arrays were scanned and analysed using Microarray Suite 5.0 software (Affymetrix). Internal control gene expression analysis was used to confirm data quality. Comparative gene expression analysis was performed on median-normalised, log-transformed data using Genesifter software (www.genesifter.net). The gene expression profile depicted above (presented as mean signal intensity of the ERα transcript of WTMCF-7 and FAS-R cells) shows higher ERα expression in WTMCF-7 cells compared to FAS-R cells. Data was provided courtesy of J. Gee and R. McClelland.

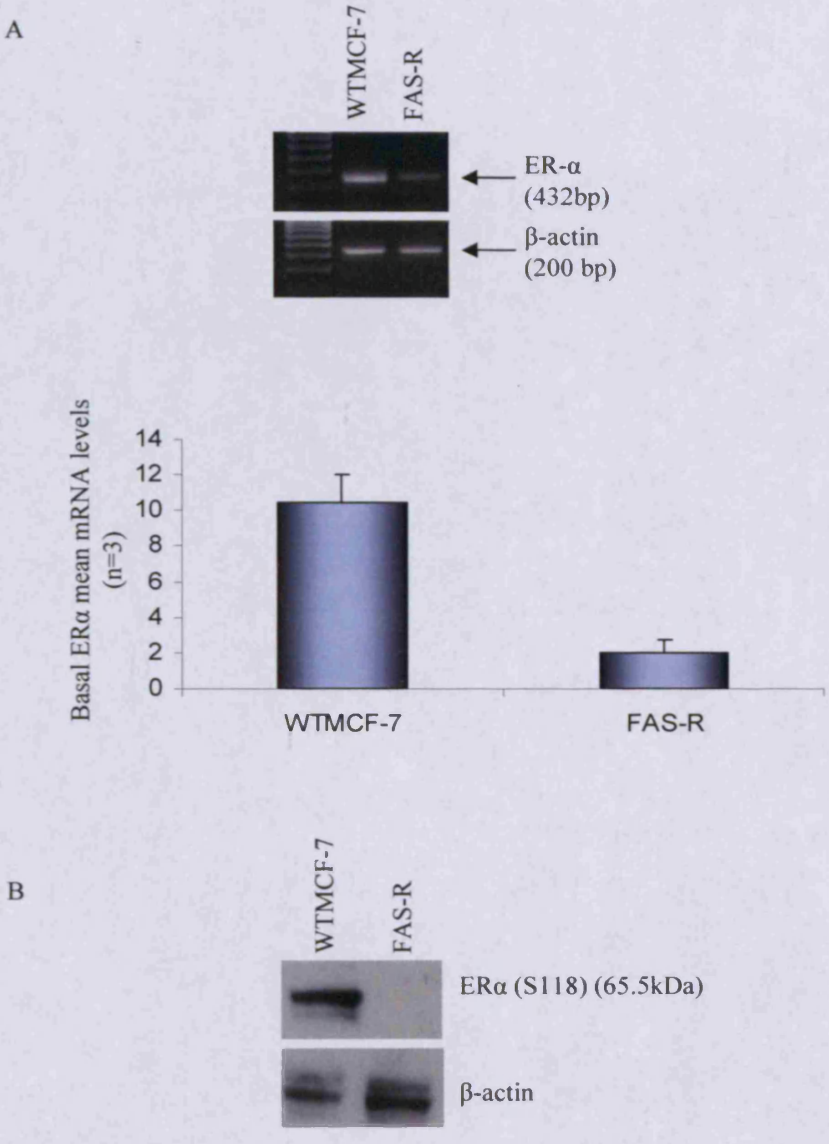


Figure 3.6 ERα mRNA and protein expression levels in WTMCF-7 and FAS-R cells.

RT-PCR analysis revealed higher ERα expression levels in WTMCF-7 cells as compared to FAS-R cells (A), as already indicated by prior Affymetrix gene expression analysis (previous page, figure 3.5). At protein level, the ERα status of FAS-R cells as compared to WTMCF-7 cells was found to be negative as shown by WB (B) and immunohistochemical staining (C) (see next page).

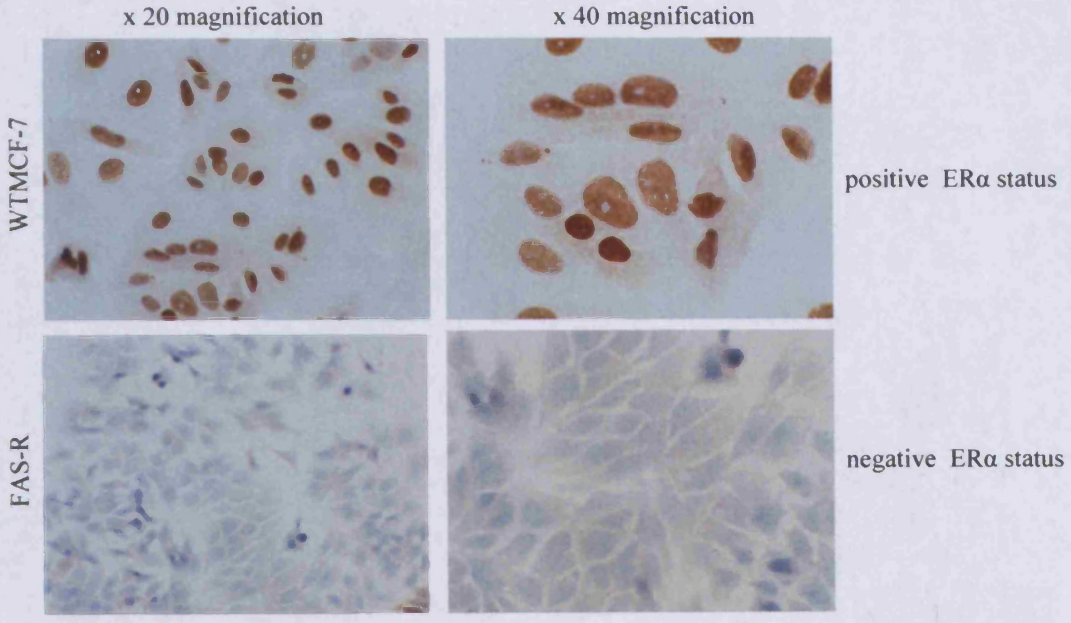


Figure 3.6 continued.

Chapter Four

Results

MMP Expression and Role in Invasiveness of FAS-R Cells

"There ain't no rules around here! We're trying to accomplish something!"

Thomas Alva Edison (1847-1931)

4 MMP Expression and Role in Invasiveness of FAS-R Cells

4.1 Introduction and Aims

As established in the previous chapter, acquisition of faslodex resistance in WTMCF-7 cells resulted in increased cell motility and overall aggressive behaviour. Although clearly a significant observation, the molecular changes underlying this phenotypical change remain largely unknown.

Tumour cell invasion and metastasis is a multi-step process which involves proteolytic degradation of the ECM, alteration of cell-cell and cell-matrix attachments and migration through the basement membrane (Kleiner and Stetler-Stevenson, 1999, Liotta *et al.*, 1980b). The metastatic potential of various tumours had been correlated to the ability of the tumour cells to degrade basement membrane collagen early on (Liotta *et al.*, 1980a, Liotta *et al.*, 1980b), and since several *in vivo* and *in vitro* studies have demonstrated the importance of MMPs in the invasive process. Numerous studies on clinical specimens have revealed increased MMP expression in virtually all malignancies compared to normal tissue (Konstantinopoulos *et al.*, 2008); furthermore, augmented MMP proteolytic activity has been associated with acquisition of an invasive phenotype, advanced stage metastatic disease and poor prognosis (Konstantinopoulos *et al.*, 2008).

Based on the widely acknowledged role of MMPs in invasion, this study aimed to investigate whether aberrant MMP and TIMP expression regulation might contribute to the molecular changes that promote development of an aggressive phenotype in faslodex resistance.

4.2 Results

4.2.1 The Role of Differential MMP Expression in FAS-R Cell Invasion

As demonstrated in chapter 3, FAS-R cells are more invasive as compared to WTMCF-7 cells. In order to be able to demonstrate a role for MMP proteolytic

activity in FAS-R cell invasion, invasion assays were carried out in the presence of a broad-spectrum inhibitor, the premise being that decreased invasion following MMP inhibition would indicate an involvement of MMPs in FAS-R cell invasion.

A range of concentrations were used, with 40μM being chosen as the optimum concentration (**figure 4.1**). Growth curves performed in parallel confirmed the MMP inhibitor to be non-toxic to the cells at that concentration (**figure 4.2**). Under these conditions, a significant reduction in FAS-R cell invasion was observed (**figure 4.1**). This suggested that MMPs might play a role in the adverse phenotype displayed by FAS-R cells.

After the initial comparison between invasive capacities of WTMCF-7 and FAS-R cells (section 3.2.1; **figure 3.4**), all further invasion studies focused solely on FAS-R cells; the poor intrinsic invasiveness of WTMCF-7 cells unfortunately rendering inhibition studies unfeasible.

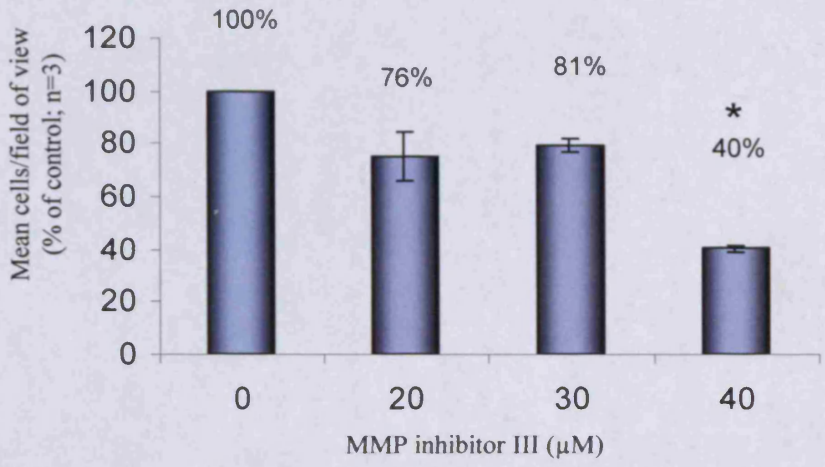


Figure 4.1 The effect of broad-spectrum MMP inhibition on invasion rates of FAS-R cells.

MMP inhibition was achieved by using a broad-spectrum MMP inhibitor at a range of doses (20, 30 and 40μM). The effect of MMP inhibition on the invasive capacities of FAS-R cells was determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera. Quantification was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of control ± S.D. (* $p<0.001$ vs. FAS-R control). As can be seen MMP inhibition at 40μM significantly reduced the invasive capacity of FAS-R cells.

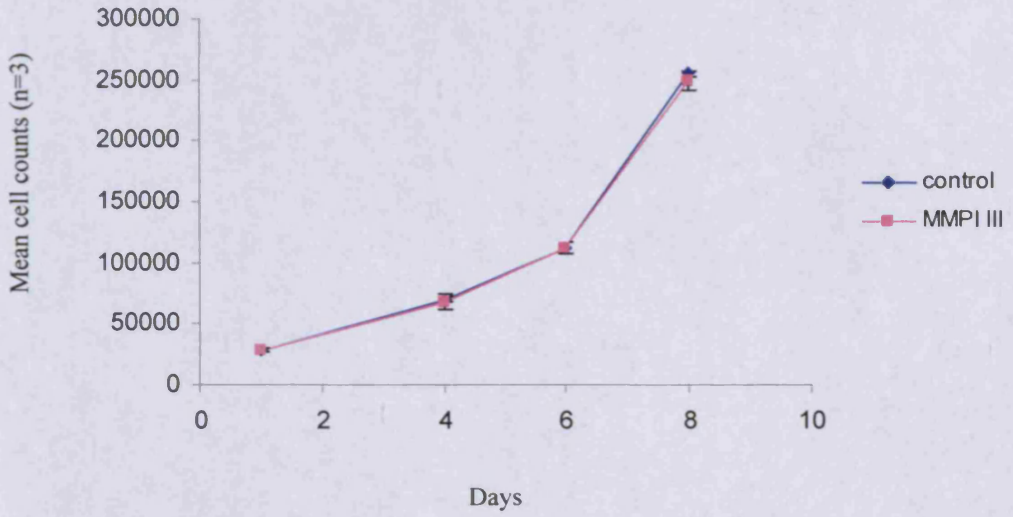


Figure 4.2 The effect of MMP inhibitor III on FAS-R cell growth.
FAS-R cells were seeded in EM ± MMP inhibitor III (40μM). Cell counts were performed as before on day 1, 4, 6 and 8. Cell numbers at each time point were determined in triplicate and the experiment repeated 3 times; a representative graph displaying mean cell counts ± S.D. is shown above. As can be seen, MMPI III did not affect FAS-R cell growth.

4.2.2 Differential Expression of MMPs and TIMPs in Basal Endocrine-Sensitive and Endocrine-Resistant Breast Cancer Cells

Next, the MMP and TIMP profiles of FAS-R cells as compared to WTMCF-7 cells under basal conditions were established; the choice of MMP enzymes to be studied being governed by which family members had primarily been associated with invasion in the literature. Serum-starved WTMCF-7 and FAS-R cells were probed for mRNA expression of MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, MMP-11, and MMP-13 as well as for mRNA expression of TIMP-1 to TIMP-4 by RT-PCR (**figure 4.3**). MMP-7 was the only gene undetected in both cell lines (data not shown). WTMCF-7 cells expressed all the other MMPs listed above with the exception of MMP-2; furthermore, expression of individual MMP family members was variable. Interestingly, high levels of MMP-2 were detected in FAS-R cells, along with modest expression of MMP-11 and MMP-13. Whilst WTMCF-7 cells expressed all four TIMP family members, only TIMP-2 to TIMP-4 were detected in FAS-R cells. FAS-R cells displayed lower TIMP-2 mRNA levels compared to WTMCF-7 cells while TIMP-3 expression was modestly increased in FAS-R cells. To establish whether this differential MMP gene expression observed at mRNA expression level was also apparent at protein level, gelatin zymography was performed on the conditioned supernatants of basal serum-starved WTMCF-7 and FAS-R cells. As outlined in section 2.7, this method allows detection of secreted active and latent forms of gelatinases, MMP-9 and MMP-2. A commercially available MMP-2/MMP-9 standard was included in each experiment as in internal assay control. It also served to ascertain whether the proteins detected were of the correct molecular weight and hence allowed them to be clearly identified as MMP-2 and MMP-9.

Adjusting the amount of supernatant loaded for both samples according to cell numbers allowed direct comparison of the MMP secretion levels of basal WTMCF-7 and FAS-R cells. As **figures 4.4A** and **4.4C** show only one protein was detected; using the standard this was identified to be the latent form of MMP-9 (92kDa) on the basis of its molecular weight.

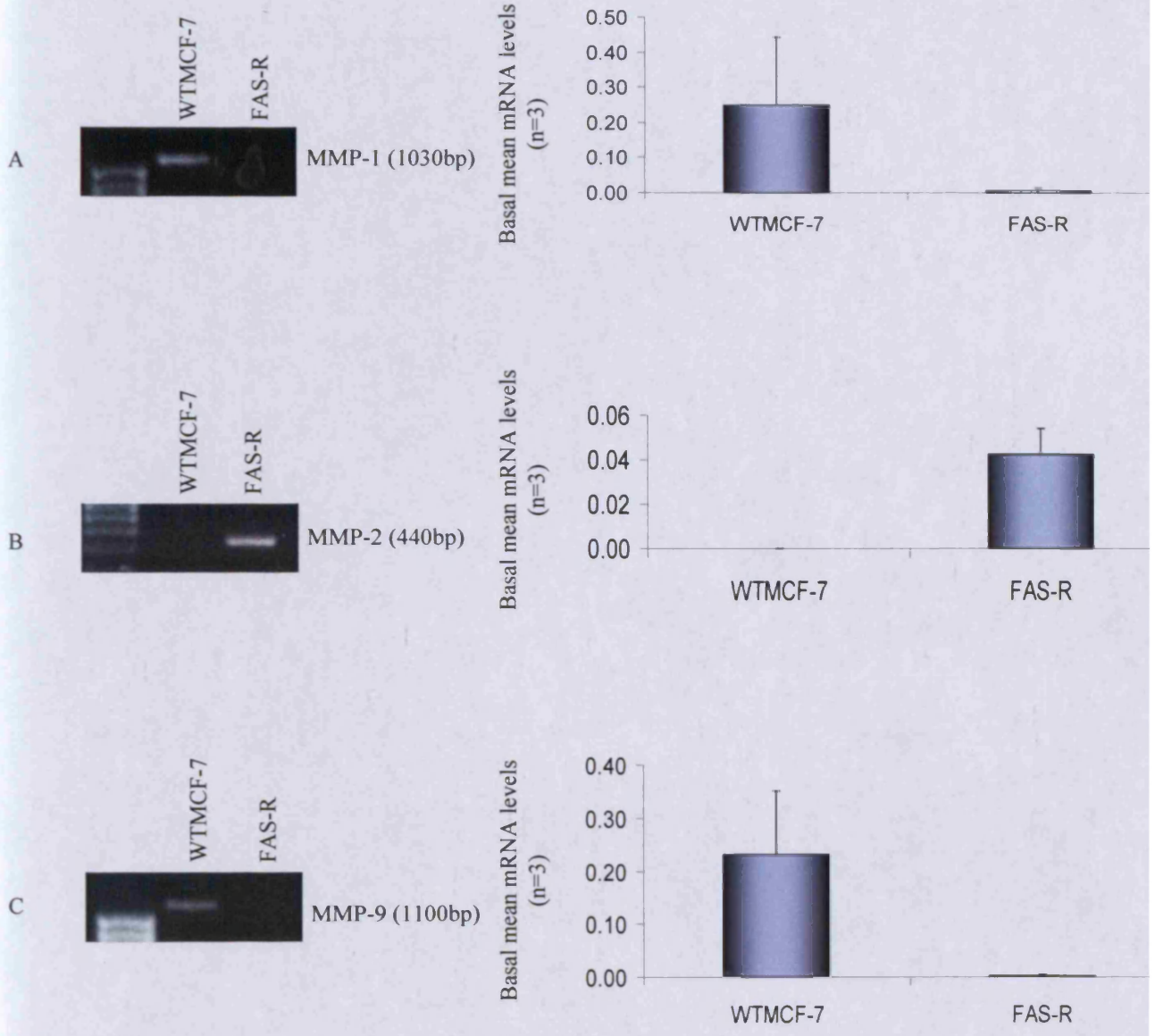


Figure 4.3 Basal MMP and TIMP mRNA expression levels in WTMCF-7 and FAS-R cells as determined by semi-quantitative RT-PCR.
WTMCF-7 and FAS-R cells were cultured in EM ± Faslodex (10^{-7}M) until log-phase growth was reached, serum-starved in DCCM O/N and lysed. Then gene expression analysis was performed as outlined in section 2.5 for MMP-1, MMP-2, MMP-9, MMP-10, MMP-11 and MMP-13 and for TIMP-1 to TIMP-4 using specially designed primers. Data was normalised using β-actin and presented as mean expression levels ± S.D. (n=3) (A-K). Representative images (A-K) are shown above and on following pages.

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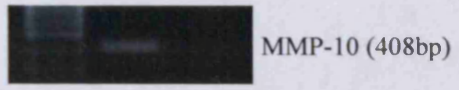
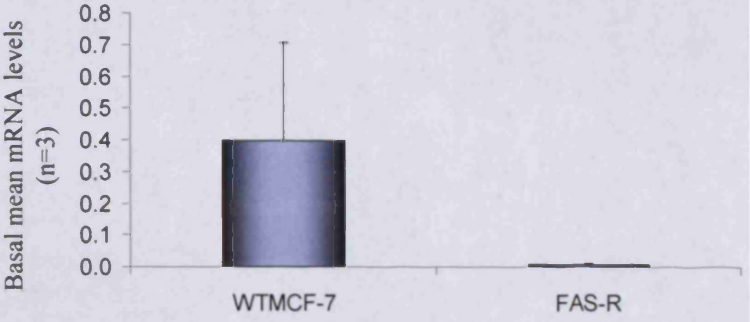


MMP-10 (408bp)



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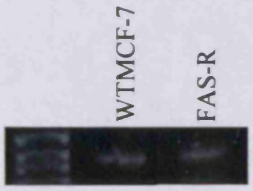
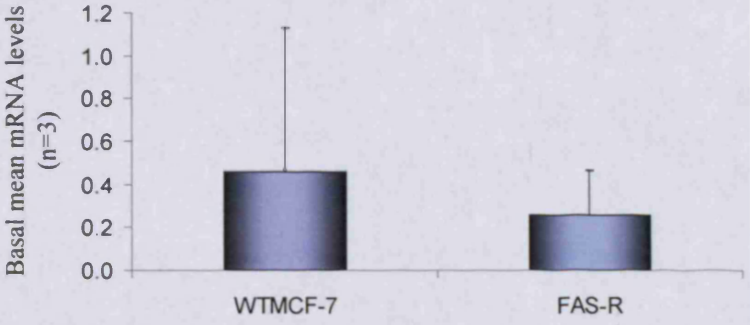


MMP-11 (700bp)



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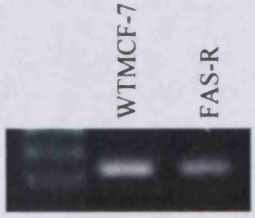
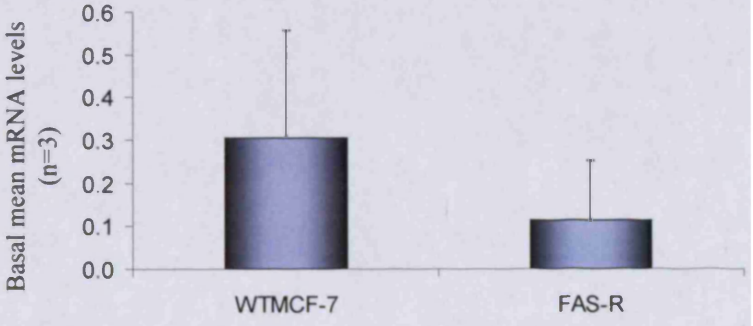


MMP-13 (359bp)



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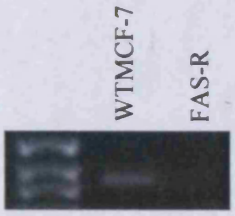
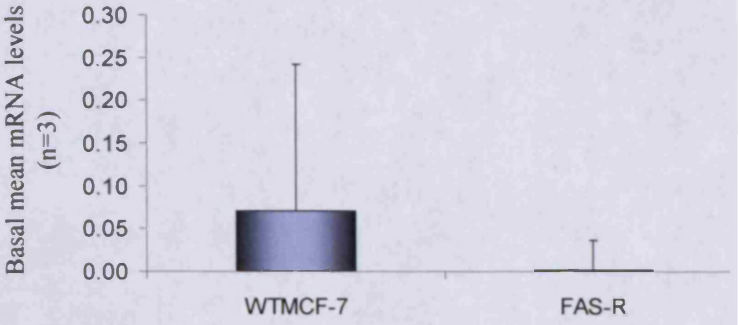


TIMP-1(655bp)



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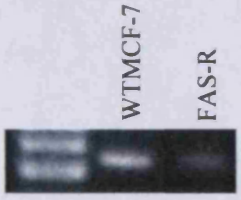
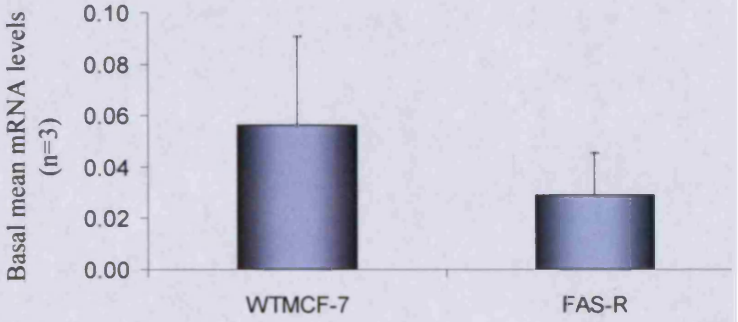


TIMP-2 (335bp)



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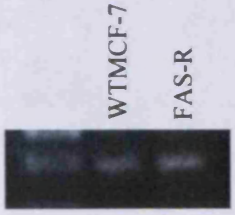
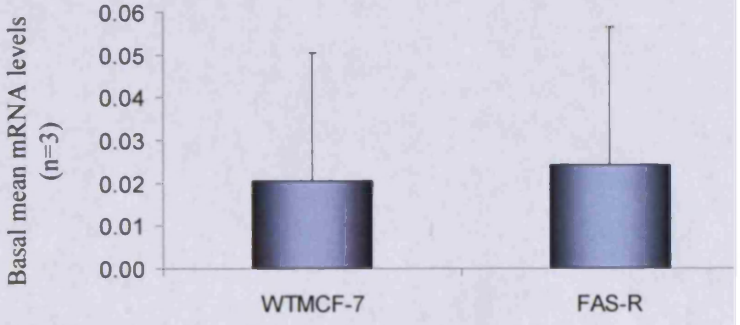


TIMP-3 (201bp)



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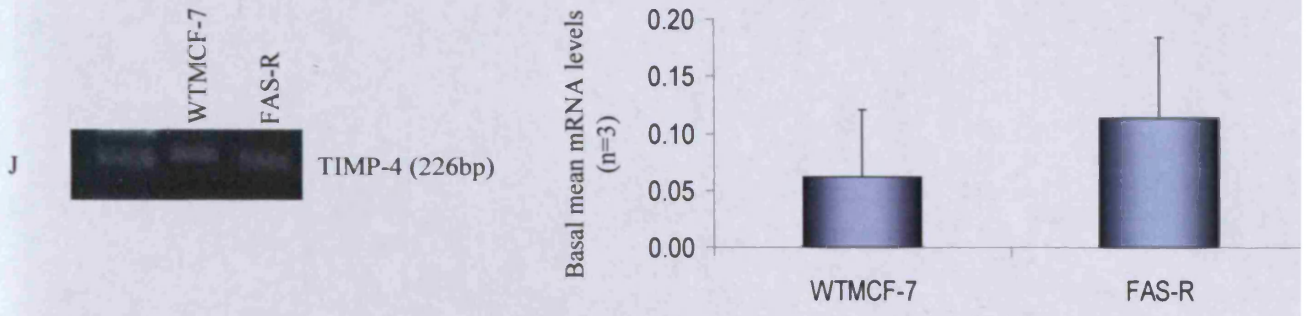

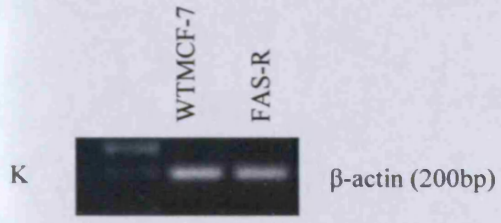

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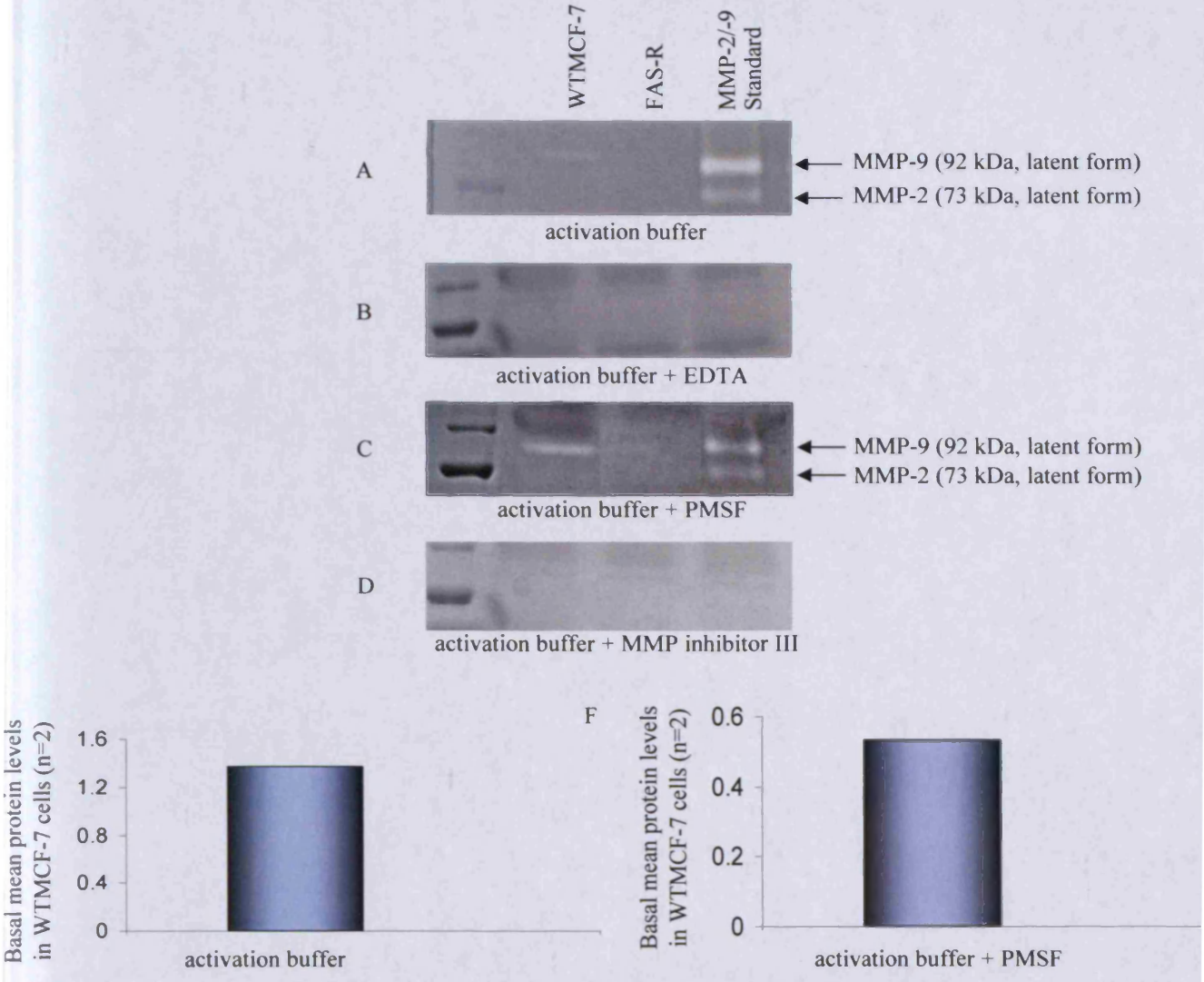


Figure 4.4 Basal MMP-9 and MMP-2 protein expression levels in WTMCF-7 and FAS-R cells as determined by zymography.

WTMCF-7 and FAS-R cells were cultured until log-phase growth was reached and then transferred to DCCM and serum-starved for 24h. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7. A commercial MMP-2/-9 standard was run alongside test samples as an internal assay control. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels (n=2). Representative images are shown above. Latent MMP-9 was detected in WTMCF-7 cells but not FAS-R cells while latent MMP-2 was not detected in either cell type (A and E). In order to confirm the MMP nature of the protein detected, gels were incubated with the chelating agent EDTA (10μM) (B), with the serine proteinase inhibitor PMSF (1mM) (C) and a broad-spectrum MMP inhibitor (40μM) (D). Incubation with both EDTA and the inhibitor abolished latent MMP activity (B and D) while incubation with PMSF did not (C and F).

Also, MMP-9 expression was restricted to WTMCF-7 cells. So while MMP-2 was not detected in either cell line, latent MMP-9 was only expressed by WTMCF-7 cells.

Biochemical validation of the MMP nature of the protein observed was carried out by standard confirmatory tests. The catalytic action of MMPs is dependent on both the presence of a zinc ion in the active site and stabilisation of the enzyme by calcium ions, therefore incubation with an agent such as EDTA that chelates divalent cations leads to loss of MMP activity; this has been demonstrated by several investigators (Murphy and Crabbe, 1995, Knowlden *et al.*, 1995). Hence EDTA incubation was performed to confirm the MMP nature of the protein detected. As **figure 4.4B** illustrates, incubation in activating buffer containing EDTA (10μM) resulted in complete loss of MMP activity, proving that the clear band had been caused by proteolytic activity of latent MMP-9.

Incubation was also performed in the presence of the serine protease inhibitor PMSF to further confirm specific MMP presence rather than general protease activity. Since presence of the band was unaffected by PMSF (**figure 4.4C**) it was concluded that the protease detected was not a serine protease.

Moreover, incubation was additionally carried out in the presence of the broad-spectrum MMP inhibitor, MMP inhibitor III, which had previously been shown to reduce invasiveness of faslodex-resistant cells. Incubation in activating buffer containing MMP inhibitor III (40μM) resulted in complete loss in MMP activity (**figure 4.4D**).

As gelatin zymography only permits gelatinase detection, and also to further corroborate the above observations, Western Blot analysis of MMP protein expression in basal WTMCF-7 and FAS-R cells was carried out. Both cell lysates and cell-conditioned supernatants were subjected to immunoblotting using antibodies specific to MMP-2, MMP-9, and MMP-1. However, none of the three MMPs were detectable; neither in the lysate nor the supernatants

despite the use of at least two different antibodies for each of the three proteases.

Since the presence of MMP-2 and MMP-9 could not be established by Western Blot analysis, immunofluorescent detection of the proteases was attempted. However, as before, neither MMP-2 and MMP-9 nor MMP-1 was detectable with the antibodies employed.

4.2.3 The Effect of Matrix on MMP and TIMP Expression in WTMCF-7 and FAS-R Cells

The data presented so far suggests only modest expression of most MMPs and TIMPS in FAS-R cells. However, during migration and invasion assays, cells are in prolonged contact with fibronectin and the reconstituted basement membrane, matrigel. Fibronectin is an ECM component integral to adhesion and migration processes while matrigel is a basement membrane preparation rich in laminin, collagen IV and many other ECM proteins. Since these ECM components are akin to the *in vivo* environment, they stimulate complex cellular behaviour *in vitro* and have been reported to increase MMP and TIMP expression (Balduyck *et al.*, 2000, Bafetti *et al.*, 1998). Therefore, matrigel and fibronectin coating was chosen to simulate the *in vivo* cell environment in the following experiments. Thus, WTMCF-7 and FAS-R cells were grown on these different matrices (plastic, matrigel and fibronectin) before mRNA and protein levels were analysed using RT-PCR and zymography, respectively, to determine the effect of cell environment on MMP and TIMP expression (**figures 4.5** and **4.6**). In the case of matrigel, both a thin gel layer and a thick gel matrix were employed where cells would predominantly either rest on top of the gel layer or invade the matrix; the rationale being that exposure to differing cell environments which reflect separate aspects of the *in vivo* tumour microenvironment environment may result in different MMP expression profiles. In WTMCF-7 cells both culture on thin and thick matrigel layers did not result in increased MMP and TIMP expression levels, but instead markedly decreased the expression levels of MMP-1, MMP-9 and MMP-10 (**figure 4.5**).

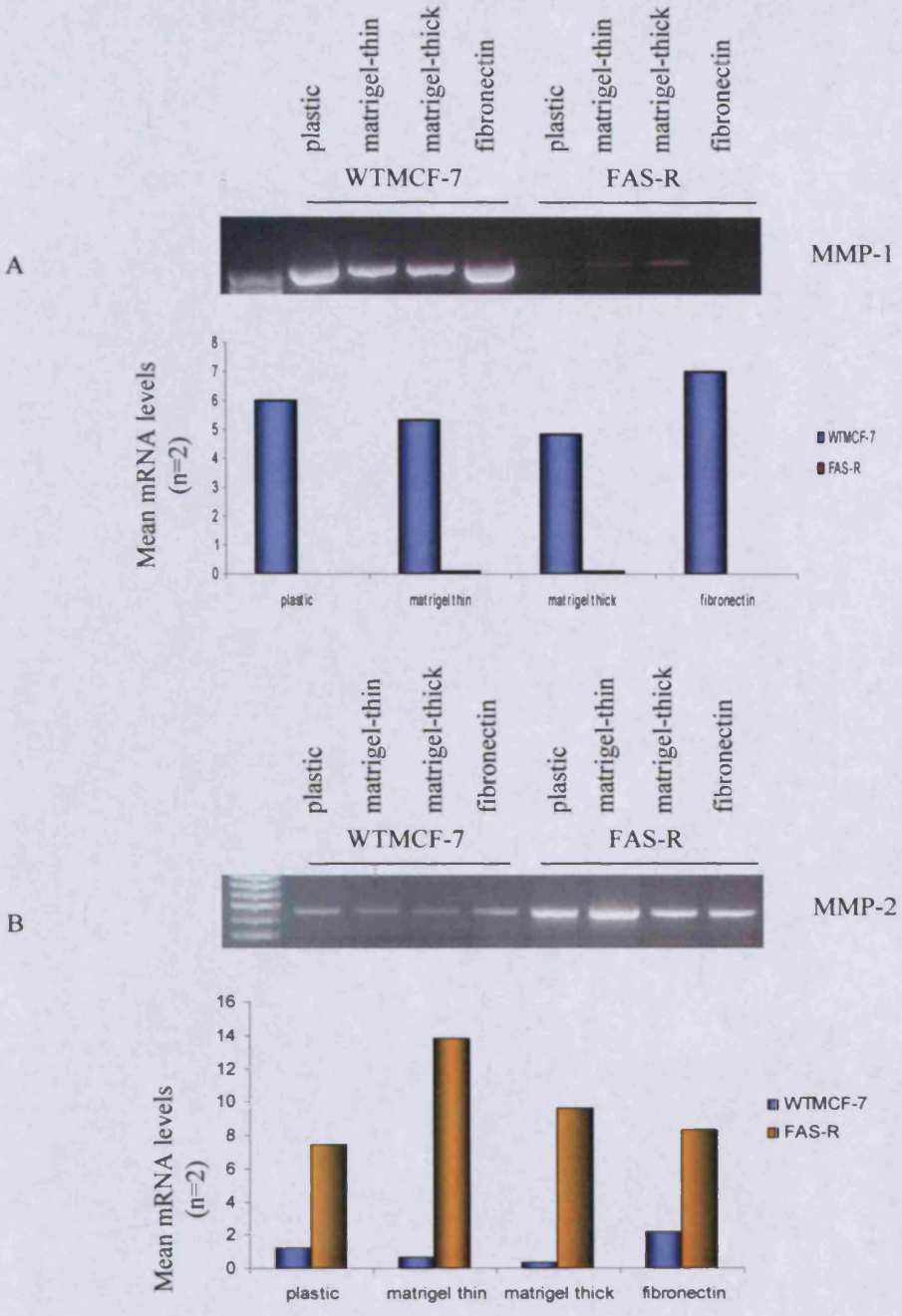
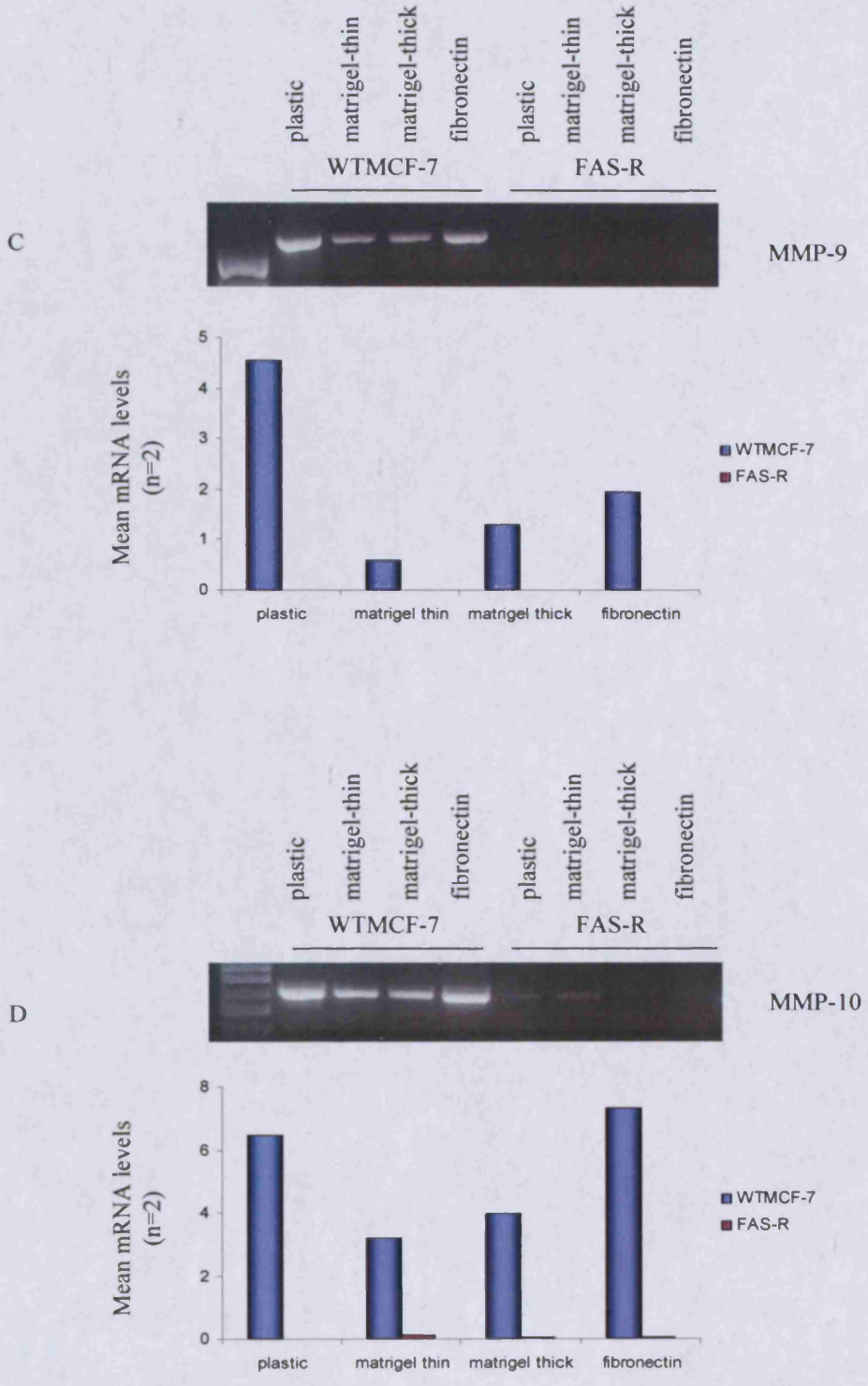


Figure 4.5 MMP and TIMP mRNA expression levels of WTMCF-7 and FAS-R cells grown on different matrices as determined by semi-quantitative RT-PCR.
WTMCF-7 and FAS-R cells were cultured on plastic, matrigel and fibronectin under serum-free conditions in DCCM until log-phase growth was reached. In the case of matrigel, both a thin and a thick coating method were employed. The effect of different matrices on MMP-1, MMP-2, MMP-9, MMP-10, MMP-11 and MMP-13 and TIMP-1 to TIMP-4 expression was determined using gene expression analysis as outlined in section 2.5. Data was normalised using β-actin and presented as mean expression levels (n=2) (A-K). Representative images (A-K) are shown above and on following pages.



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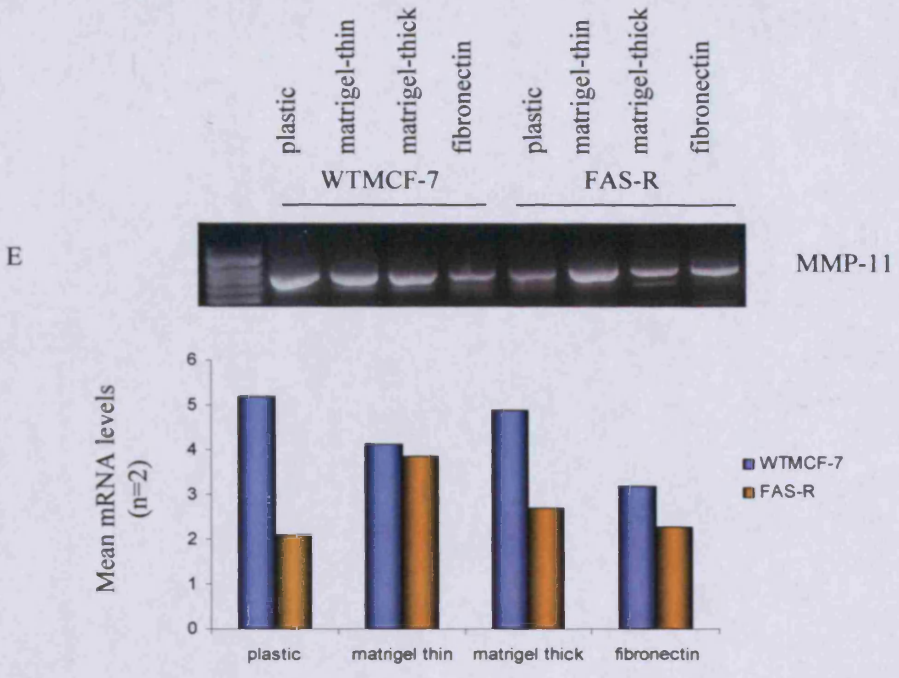

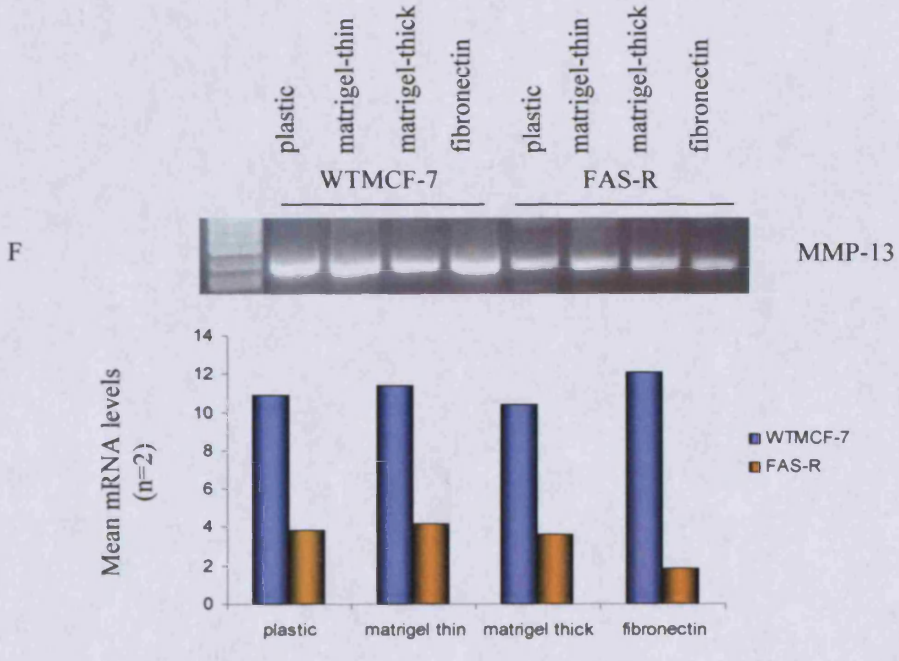

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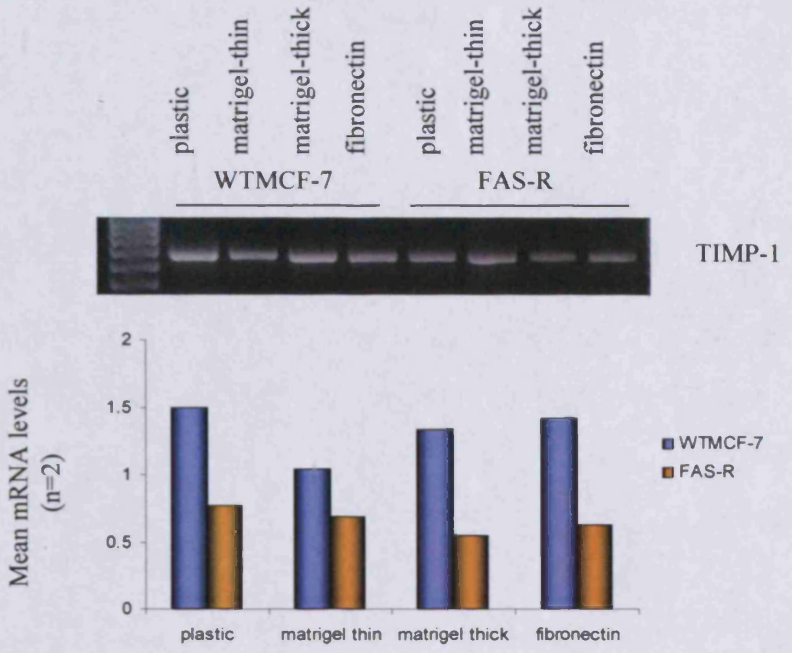

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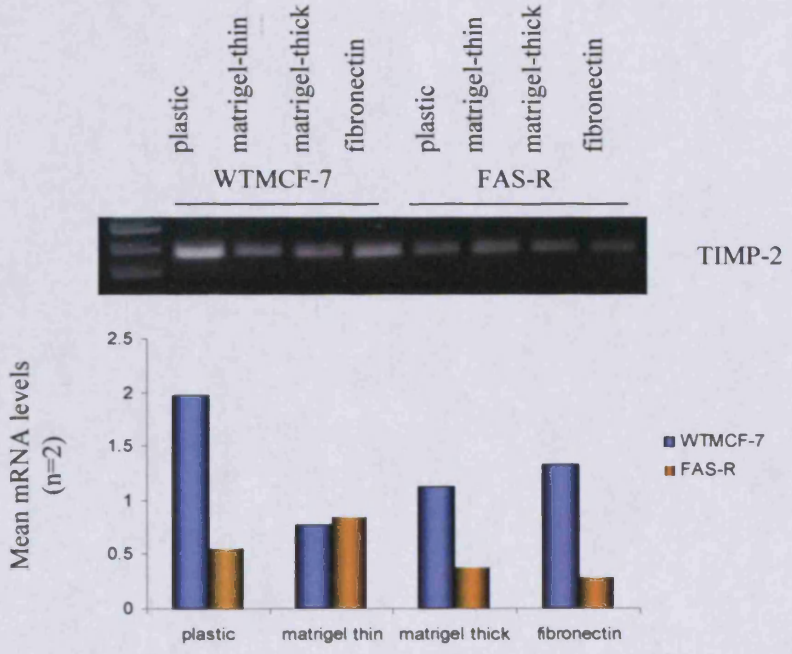

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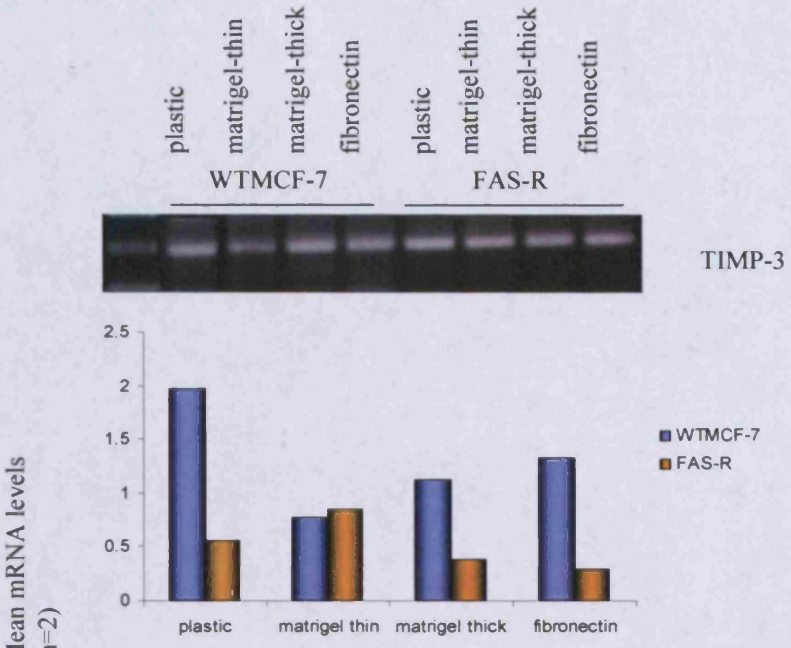

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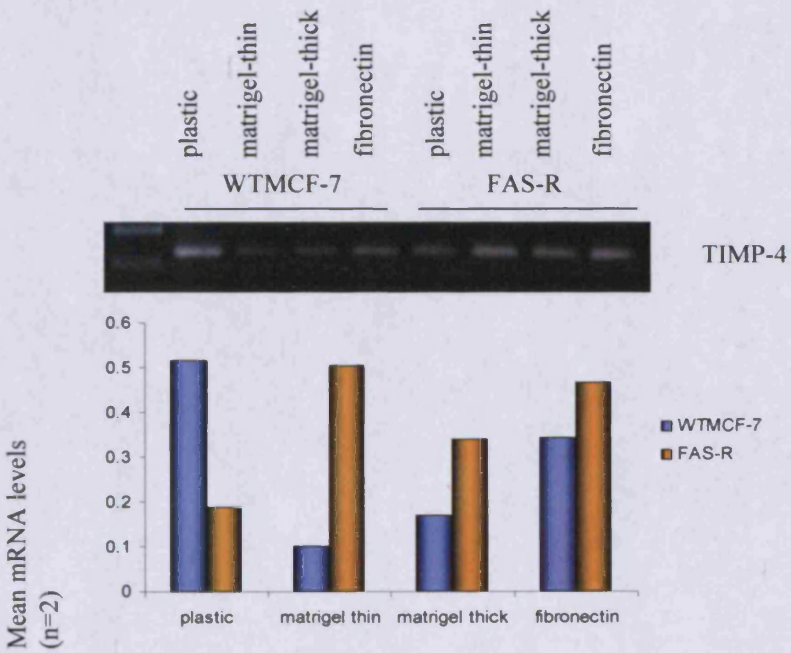

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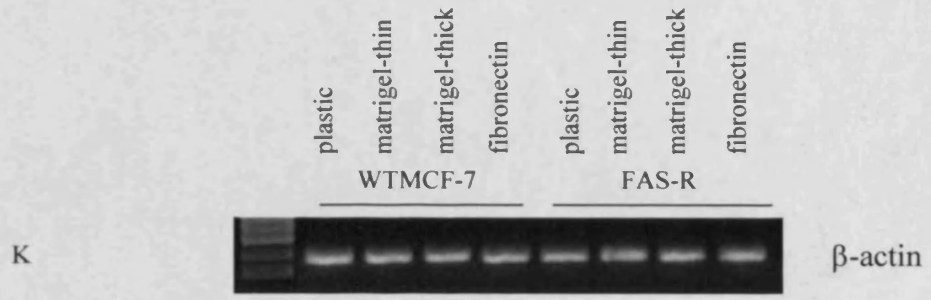


Figure 4.5 continued.

As was observed throughout the course of this study the expression of MMP-2 in WTMCF-7 cells was detectable in some sample sets but not in others; this suggested that levels are exceedingly low and at detection threshold. Low levels of MMP-2 were detected under control conditions; and culture on both matrigel layers seemed to reduce its expression. Culture on both matrigel layers furthermore distinctly lowered TIMP-2 and TIMP-4 expression.

Similarly, culture on fibronectin did not cause a marked increase in MMP and TIMP expression levels. In fact, a noticeable decrease in mRNA expression levels of MMP-9, MMP-11, and TIMP-4 was observed on culture on fibronectin as compared to culture on plastic.

Basal characterisation of MMP expression has already shown that MMP expression levels in FAS-R cells are lower than those observed for WTMCF-7 cells, with the exception of MMP-2. This trend continued on culture on matrix. MMP -9, which was not expressed in basal FAS-R cells, remained undetected on culture on matrigel and fibronectin while the expression levels for MMP-13 following culture on matrix remained almost unchanged. Only MMP-2 and MMP-11 showed an upregulation in expression levels following culture on matrigel compared to plastic, whilst MMP-1 and MMP-10 gene expression seemed to be induced by matrigel. Whilst MMP-10 was undetected in FAS-R cells previously (section 4.2.2; **figure 4.3D**), expression was observed in these samples. It may be that this apparent inconsistency is due to the fact that expression levels of MMP-10 in basal FAS-R cells are extremely low and hence just at detection threshold.

In conclusion, with the exception of MMP-2, all MMPs displayed lower expression in FAS-R cells despite culture on different matrices. Similarly, culture on matrix did not distinctly alter TIMP-1 to TIMP-4 expression in FAS-R cells. Collectively these results suggested that cell environment did not significantly increase or decrease MMP or TIMP expression levels, respectively.

Zymographic analysis was used to corroborate the above results with respect to MMP-9 at protein level (**figure 4.6**). Unlike before, where the amount of supernatant assayed for each sample had been adjusted according to cell numbers, for this experiment and subsequent zymographic studies the same volume (50µl) was assayed for all samples, and data subsequently normalised using protein concentration values obtained from respective cell lysates as these were required for downstream assays. As can be seen from figure **4.6C**, the MMP-9 protein expression profile of FAS-R cells correlated directly to the mRNA expression profile. This confirmed that culture on matrix induced neither the active nor the latent form of the MMP-9 protein in FAS-R cells. Zymography also showed that MMP-2 protein remained undetected following culture on matrix despite MMP-2 being expressed at mRNA level.

4.2.4 Role of Growth Factor Signalling in Endocrine-Resistant Growth and in MMP and TIMP Expression and Invasion

Basal MMP expression of FAS-R cells has been determined previously (**figure 4.3**). In the *in vivo* setting, however, cells are exposed to a plethora of growth factors, which activate various signalling pathways thus altering expression levels of target genes. Furthermore, it is now widely acknowledged that breast cancer progression involves complex interactions between steroidal and growth factor signalling pathways, and several studies have demonstrated the importance of aberrant growth factor signalling in the development of endocrine therapy resistance (Nicholson and Johnston, 2005, Nicholson *et al.*, 2005). Signalling studies carried out on our in-house cell models have demonstrated an important role for deregulated growth factor signalling in endocrine-resistant growth and invasive behaviour. More specifically, our TAM-R and double-resistant TAM/TKI-R cell model systems have revealed that endocrine-resistant growth and invasion in these cells is driven by EGFR and its ligands EGF and TGF-α and by IGF-1R and its ligand IGF-II, respectively (Knowlden *et al.*, 2003, Jones *et al.*, 2004).

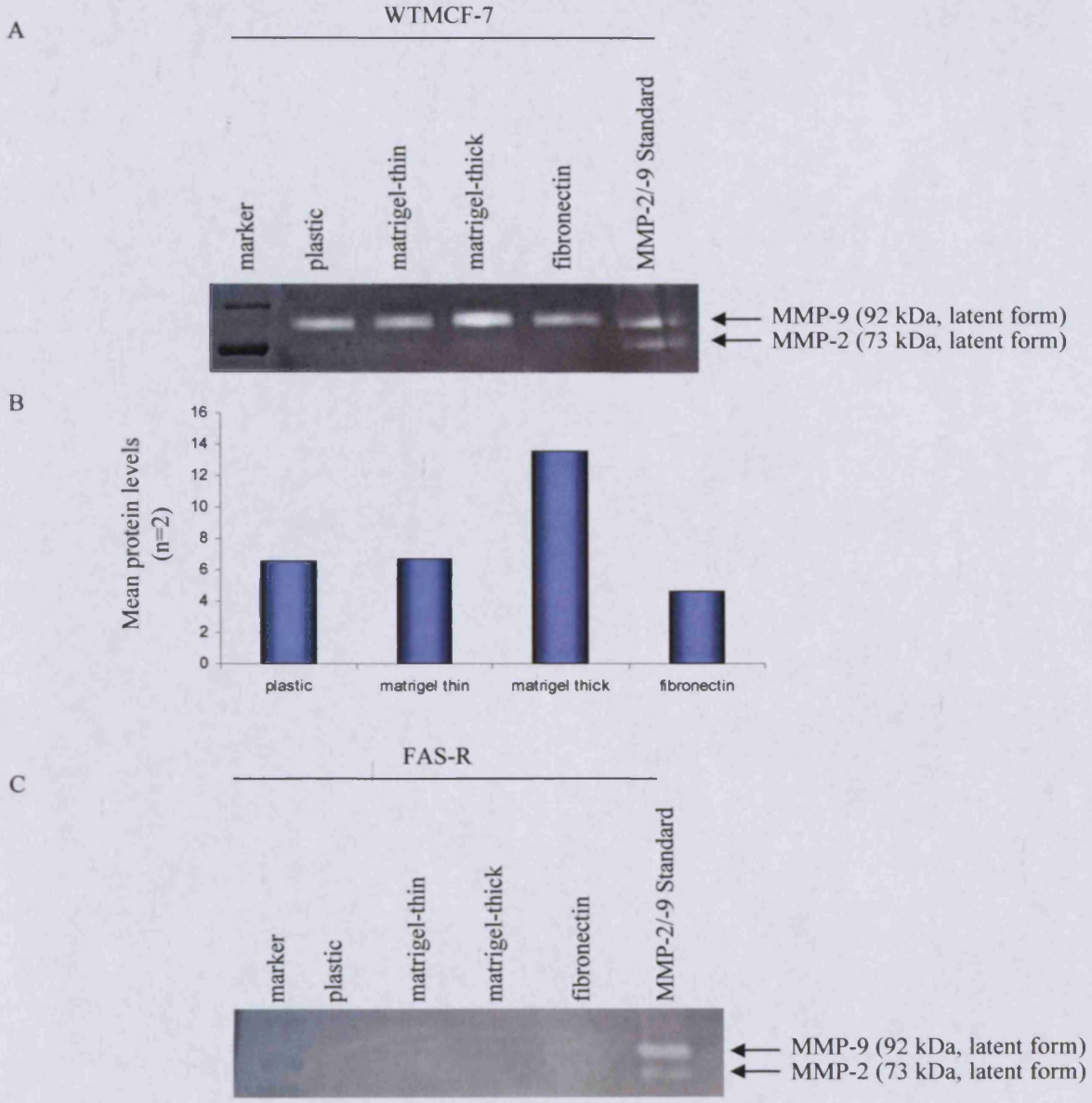


Figure 4.6 MMP-2 and MMP-9 protein expression levels of WTMCF-7 and FAS-R cells grown on different matrices as determined by zymography.
WTMCF-7 (A) and FAS-R cells (C) were cultured on plastic, matrigel and fibronectin under serum-free conditions in DCCM until log-phase growth was reached. In the case of matrigel, both a thin and a thick coating method were employed. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7 to determine the effect of different matrices on MMP-2 and MMP-9 protein expression. A commercial MMP-2/-9 standard was run alongside test samples as an internal assay control. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels (n=2) (B). Representative images are shown above (A and C).

The protein expression profile and signalling activity of these RTKs in FAS-R cells has been investigated previously (**figure 4.7**; [data provided courtesy of J. Gee and J. Knowlden]). While both EGFR and IGF-1R are expressed in FAS-R cells, phospho-EGFR and phospho-IGF-1R levels are very low compared to their wildtype counterpart, suggesting their signalling activity to be only modest. It is therefore unlikely that these two RTKs should play a vital role in the invasive behaviour of FAS-R cells.

However, parallel studies employing Affymetrix cDNA array technology had recently revealed a distinct increase in c-Met mRNA levels in FAS-R cells as compared to in WTMCF-7 cells (**figure 4.8A**; [data provided courtesy of J. Gee and R. McClelland]). This pro-invasive RTK is another receptor commonly overexpressed in a wide variety of malignancies. Therefore, analysis of c-Met mRNA and protein levels of FAS-R cells was undertaken to confirm that c-Met expression was altered in our endocrine-resistant cells. As can be seen in **figure 4.8B**, RT-PCR analysis corroborated the microarray-generated mRNA profile for c-Met. Protein expression levels of the receptor were estimated using Western Blotting. Whilst WTMCF-7 cells did not express the c-Met receptor protein, expression of the pro-invasive RTK was detected in FAS-R cells (**figure 4.9A**). This overexpression was further confirmed by immunocytochemical analysis as can be seen in **figure 4.9B**.

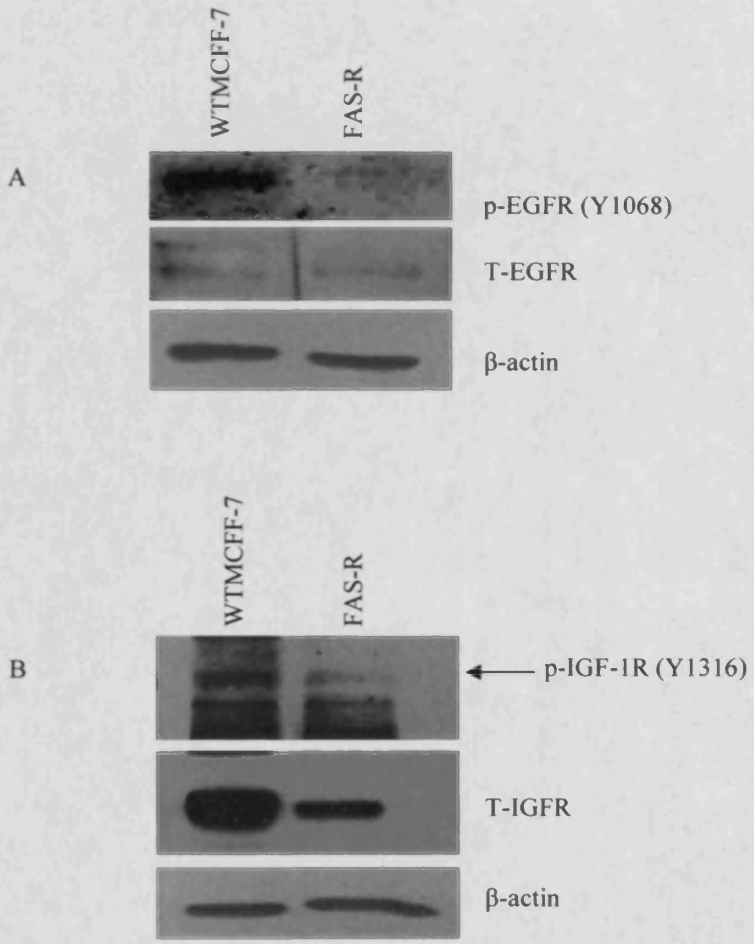
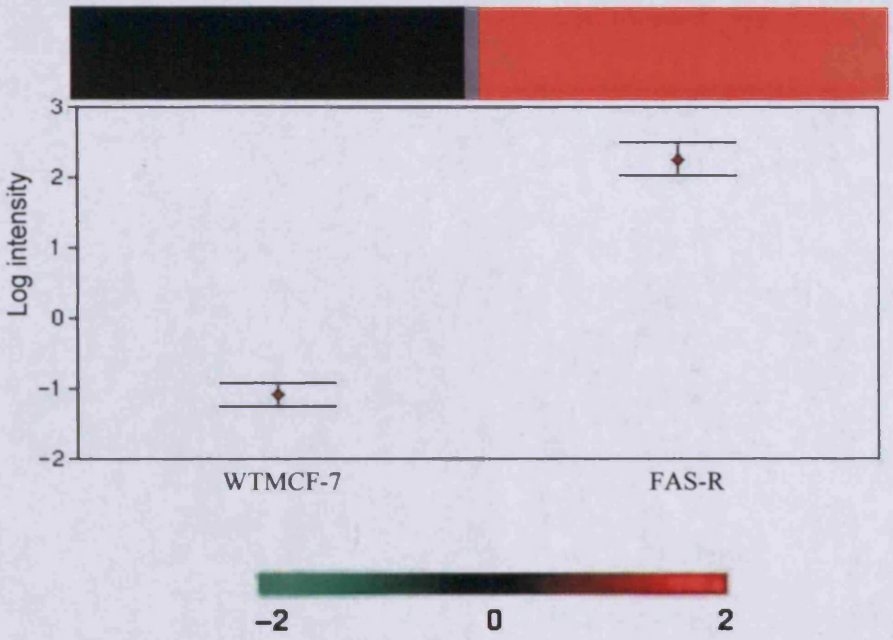


Figure 4.7 Comparison of EGFR and IGFR signalling activity in WTMCF-7 and FAS-R cells.
Western blot analysis of total and phosphorylated levels of EGFR (A) and IGF-1R (B) proteins in WTMCF-7 and FAS-R cells (data provided courtesy of J. Gee and J. Knowlden).

A



Probe ID: 203510_at

Accession No.: BG170541

t-test: $p<0.01$

B

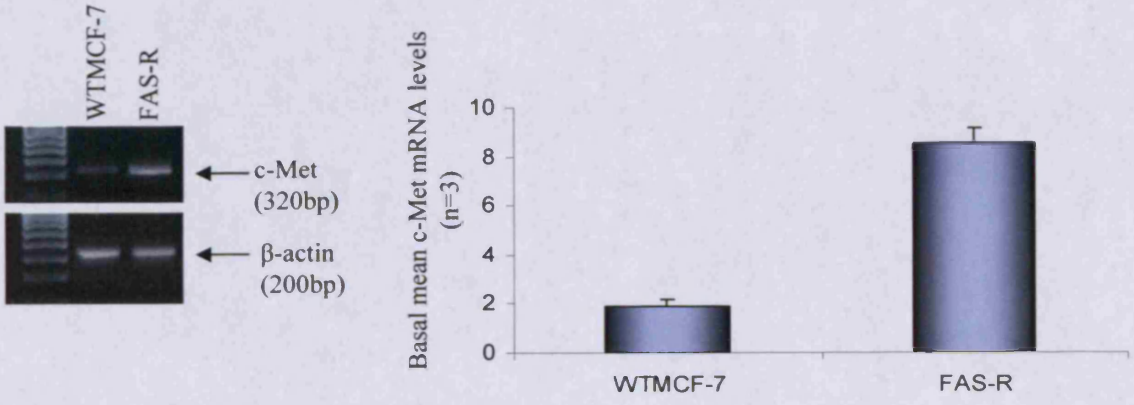
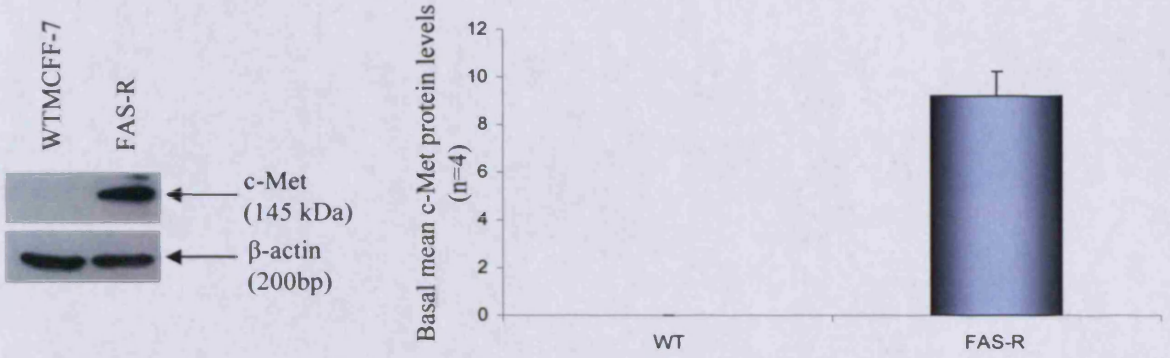


Figure 4.8 c-Met mRNA expression levels in WTMCF-7 and FAS-R cells.
mRNA expression levels of c-Met were increased in FAS-R cells as compared to WTMCF-7 cells as shown by Affymetrix cDNA array analysis (A (data provided courtesy of J. Gee and R. McClelland)) and RT-PCR (B).

A



B

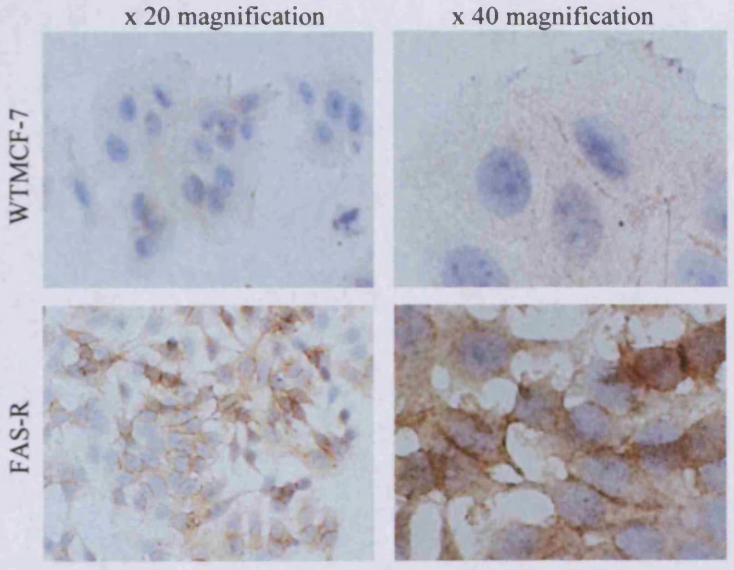


Figure 4.9 c-Met protein expression levels in WTMCF-7 and FAS-R cells.
Protein expression levels of c-Met were increased in FAS-R cells as compared to WTMCF-7 cells as shown by Western Blotting (A) and immunohistochemical staining (B (Hiscox *et al.*, 2006)).

While FAS-R cells displayed high levels of membranous c-Met expression with predominant staining at intercellular junctions, only very faint staining was observed in WTMCF-7 cells. Taken together, these data demonstrated distinct overexpression of the pro-invasive RTK c-Met by FAS-R cells.

In order to prove that this receptor was functional, the effect of HGF/SF stimulation (20ng/ml for 5 minutes) on c-Met phosphorylation was examined by Western Blot analysis. While no c-Met phosphorylation was observed in the wildtype, HGF/SF clearly activated both phosphorylation sites, Y1349/1356 and Y1234/1235, in FAS-R cells (**figure 4.10**).

Accompanying this activation was an augmentation of the motile capacities of FAS-R cells as demonstrated by scatter and migration assays: as **figure 4.11** illustrates, exposure to exogenous HGF/SF (20ng/ml) elicited a strong scatter response in FAS-R cells while virtually no colony dispersion was observed in WTMCF-7 cells. Similarly, as demonstrated in **figure 4.12**, FAS-R cell migration rates were significantly increased in the presence of exogenous HGF/SF (20ng/ml) (6.23-fold) whilst the response of WTMCF-7 cells was only modest in comparison (2.42-fold).

As outlined above, the RTKs EGFR, IGF-1R and c-Met are of paramount importance to endocrine-resistant cell growth; this warranted further investigation to determine whether they played a role in the invasive behaviour of FAS-R cells. Invasion assays were therefore performed in the presence of their respective ligands EGF, TGF-α, IGF-II and HGF/SF. While both the EGFR ligands EGF and TGF-α and the IGF-1R ligand IGF-II did not significantly increase the invasive capacity of FAS-R cells, HGF/SF treatment resulted in a 2.3-fold increase in FAS-R cell invasion (**figure 4.13**).

Excitingly, when invasion assays were carried out in the presence of the broad-spectrum MMP inhibitor used earlier (again at 40μM), a significant reduction in HGF/SF-augmented invasive rates was observed (**figure 4.14**). This finding strongly implicated MMP function in HGF/SF-enhanced invasive behaviour.

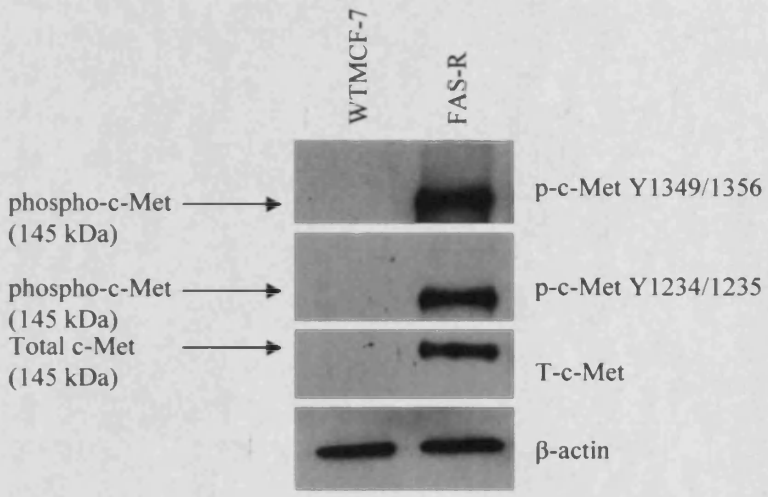


Figure 4.10 HGF/SF activates c-Met receptor phosphorylation in FAS-R cells.
Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) for 5 minutes. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-c-Met Y1349/1356 and Y1234/1235 and total c-Met as outlined in section 2.6. As can be seen, HGF/SF activated both c-Met phosphorylation sites in FAS-R cells.

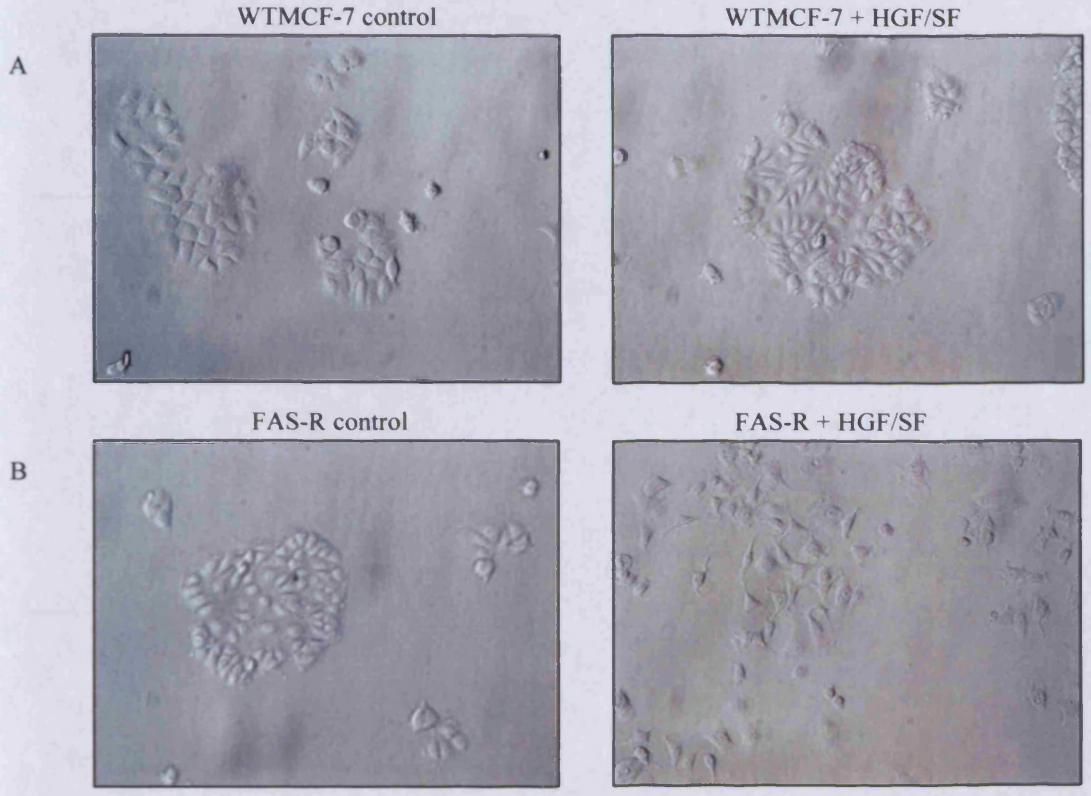
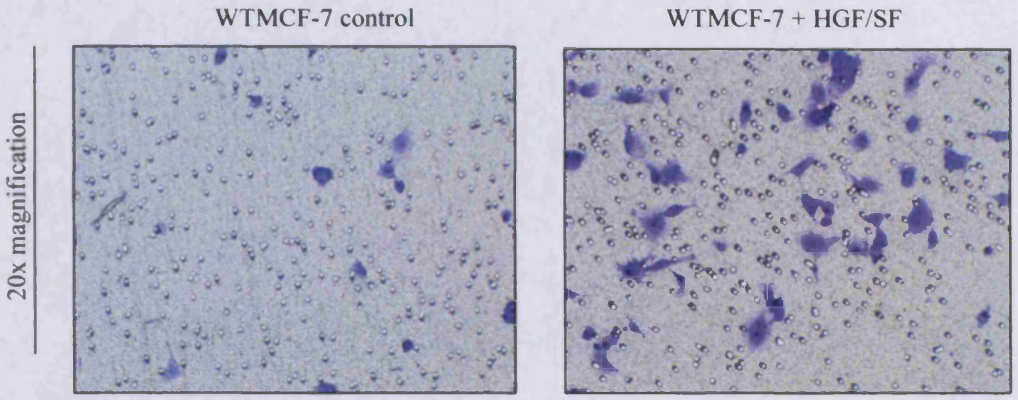


Figure 4.11 Colony dispersion in WTMCF-7 and FAS-R cells following HGF/SF challenge as determined by scatter assays.
WTMCF-7 (A) and FAS-R cells (B) were seeded in EM ± Faslodex (10^{-7}M) and allowed to form discrete colonies prior to 24h HGF/SF (20ng/ml) challenge in DCCM. The extent to which HGF/SF induced colony dispersion was determined using cell scatter assays as described in section 2.10. Scatter response was assessed visually and selected colonies were photographed using a Leica DM-IRE2 inverted microscope; representative images are shown above. As can be seen, HGS/SF induced extensive colony dispersion in FAS-R cells (B).

A



B

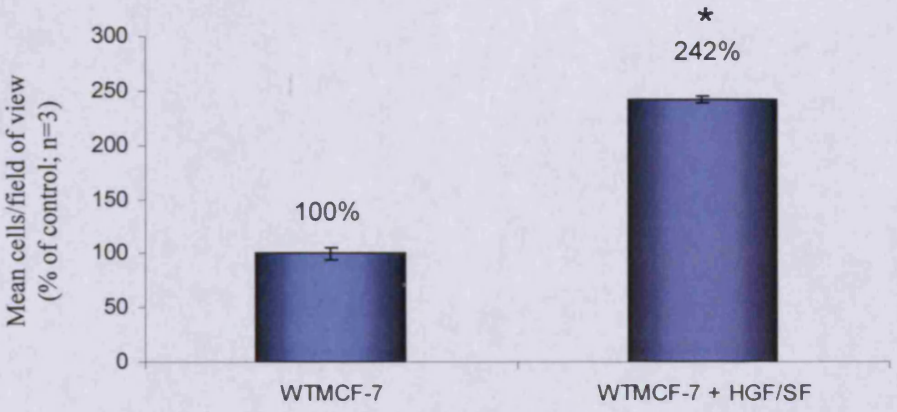
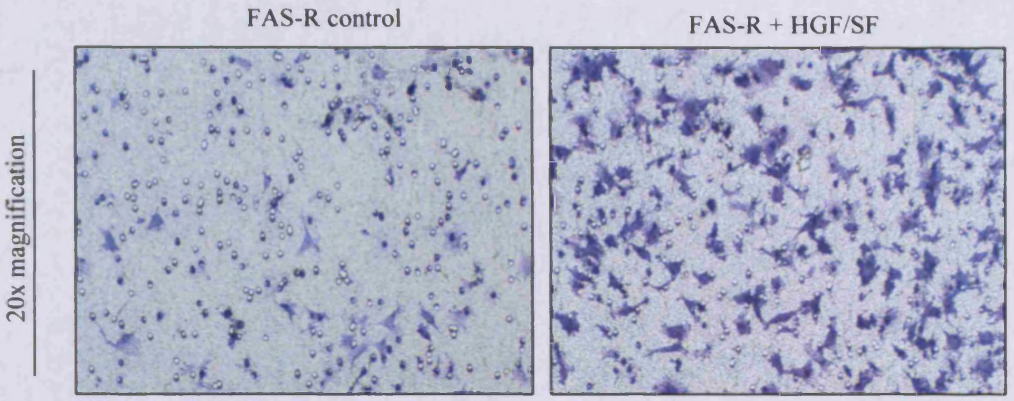


Figure 4.12 The effect of HGF/SF on migration rates of WTMCF- and FAS-R cells. The effect of HGF/SF treatment (20ng/ml) on the migratory capacities of WTMCF-7 and FAS-R cells was determined using *in vitro* cell migration assays as described in section 2.9. Crystal-violet-stained cells were photographed at 20x magnification using an Olympus BH-2 phase contrast microscope; representative images are shown above and on the following page (A and C). Quantification was by counting the number of migratory cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of WTMCF-7 ± S.D. (*$p<0.001$ vs. WTMCF-7 control) and as % of FAS-R cells ± S.D. (*$p<0.005$ vs. FAS-R control) (B and D). As can be seen HGF/SF caused a significant increase in the migratory capacity of FAS-R cells.

C



D

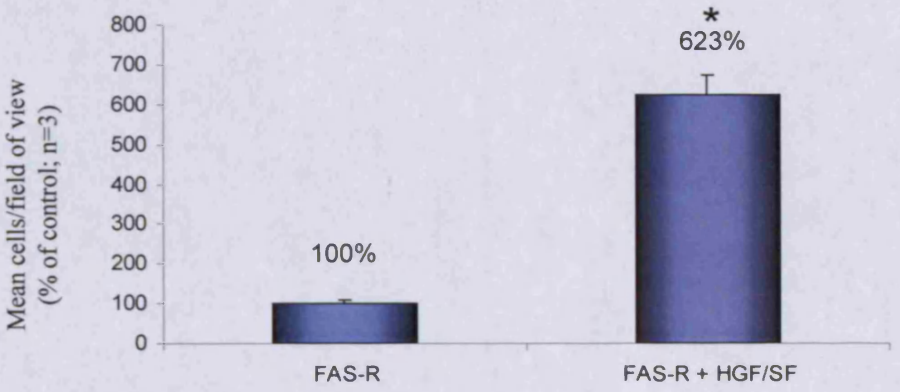
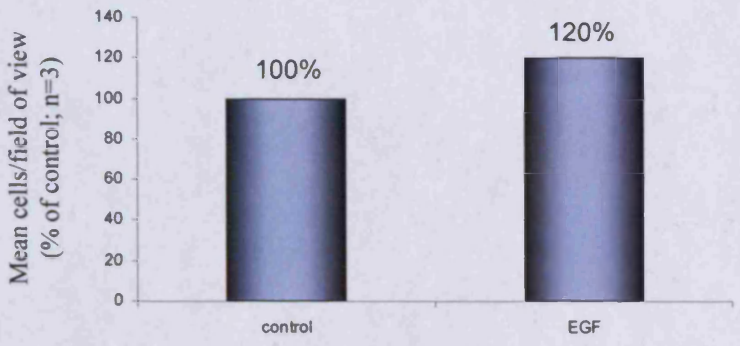


Figure 4.12 continued.

A



B

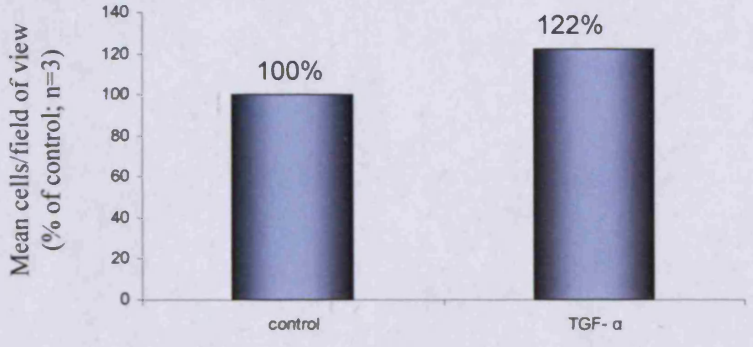
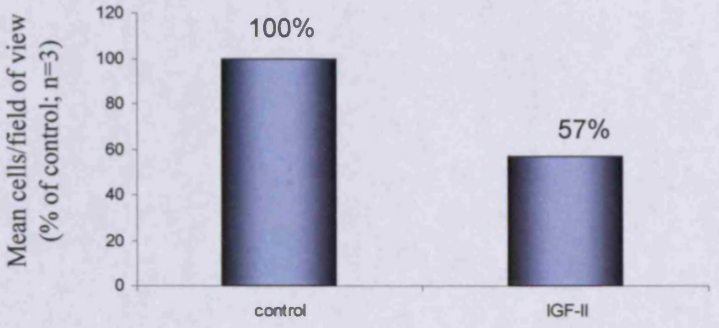


Figure 4.13 The effect of growth factor treatment on invasion rates of FAS-R cells. The effect of EGF (10ng/ml) (A), TGF-α (10ng/ml) (B), IGF-II (10ng/ml) (C) and HGF/SF (20ng/ml) (D) treatment on the invasive capacities of FAS-R cells was determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera. Quantification was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of control ± S.D. As can be seen on the following page HGF/SF clearly increased the invasive capacity of FAS-R cells (D).

C



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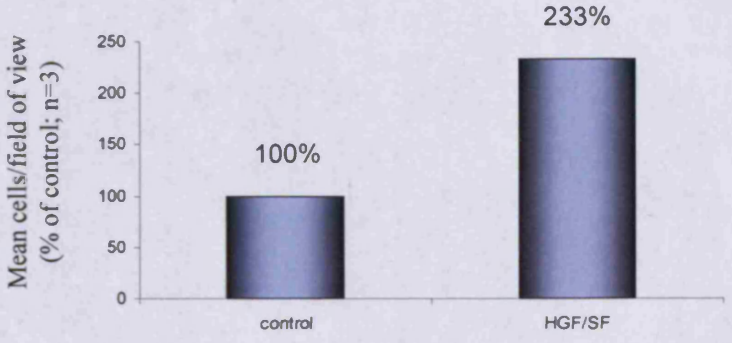


Figure 4.13 continued.

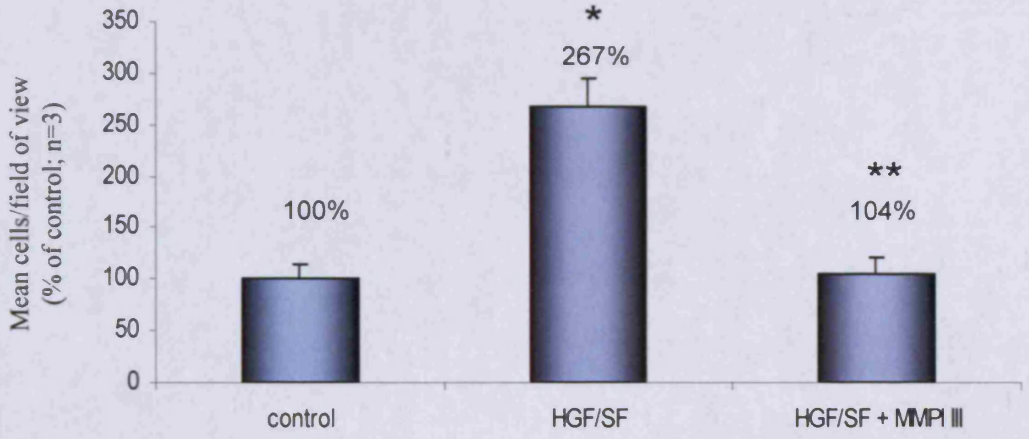


Figure 4.14 The effect of broad-spectrum MMP inhibition on HGF/SF-modulated invasion rates of FAS-R cells.

MMP inhibition was achieved by using a broad-spectrum MMP inhibitor (40μM). The effect of MMP inhibition on HGF/SF-modulated invasive capacities of FAS-R cells was determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera. Quantification was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of control ± S.D. (*$p<0.001$ vs. FAS-R control **$p<0.001$ vs. FAS-R + HGF/SF). As can be seen, MMP inhibition significantly reduced HGF/SF-modulated invasion rates of FAS-R cells.

Following assessment of the contribution of the above RTKs to the regulation of invasive behaviour, the effect of their respective ligands on MMP and TIMP expression was studied. Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with TGF-α (10ng/ml), EGF (10ng/ml), HGF/SF (20ng/ml), IGF-II (10ng/ml) and TPA (100nM) overnight and subsequently probed for mRNA expression of MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, MMP-11 and MMP-13 as well as for mRNA expression of TIMP-1 to TIMP-4 by RT-PCR (**figure 4.15**). TPA-treated samples served as positive controls for MMP and TIMP expression. The gene expression data shown in graphs and gel photos in **figure 4.15** has been summarised in tables for each individual target gene in the appendix (see p. 247).

EGF and TGF-α clearly augmented MMP-1, MMP-9, MMP-10, TIMP-1 and, to a lesser extent, TIMP-4 expression in WTMCF-7 cells suggesting that expression regulation of these genes underlies the EGFR signalling pathway (**figure 4.15**). Conversely, in FAS-R cells all the above genes (with the exception of MMP-10 at this early time-point) were upregulated by HGF/SF, suggesting that in these cells expression regulation of these genes underlies the c-Met signalling pathway instead. Moreover, while EGF and TGF-α also increased TIMP-1 and TIMP-4 expression in FAS-R cells, a much more distinct upregulation was observed upon HGF/SF stimulation. These results seem to imply a switch in MMP and TIMP expression regulation from the EGFR pathway in WTMCF-7 cells to the HGF/SF/c-Met signalling axis in FAS-R cells. The only exception to this was in the form of MMP-2, the expression of which seemed to be upregulated in the presence of EGF but suppressed following HGF/SF treatment in FAS-R cells. MMP-7 was undetectable in both cell types. The apparent inconsistency of sudden MMP-9 detection in basal FAS-R cells is thought to be due to mRNA levels at detection threshold that are detected in some samples but not in others.

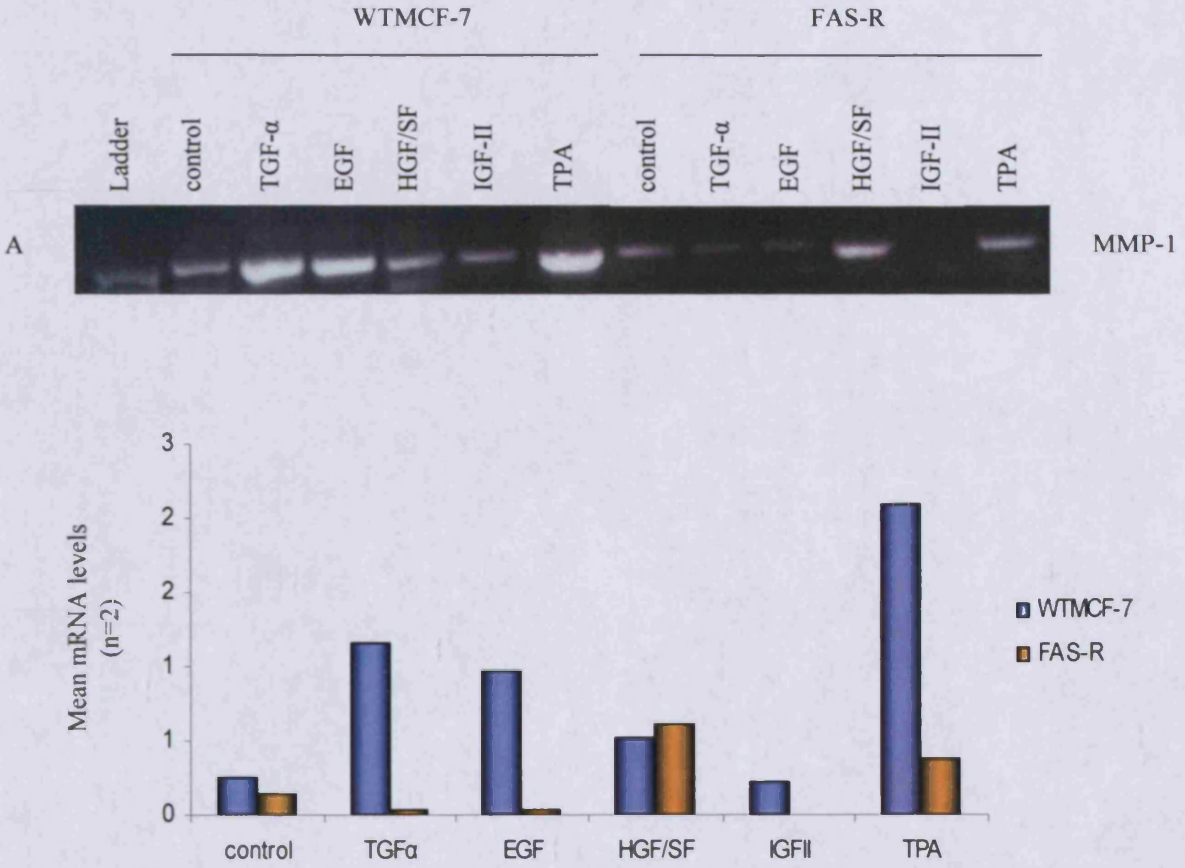
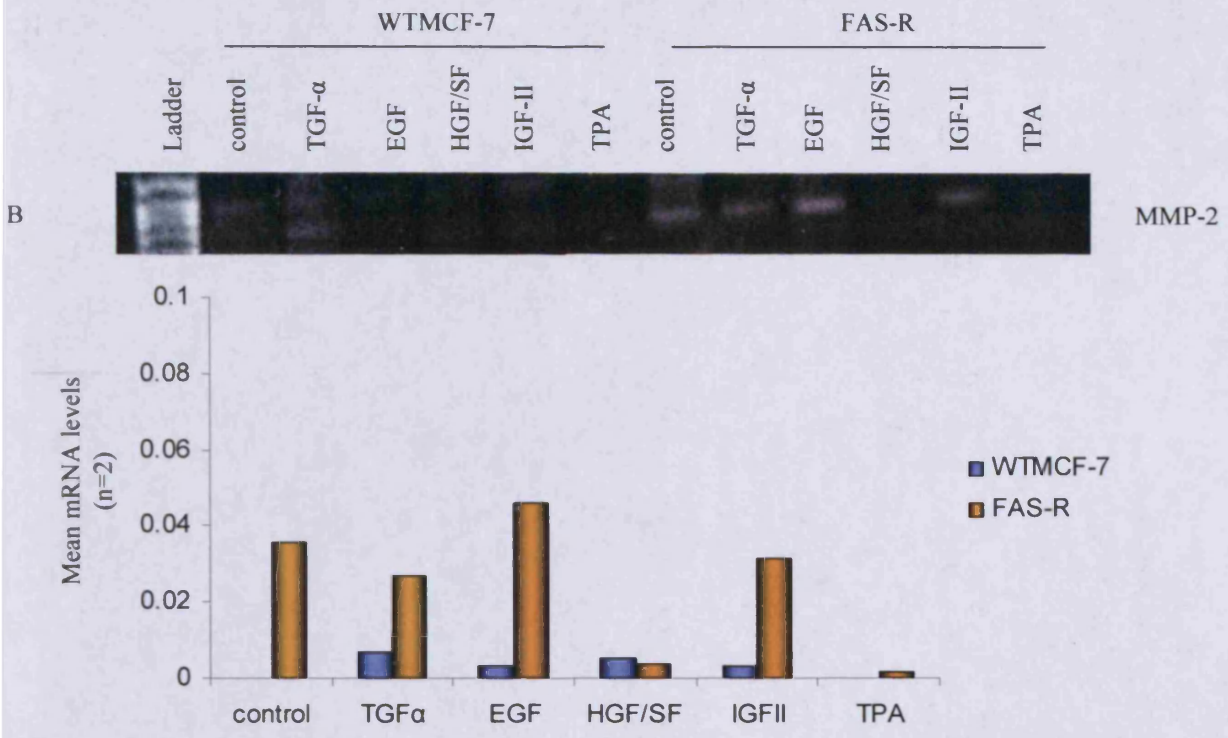
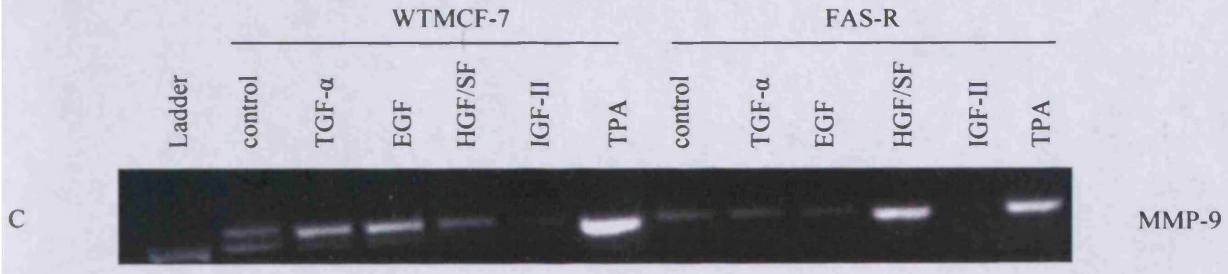


Figure 4.15 MMP and TIMP mRNA expression levels of WTMCF-7 and FAS-R cells following growth factor challenge as determined by semi-quantitative RT-PCR.
Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with TGF-α (10ng/ml), EGF (10ng/ml), HGF/SF (20ng/ml), IGF-II (10ng/ml) and TPA (100nM) for 16 hours. The effect of these growth factors on MMP-1, MMP-2, MMP-9, MMP-10, MMP-11 and MMP-13 and TIMP-1 to TIMP-4 expression was determined using gene expression analysis as outlined in section 2.5. Data was normalised using β-actin and presented as mean expression levels (n=2) (A-K). Representative images (A-K) are shown above and on following pages.



B

MMP-2



C

MMP-9

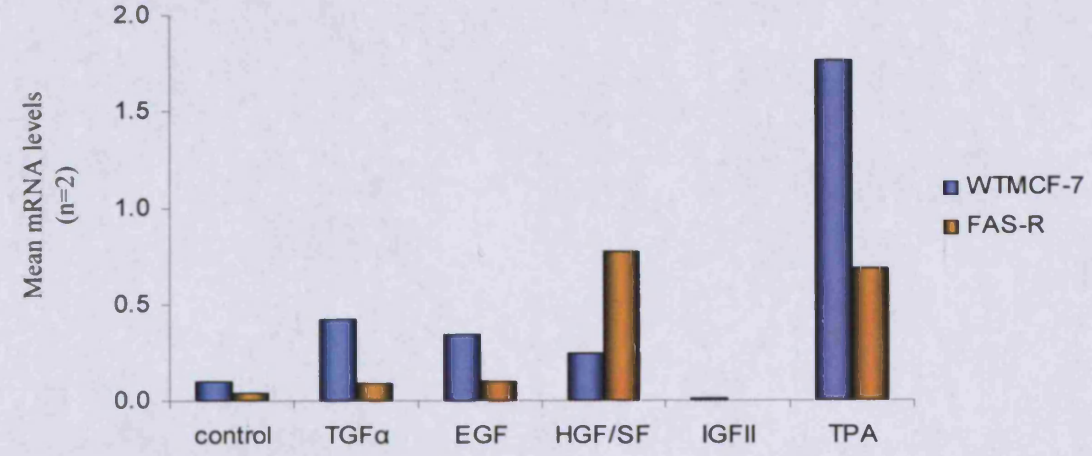
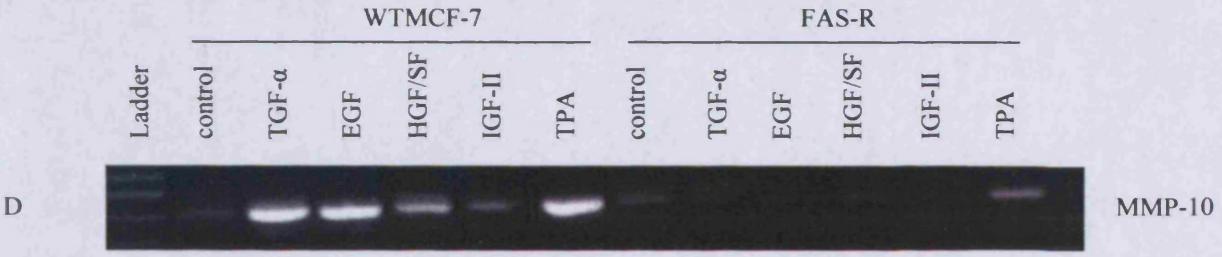
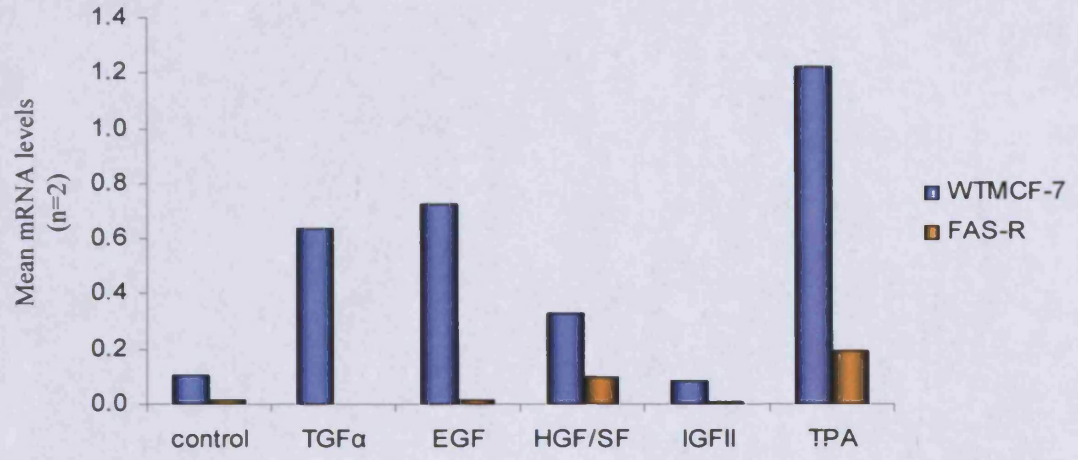


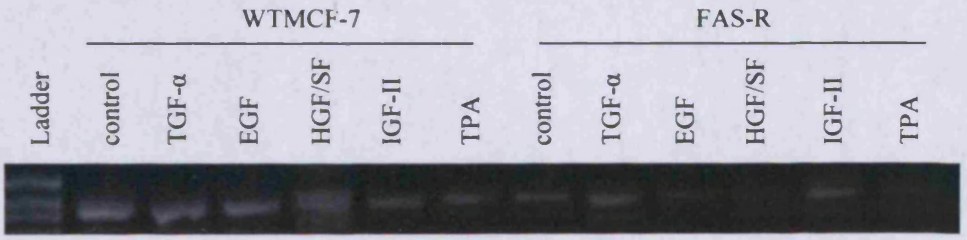
Figure 4.15 continued.



D

MMP-10





E

MMP-11

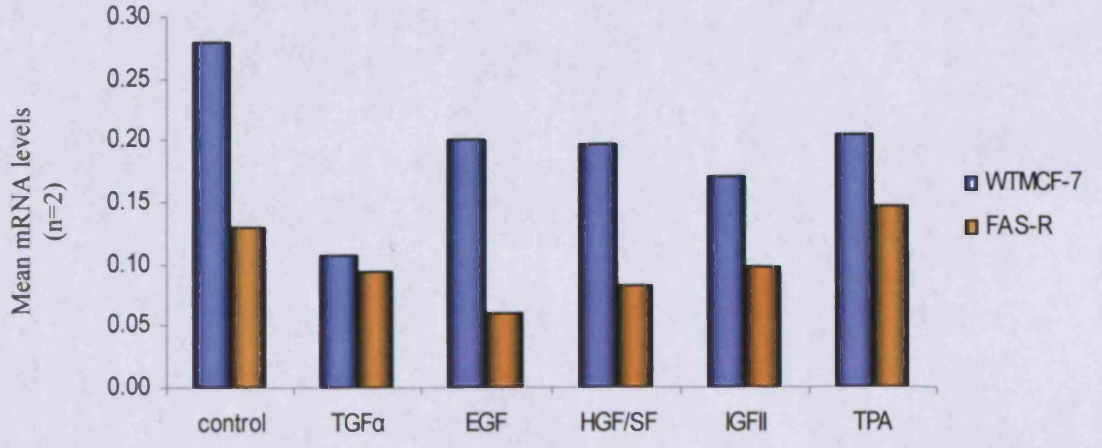
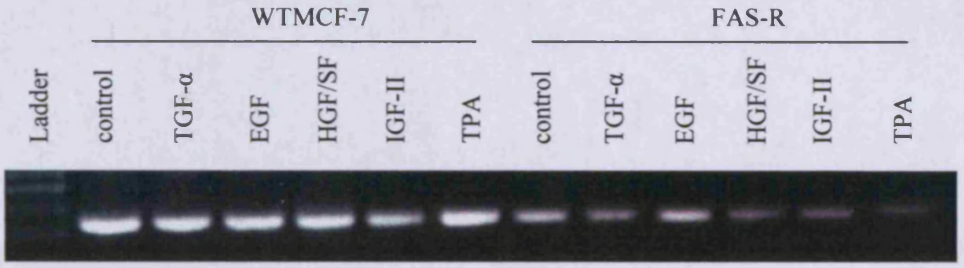
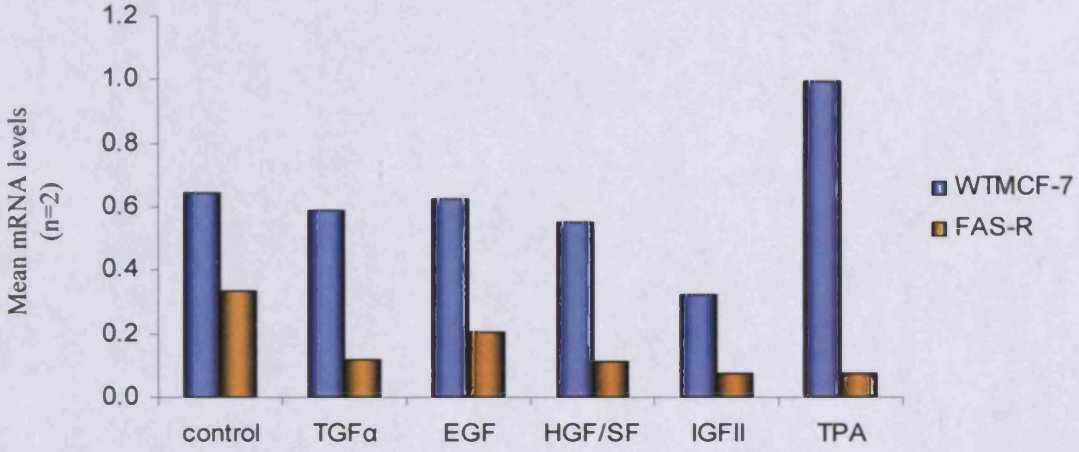


Figure 4.15 continued.

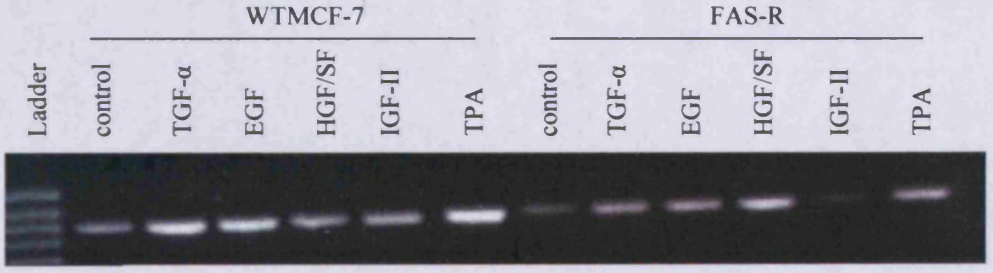
F



MMP-13



G



TIMP-1

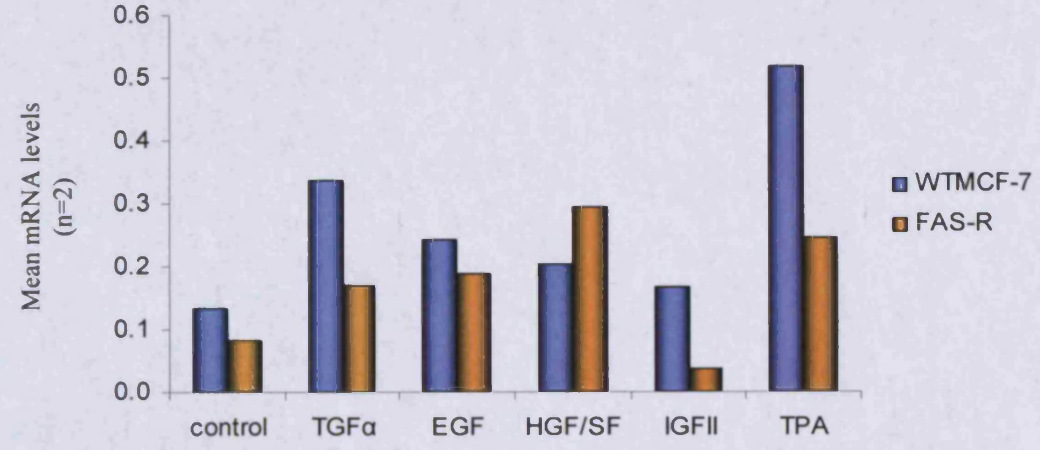
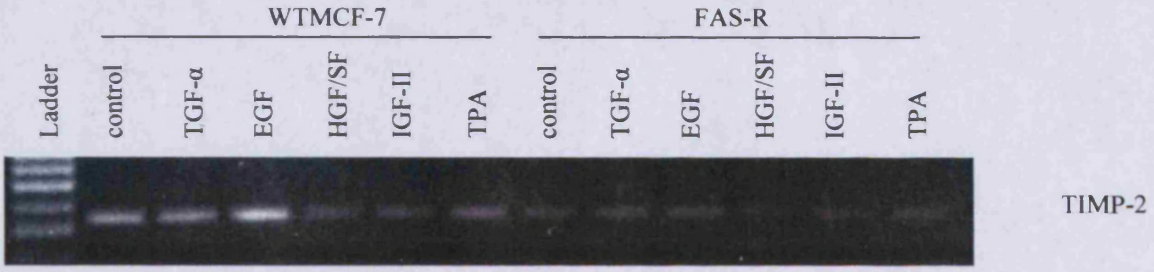
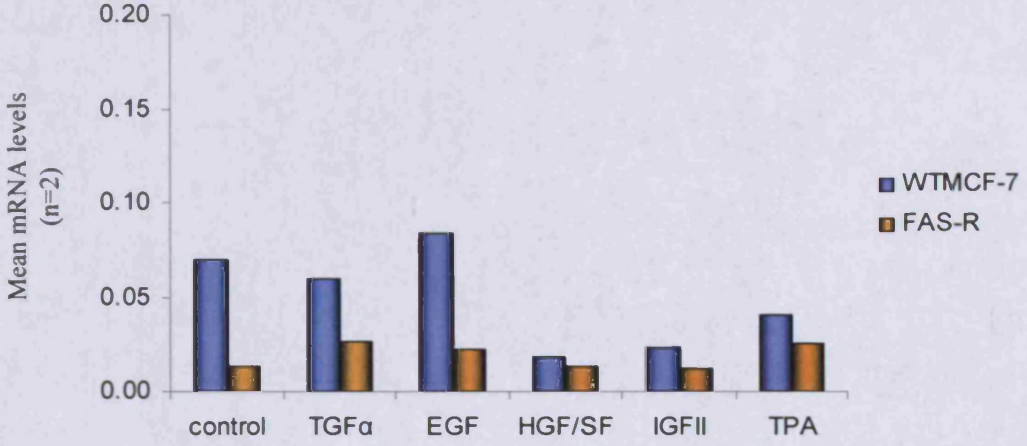


Figure 4.15 continued.

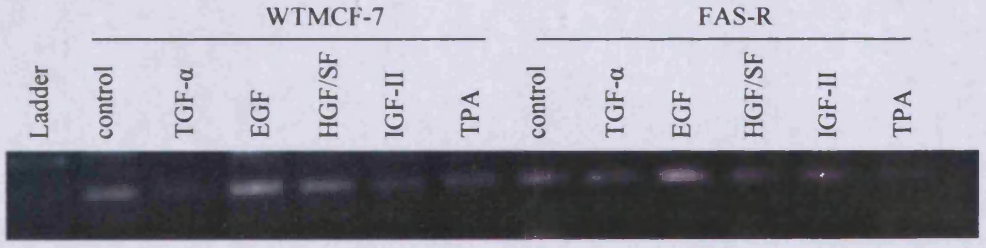
H



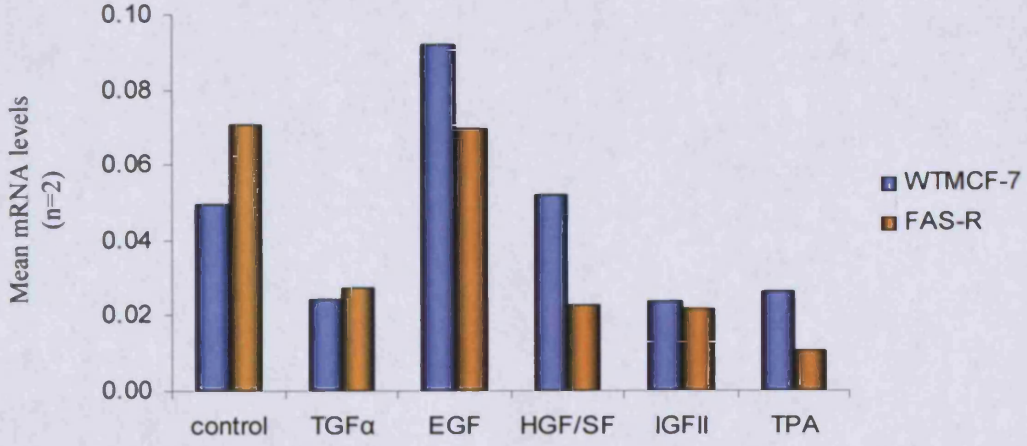
TIMP-2



I



TIMP-3



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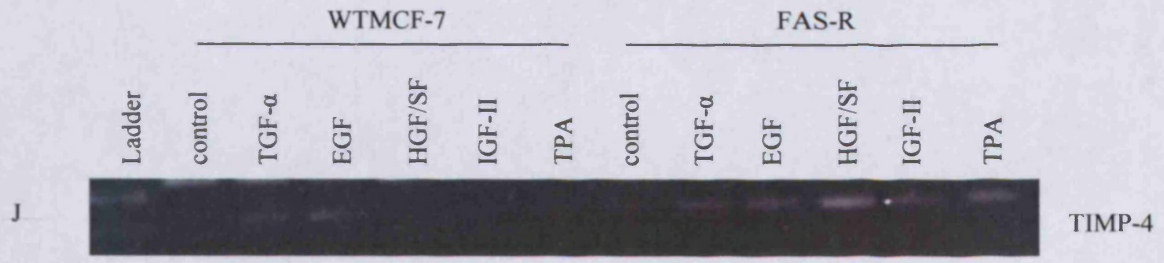
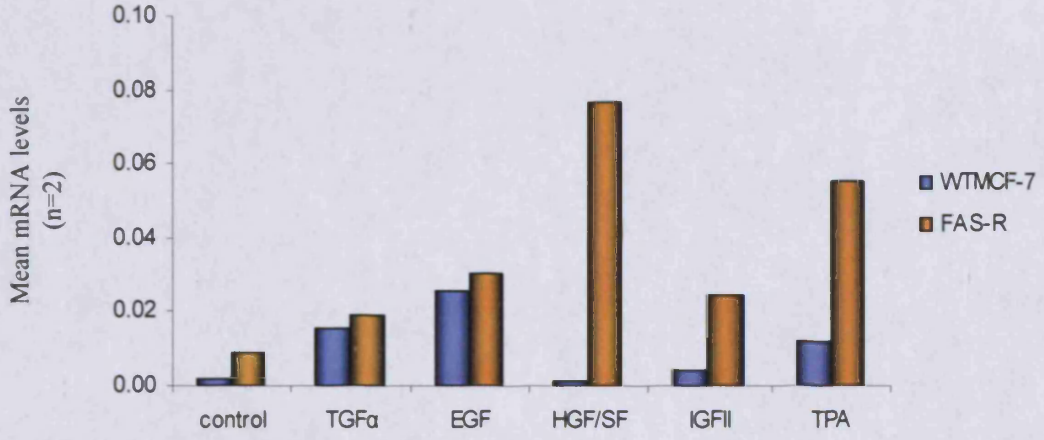
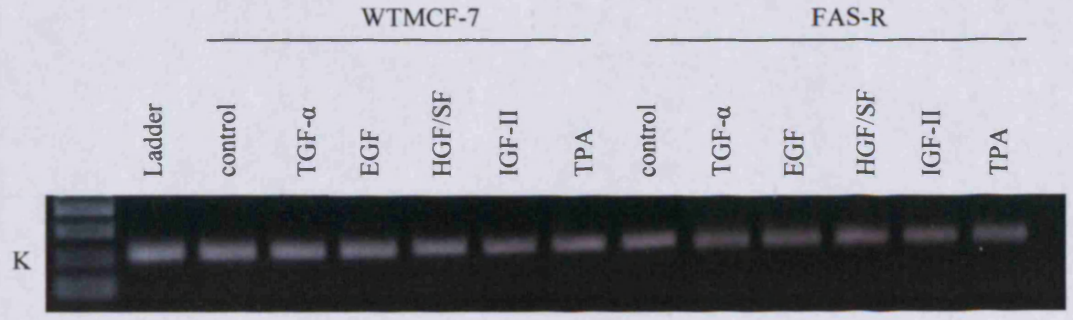


J

TIMP-4





K

β-actin

Figure 4.15 continued.

Again, zymography was used to corroborate the above results at protein level (**figure 4.16**). As explained above, data was normalised using protein concentration values obtained from respective cell lysates. Analysis of MMP-9 protein expression by this method confirmed the observations made at mRNA level, with the EGFR ligands EGF and TGF-α augmenting expression in the wild-type whilst HGF/SF significantly increased MMP-9 secretion in FAS-R cells. As before, only latent MMP-9 was identified while MMP-2 remained undetectable. This further supported the hypothesis of regulatory pathway switching from EGFR signalling in the wildtype to HGF/SF/c-Met signalling in FAS-R cells.

Treatment of WTMCF-7 and FAS-R cells with the c-Met ligand HGF/SF for 16 hours caused a marked upregulation in MMP-1 and MMP-9 mRNA levels and also significantly increased secretion of latent MMP-9. In view of the widely accepted role for the HGF/SF/c-Met signalling axis and MMP proteolytic activity in invasive cancer in general and the reduction in HGF/SF-augmented FAS-R cell invasion following MMP activity inhibition in particular, these findings warranted further investigation. The effect of HGF/SF treatment (20ng/ml) on MMP expression in FAS-R cells as compared to WTMCF-7 cells was thus further analysed at two more time-points, namely 24h and 4 days, to mirror duration of migration and invasion assays, respectively (**figure 4.17**).

Up to this point, TIMP mRNA levels have been analysed alongside MMP expression profiles as it was considered that it was the balance between MMPs and their specific inhibitors that ultimately determined overall proteolytic behaviour. However, the data resulting from TIMP expression analysis proved inconclusive. Furthermore, in light of the multi-faceted and paradoxical role ascribed to TIMP family members in carcinogenesis and metastasis it was recognised that putting findings into context with regard to these MMP inhibitors would be beyond the scope of this project, and analysis of TIMP expression was accordingly discontinued at this point.

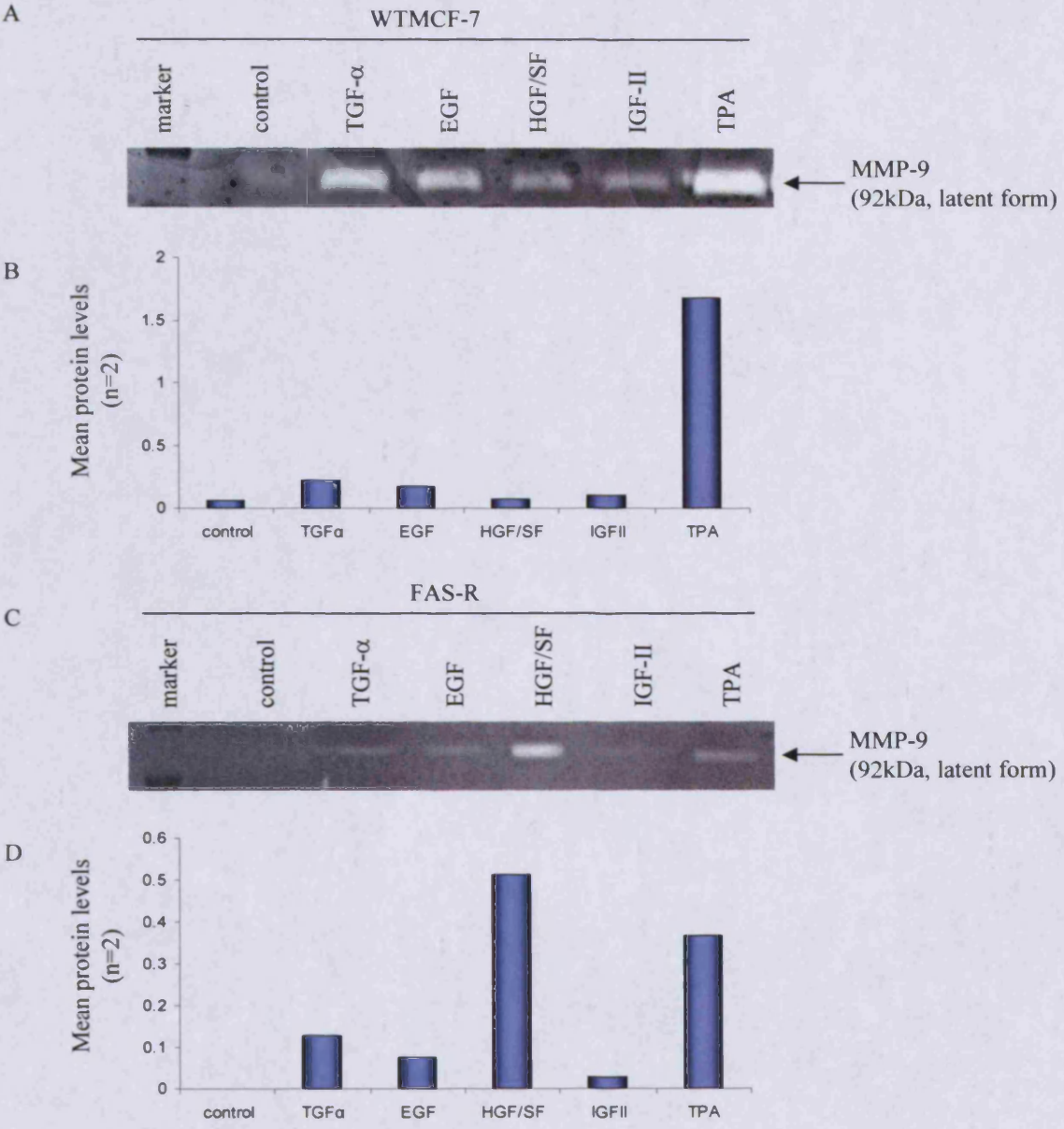


Figure 4.16 MMP-9 protein expression levels of WTMCF-7 and FAS-R cells following growth factor challenge as determined by zymography.
Serum-starved subconfluent WTMCF-7 (A) and FAS-R cells (C) were treated with TGF-α (10ng/ml), EGF (10ng/ml), HGF/SF (20ng/ml) and IGF-II (10ng/ml) for 16h. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7 to determine the effect of growth factor challenge on MMP-2 and MMP-9 protein expression. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels (n=2) (B and D). Representative images are shown above (A and C).

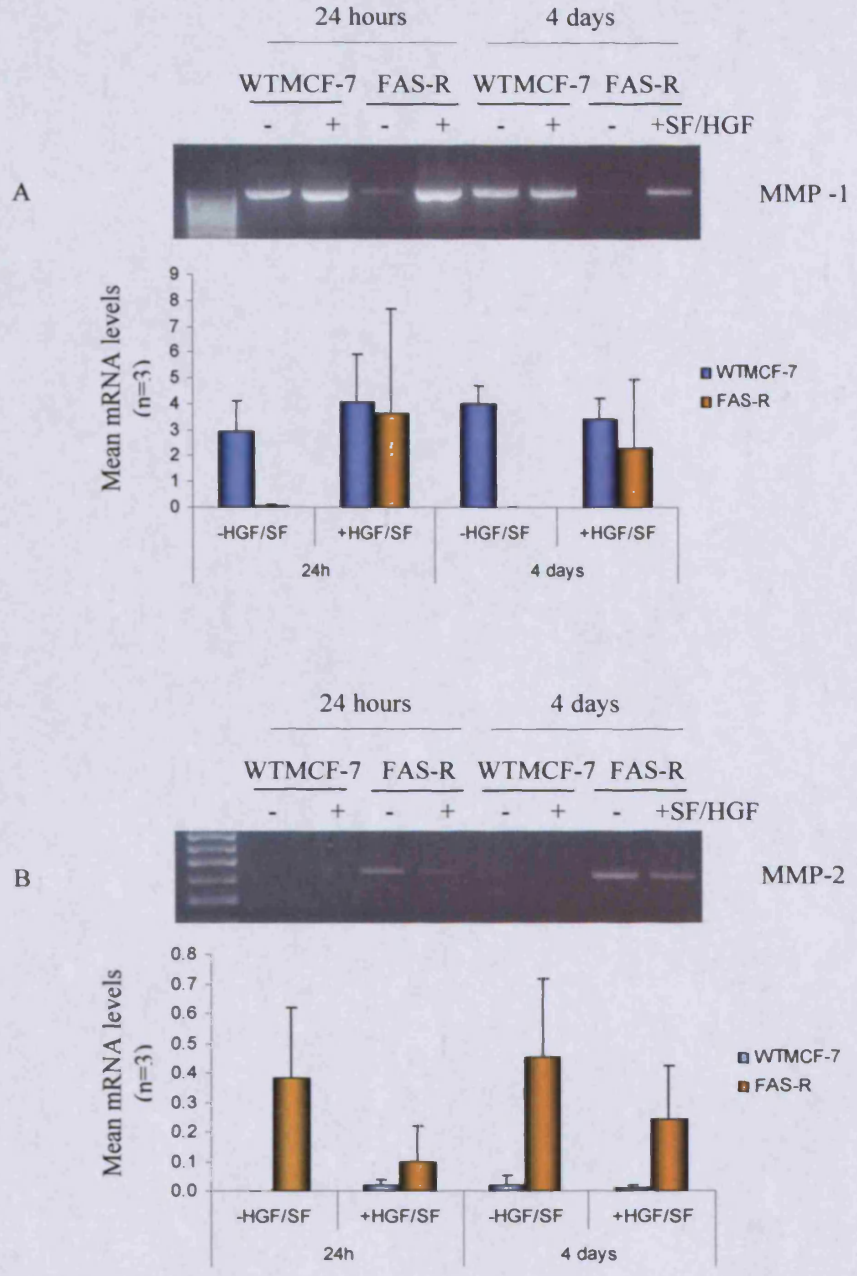
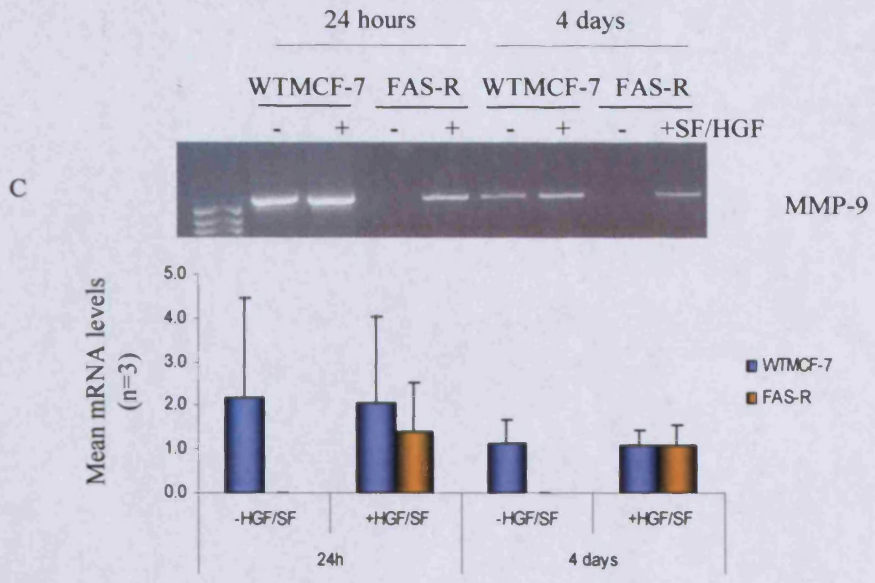
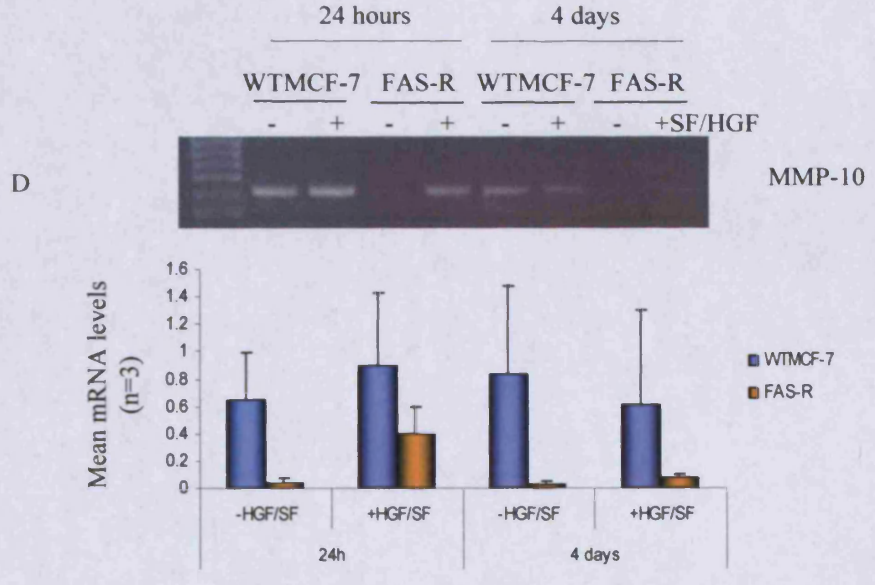


Figure 4.17 MMP mRNA expression levels of WTMCF-7 and FAS-R cells following HGF/SF challenge as determined by semi-quantitative RT-PCR.
Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) for 24h and 4 days respectively. The effect of HGF/SF on MMP-1, MMP-2, MMP-9, MMP-10, MMP-11 and MMP-13 expression was determined using gene expression analysis as outlined in section 2.5. Data was normalised using β-actin and presented as mean expression levels ± S.D. (n=3) (A-G). Representative images (A-G) are shown above and on following pages.





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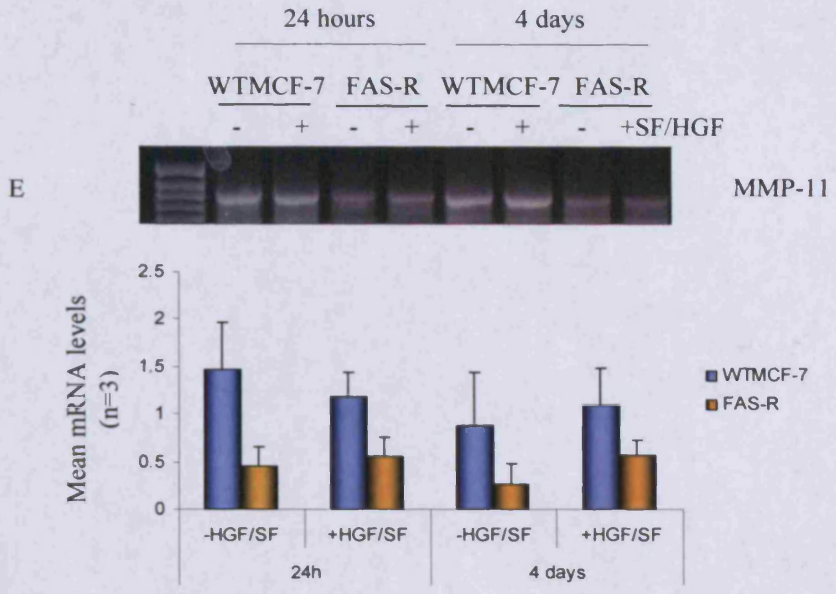

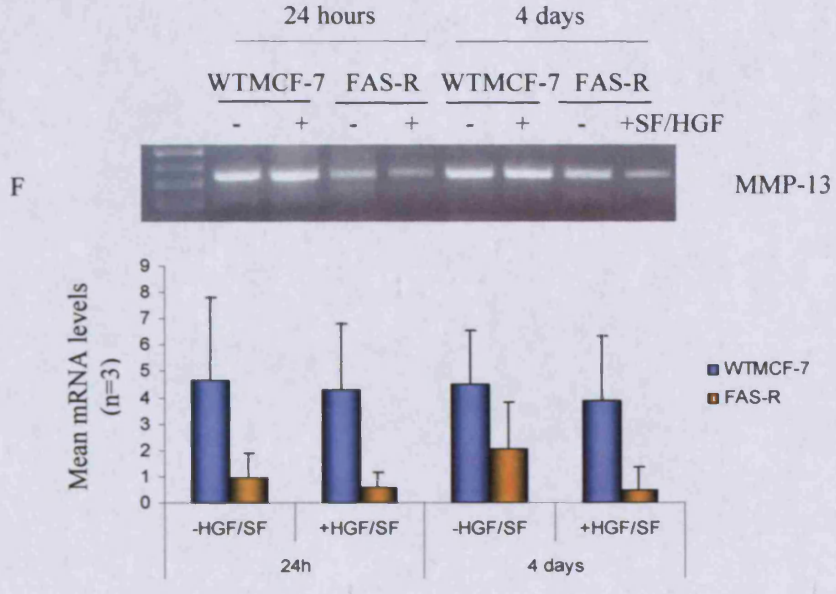

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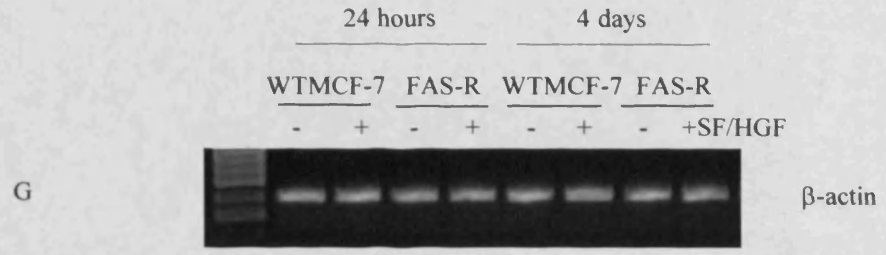


Figure 4.17 continued.

As before, HGF/SF addition induced MMP-1 and MMP-9 in FAS-R cells at both 24h and 4 days (**figure 4.17**). Interestingly, at these two later time-points HGF/SF treatment also resulted in induction of MMP-10 mRNA expression in FAS-R cells, another MMP that was augmented following EGF and TGF-α treatment in the wildtype and that therefore presumably underlies control of the EGFR signalling pathway in those cells while being governed by the HGF/SF/c-Met axis in FAS-R cells. This further confirmed the pathway-switching hypothesis brought forward earlier. While MMP-1, MMP-9, and MMP-10 induction was seen at both time-points, the effect of HGF/SF treatment for all three proteins was more pronounced at 24h. As observed before, HGF/SF addition again resulted in downregulation of MMP-2 mRNA levels in FAS-R cells; this downregulation was more also prominent at the earlier time-point of 24h. A similar trend was observed for MMP-13 expression in FAS-R cells.

Again, zymography was used to screen WTMCF-7 and FAS-R cells for expression of latent and active forms of MMP-9 and MMP-2 (**figure 4.18**). MMP-2 remained undetected in all samples; this confirmed previous findings and was consistent with the above observations that HGF/SF downregulated MMP-2 gene expression in FAS-R cells. In the case of MMP-9, the protein expression profile obtained by zymography correlated directly to the mRNA expression profile and revealed induction of secretion of MMP-9 at both time points upon HGF/SF treatment of FAS-R cells. Moreover, HGF/SF did not induce secretion of active MMP-9, and MMP-9 expression levels of basal and HGF/SF-treated WTMCF-7 cells were higher than those detected in FAS-R cells for all samples.

Western Blot detection of MMP-9, MMP-2, and MMP-1 in basal WTMCF-7 and FAS-R cells had been attempted earlier without much success. Since stimulation with HGF/SF resulted in such a distinct upregulation of MMP-1 mRNA levels and MMP-9 protein secretion it was hoped that Western Blot detection of MMPs in HGF/SF-treated FAS-R cells would yield a positive

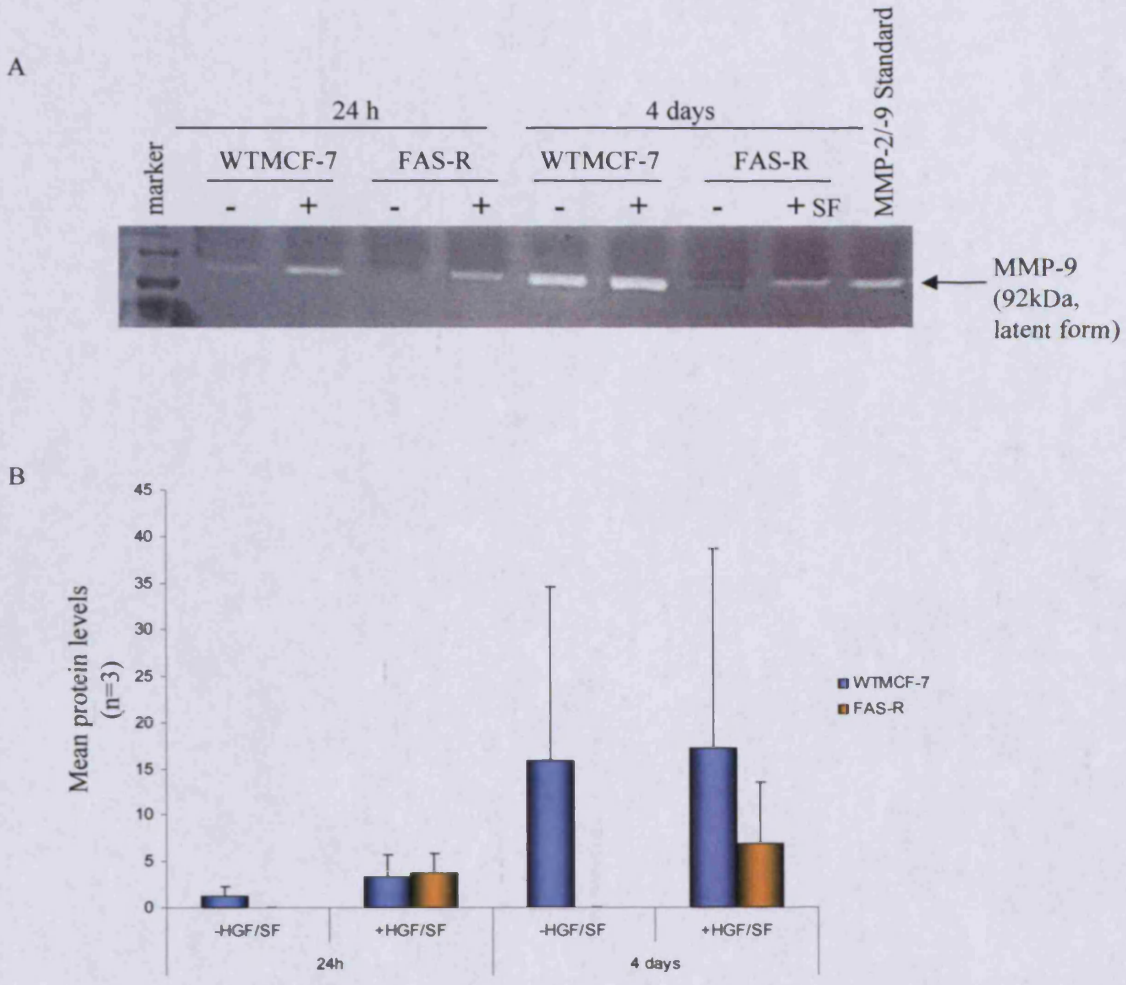


Figure 4.18 MMP-2 and MMP-9 protein expression levels of WTMCF-7 and FAS-R cells following HGF/SF challenge as determined by zymography.
Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) for 24h and 4 days respectively. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7 to determine the effect of HGF/SF challenge on MMP-2 and MMP-9 protein expression. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels ± S.D. (n=3) (B). A representative image is shown above (A).

signal. However, as **figure 4.19** illustrates, MMP-9 protein was only visible in the TPA-treated TAM/TKI-R sample and in the MMP-2/MMP-9 standard (A), whilst MMP-2 was only visible in the standard (B) and MMP-1 was not detected at all (C). It was therefore concluded that the commercially available antibodies were unsuitable for MMP detection in our model systems and Western Blot analysis of MMP expression was accordingly discontinued. Casein zymography for the expression analysis of MMP-1 protein was performed but the enzyme could not be detected, suggesting MMP-1 protein levels to be either just below detection threshold or absent altogether.

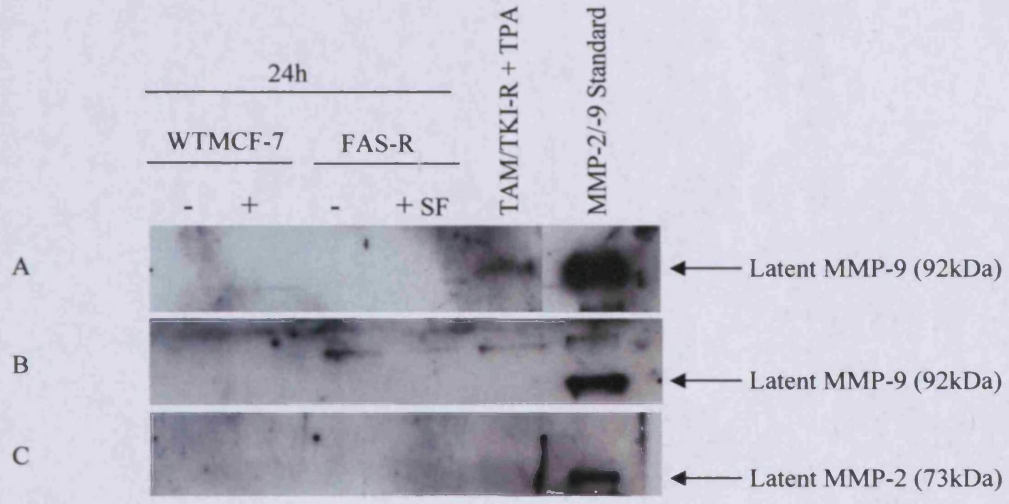


Figure 4.19 MMP-2 and MMP-9 protein expression levels of WTMCF-7 and FAS-R cells as determined by Western Blotting.
Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) for 24h. Serum-starved TAM/TKI cells were treated with TPA (100nM) for 24h. Cell-conditioned supernatants were collected and processed as described in section 2.6.2 and then subjected to SDS-PAGE analysis as outlined in section 2.6 and probed with at least 2 different antibodies specific for MMP-9, MMP-2 and MMP-1 respectively. A commercial MMP-2/-9 standard was run alongside test samples as an internal assay control. To improve chances of MMP-2 and-9 detection, cell-conditioned supernatants were concentrated following collection (B and C). As can be seen latent MMP-9 was only detected in the TPA-treated TAM/TKI-R cells and standard (A and B); latent MMP-2 only in the standard (C).

Chapter Five

Results

Regulation of HGF/SF-induced MMP Expression and its Role in the aggressive Phenotype of FAS-R Cells

"If you're not part of the solution, you're part of the precipitate."

Henry J. Tillman

pathways by many investigators (Jinnin *et al.*, 2005, Tanimura *et al.*, 2003, Tanimura *et al.*, 2002, Kermorgant *et al.*, 2001, Zeigler *et al.*, 1999, Zhang *et al.*, 2004). The aim of the present study therefore was to establish whether ERK1/2 and PI3K signalling play a role in HGF/SF-mediated MMP-1/-9 and -10 expression and in the aggressive behaviour of FAS-R cells.

5.2 Results

5.2.1 The Role of ERK1/2 signalling in HGF/SF-mediated MMP Expression and in the aggressive Phenotype of FAS-R Cells

In order to establish whether c-Met signals through the ERK1/2 pathway in our cell model as hypothesised, ERK1/2 phosphorylation levels following stimulation with HGF/SF were measured by Western Blotting. Subconfluent serum-starved WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) for 5 minutes and subsequently immunoprobed for phospho-ERK1/2. As can be seen in **figure 5.1**, phospho-ERK1/2 expression was markedly increased in FAS-R cells, proving ERK1/2 to be a target of c-Met in these cells; no noticeable increase was seen in WTMCF-7 cells.

To be able to identify a possible role for ERK1/2 signalling in HGF-mediated MMP expression and in the adverse FAS-R cell phenotype, ERK1/2 signalling was blocked using the pharmacological MEK-1 inhibitor PD98059. The effectiveness and use of this compound is well documented in the literature and has also been demonstrated in in-house studies (Knowlden *et al.*, 2003). A dose response in which WTMCF-7 and FAS-R cells were treated with a range of doses of PD98059 (5, 10, 30 and 50μM) for 24h was performed in order to determine the optimum dose for ERK1/2 inhibition (**figure 5.2**). Optimum inhibition without cytotoxic effects to both cell models was achieved at 50μM; all further experiments with PD98059 were hence carried out at this concentration. In contrast to **figure 5.1**, ERK phosphorylation was detected in basal FAS-R cells. This apparent discrepancy is due to deliberate overexposure which permitted identification of the optimum dose of PD98059 to be used.

5 Regulation of HGF/SF-induced MMP Expression and its Role in the aggressive Phenotype of FAS-R Cells

5.1 Introduction and Aims

The pivotal role of MMP proteolytic activity in *in vitro* and *in vivo* tumour cell invasion has long been acknowledged. Equally well established is the role of aberrant HGF/SF-c-Met signalling in both the acquisition of an aggressive cancer cell phenotype *in vitro* and in promoting tumour progression and metastatic disease in solid tumours *in vivo*. Moreover, many *in vitro* studies have established a clear link between MMPs and c-Met signalling, whereby HGF/SF-induced protease activity through matrix degradation and facilitation of invasion promotes the adverse cell phenotype associated with c-Met receptor overexpression. As the findings of this project have shown, the same mechanism appears to be operative in FAS-R cells.

As the data presented so far illustrates, FAS-R cells display an aggressive phenotype and express MMPs which are thought to contribute to the aforementioned adverse behaviour. Furthermore, these resistant cells display c-Met receptor overexpression and an enhanced sensitivity to exogenous HGF/SF which promotes colony dispersion and invasion. Moreover, HGF/SF was shown to promote MMP-1, MMP-9 and MMP-10 expression, whilst HGF/SF-enhanced invasive capability was reduced following inhibition of proteolytic activity of MMPs using a broad-spectrum inhibitor.

Having established that MMPs contribute to the augmented invasive behaviour of FAS-R cells, the aim was to clarify the molecular mechanisms underlying the regulation of MMP expression by HGF/SF by identifying the signalling components which control MMP expression downstream of c-Met.

Whilst the precise signalling pathways downstream of c-Met that mediate each of the diverse biological effects of HGF/SF remain somewhat obscure, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phosphoinositide-3 kinase (PI3K) have been demonstrated to be the two main target candidate

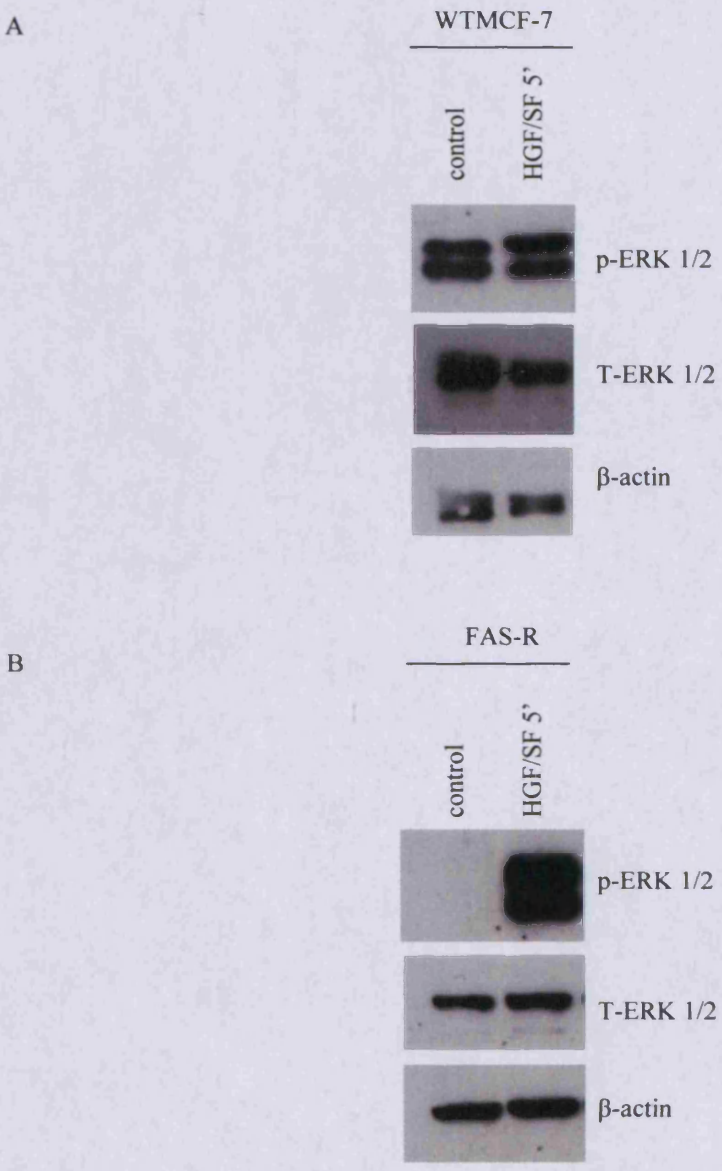


Figure 5.1 HGF/SF enhances ERK1/2 signalling in WTMCF-7 and FAS-R cells. Serum-starved subconfluent WTMCF-7 (A) and FAS-R cells (B) were treated with HGF/SF (20ng/ml) for 5 minutes. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-ERK 1/2 and total ERK1/2 as outlined in section 2.6. HGF/SF stimulation resulted in enhanced ERK1/2 in FAS-R cells (B).

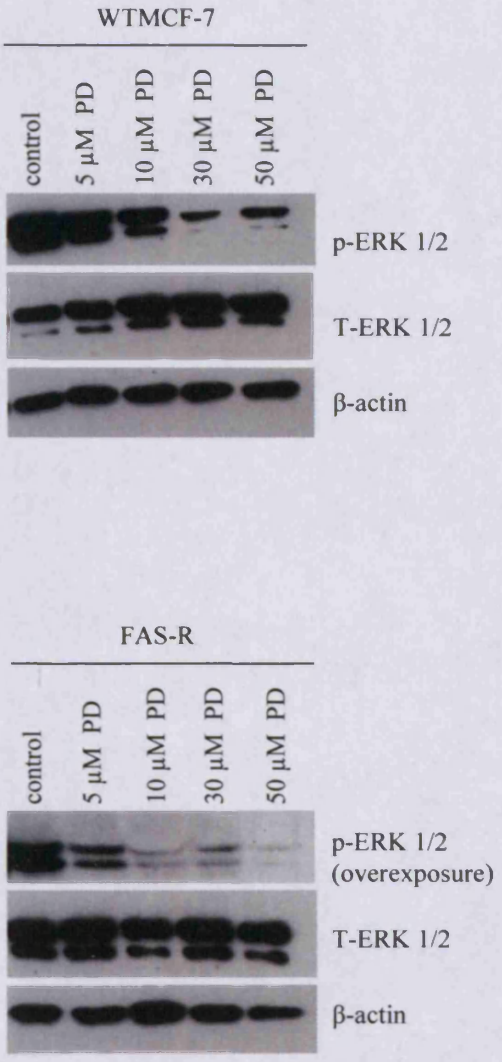


Figure 5.2 PD98059 dose response to establish optimum dose for ERK 1/2 inhibition in WTMCF-7 and FAS-R cells.
Serum-starved subconfluent WTMCF-7 (A) and FAS-R cells (B) were treated with a range of doses of PD98059 (5, 10, 30 and 50μM) for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-ERK 1/2 and total ERK1/2. As can be seen, the optimum dose for ERK 1/2 inhibition was established to be 50μM.

Next, a time course of WTMCF-7 and FAS-R cells ± PD98059 ± HGF/SF was performed to examine the stimulatory effect of HGF/SF on ERK1/2 phosphorylation and the inhibitory effect of PD98059 on HGF/SF-enhanced ERK1/2 signalling over time and to identify the optimum time point to study these events (**figure 5.3**). With the exception of the control sample and the sample treated with PD98059 alone, cells were either treated with only HGF/SF for 5, 30, 60 minutes and 24h or pre-treated with PD98059 for 30 minutes before additionally being stimulated with HGF/SF for the above lengths of time. Samples were then immediately lysed and subsequently immunoprobed for phospho-ERK1/2. As can be seen in **figure 5.3**, HGF/SF-enhanced ERK1/2 phosphorylation in FAS-R cells was sustained over 24h. Moreover, inhibition of this phosphorylation by PD98059 was most pronounced at 24h; further Western Blot analysis was therefore carried out at this time point.

Following these optimisation studies, WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± PD98059 (50μM) were grown in parallel for 24h for MMP gene and protein expression analysis by RT-PCR and zymography, respectively, and for ERK 1/2 and AKT phosphorylation level determination by Western Blotting.

As observed during the optimisation studies, HGF/SF treatment resulted in increased ERK1/2 phosphorylation in FAS-R cells (**figure 5.4**) which was effectively blocked by PD98059, re-confirming ERK1/2 to be a target of c-Met. As before, ERK1/2 phosphorylation levels in WTMCF-7 cells were overall higher compared to FAS-R cells.

Lysates were simultaneously immunoprobed for phospho-AKT to ensure that treatment with PD98059 did not interfere with the PI3K/AKT signalling pathway. As can be seen in **figure 5.5**, AKT phosphorylation levels of PD98059-treated samples were unchanged for both WTMCF-7 and FAS-R cells.

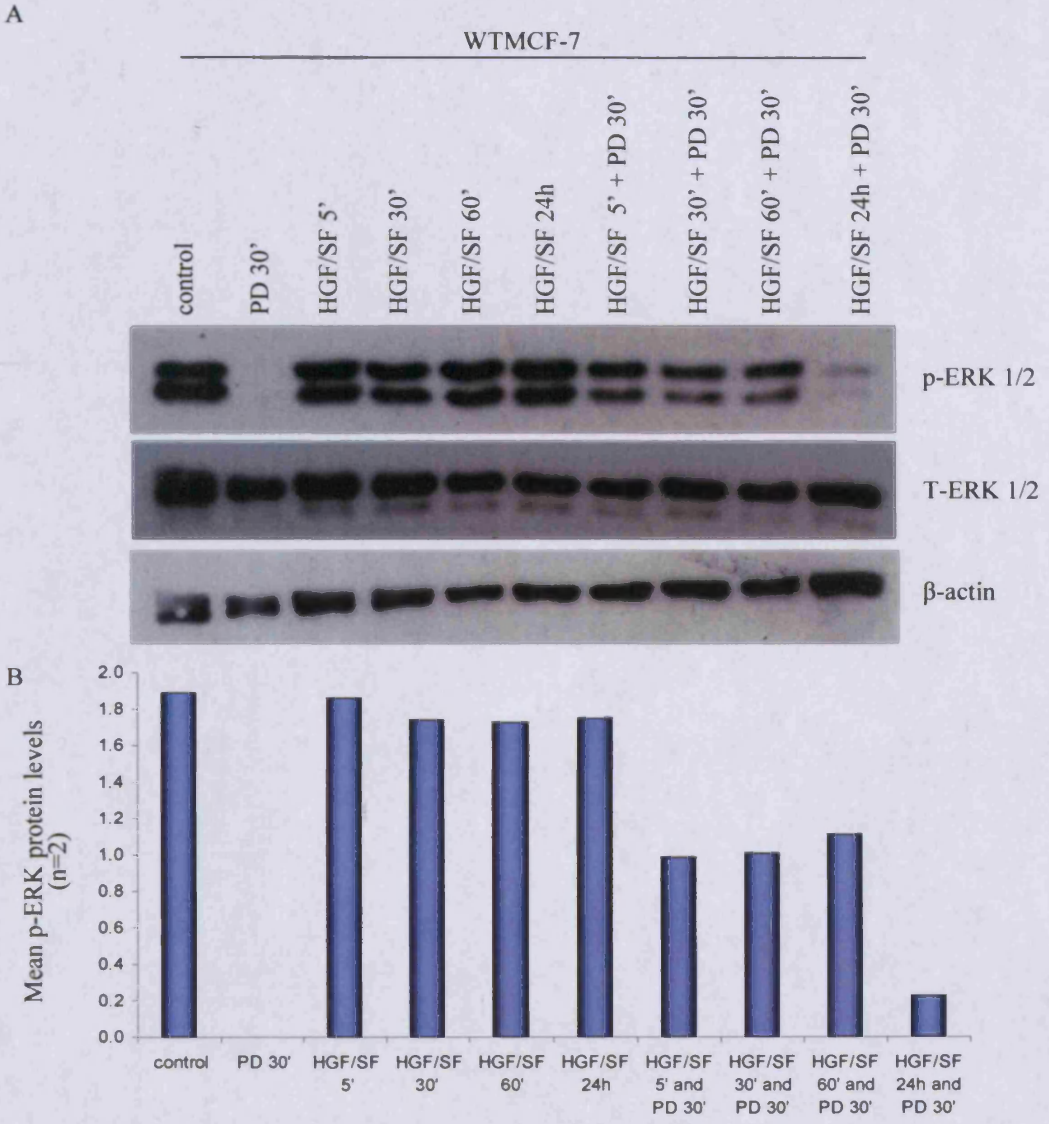
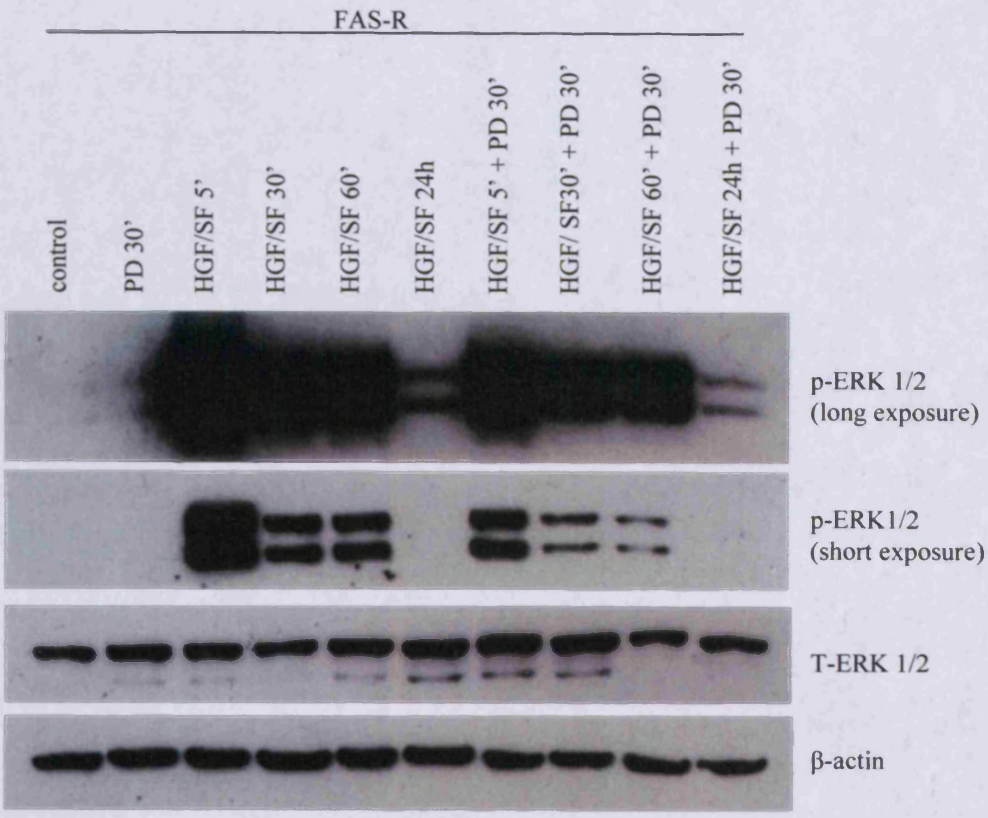


Figure 5.3 Time course with HGF/SF and PD98059 in WTMCF-7 and FAS-R cells. Subconfluent WTMCF-7 and FAS-R cells were serum-starved 24h prior to treatments. Out of 10 dishes, one set of 5 dishes was pre-treated with PD98059 (50μM) for 30 minutes with the exception of the control sample. Following this pre-treatment the remaining 4 dishes were treated with HGF/SF (20ng/ml) for 5, 30, 60 minutes and 24h respectively. With the exception of another control sample the second set of 5 dishes was also treated with HGF/SF (20ng/ml) for 5, 30, 60 minutes and 24h respectively. At the end of each time point, cell lysates were obtained immediately which were then subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-ERK 1/2 and total ERK 1/2 as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels (n=2) (B and D). Representative images (A and C) are shown above and on the next page.

C



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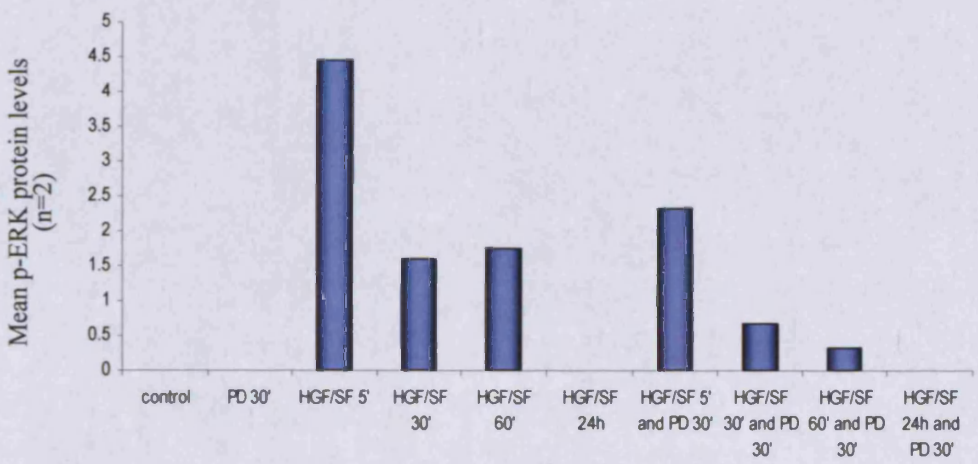

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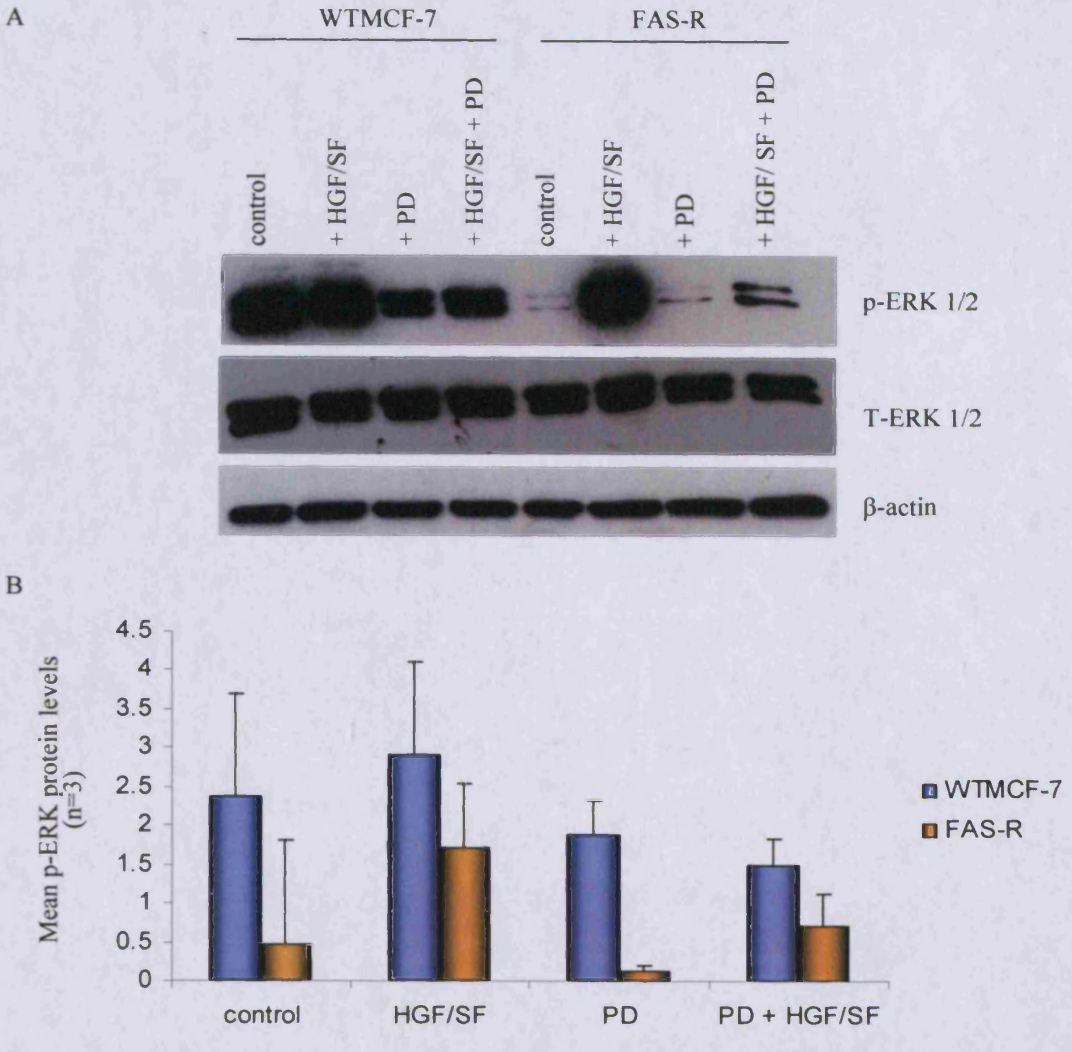


Figure 5.4 The effect of PD98059 on HGF/SF-mediated ERK 1/2 phosphorylation in WTMCF-7 and FAS-R cells as determined by Western Blotting.
Serum-starved subconfluent WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± PD98059 (50μM) were incubated for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-ERK 1/2 and total ERK 1/2 as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels ± S.D. (n=3) (B). Representative images are shown above (A). As can be seen PD98059 decreased HGF/SF-modulated ERK 1/2 phosphorylation in FAS-R cells.

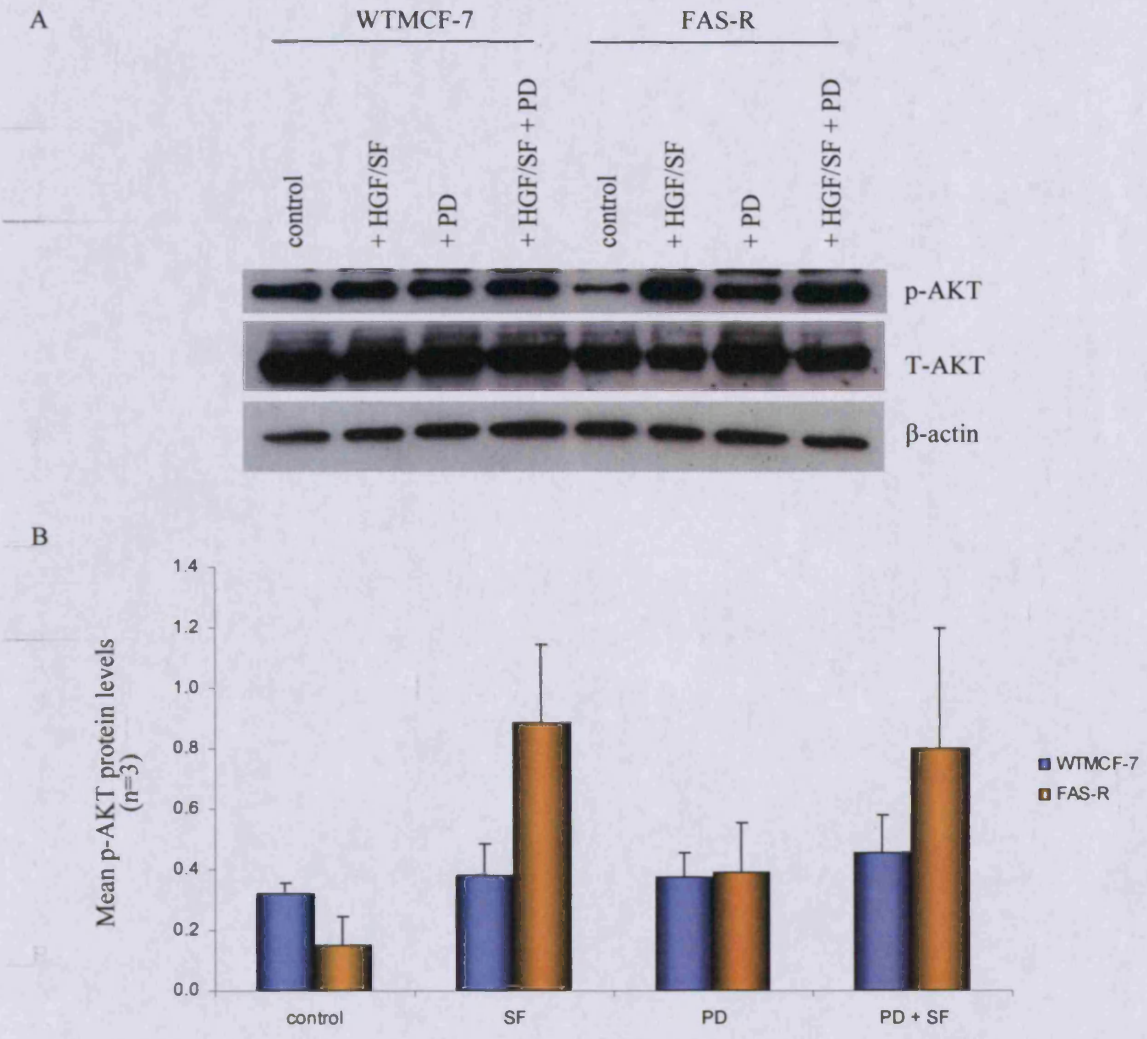


Figure 5.5 Effect of PD98059 on AKT phosphorylation in WTMCF-7 and FAS-R cells as determined by Western Blotting.

Serum-starved subconfluent WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± PD98059 (50μM) were incubated for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-AKT and total AKT as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels ± S.D. ((n=3) (B). Representative images are shown above (A). As can be seen, PD98059 did not affect AKT phosphorylation levels.

Next, RT-PCR was employed to reveal the effect of ERK1/2 inhibition upon HGF/SF- controlled MMP gene expression. As before, HGF/SF induced or enhanced MMP-1, MMP-9 and MMP-10 expression in FAS-R cells (**figure 5.6**). Interestingly, PD98059 treatment reduced HGF/SF-mediated MMP expression for all three genes, confirming the assumption that the HGF/SF/c-Met complex regulates MMP-1, MMP-9 and MMP-10 expression via ERK1/2. Zymographic analysis revealed PD98059 treatment to distinctly decrease MMP-9 protein secretion (**figure 5.7**), further confirming the role ERK1/2 in HGF/SF-mediated MMP expression. As before, HGF/SF markedly upregulated MMP-9 protein secretion, corroborating earlier data.

In order to establish whether ERK1/2 signalling was also implicated in the adverse phenotype of FAS-R cells, scattering, migration and invasion assays were performed in the presence of PD98059. As observed before, HGF/SF greatly enhanced the motile, migrative and invasive capabilities of FAS-R cells (**figures 5.8; 5.9** and **5.10**); this again validated earlier findings. Importantly, ERK1/2 inhibition dramatically reduced HGF/SF-enhanced scattering (**figure 5.8**) and migration rates (**figure 5.9**) in FAS-R cells, suggesting a role for ERK1/2 signalling in the motile behaviour of these cells. Invasion assays, however, revealed an increase in HGF/SF-augmented invasion following PD98059 treatment (**figure 5.10**); leaving the involvement of ERK1/2 in the FAS-R invasive cell phenotype inconclusive.

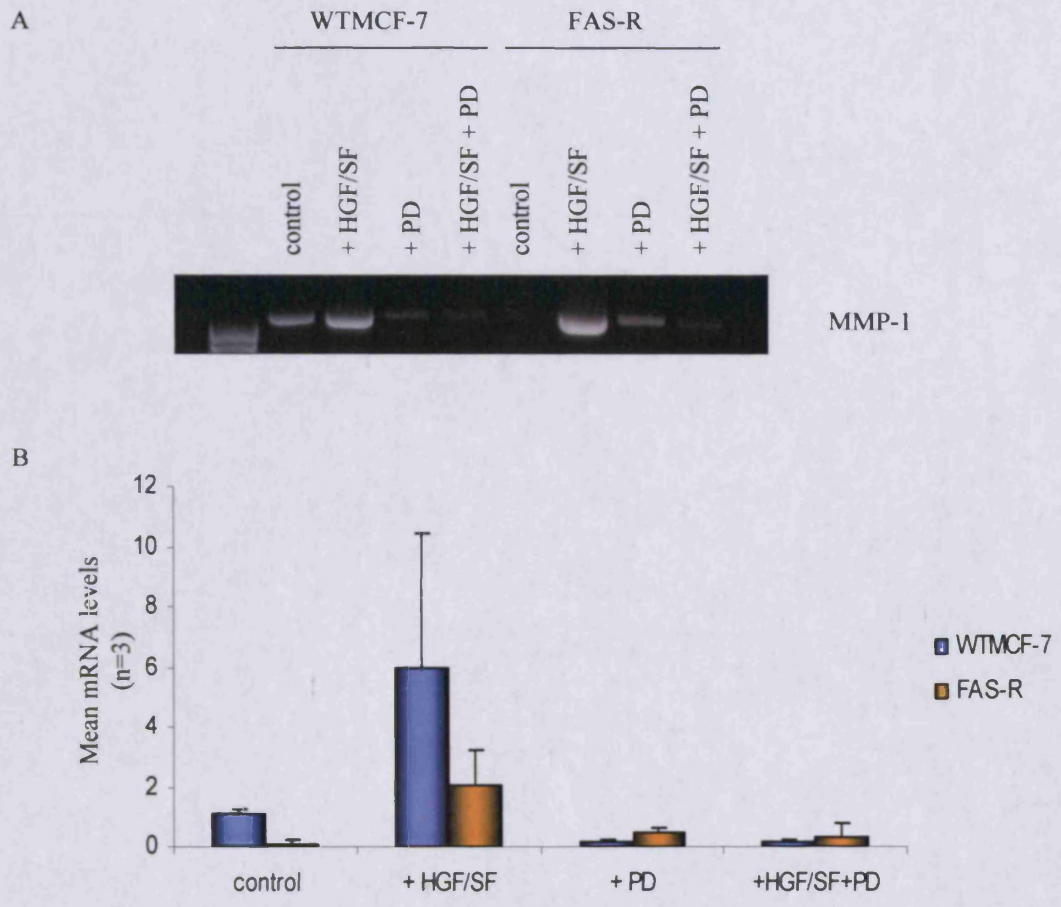
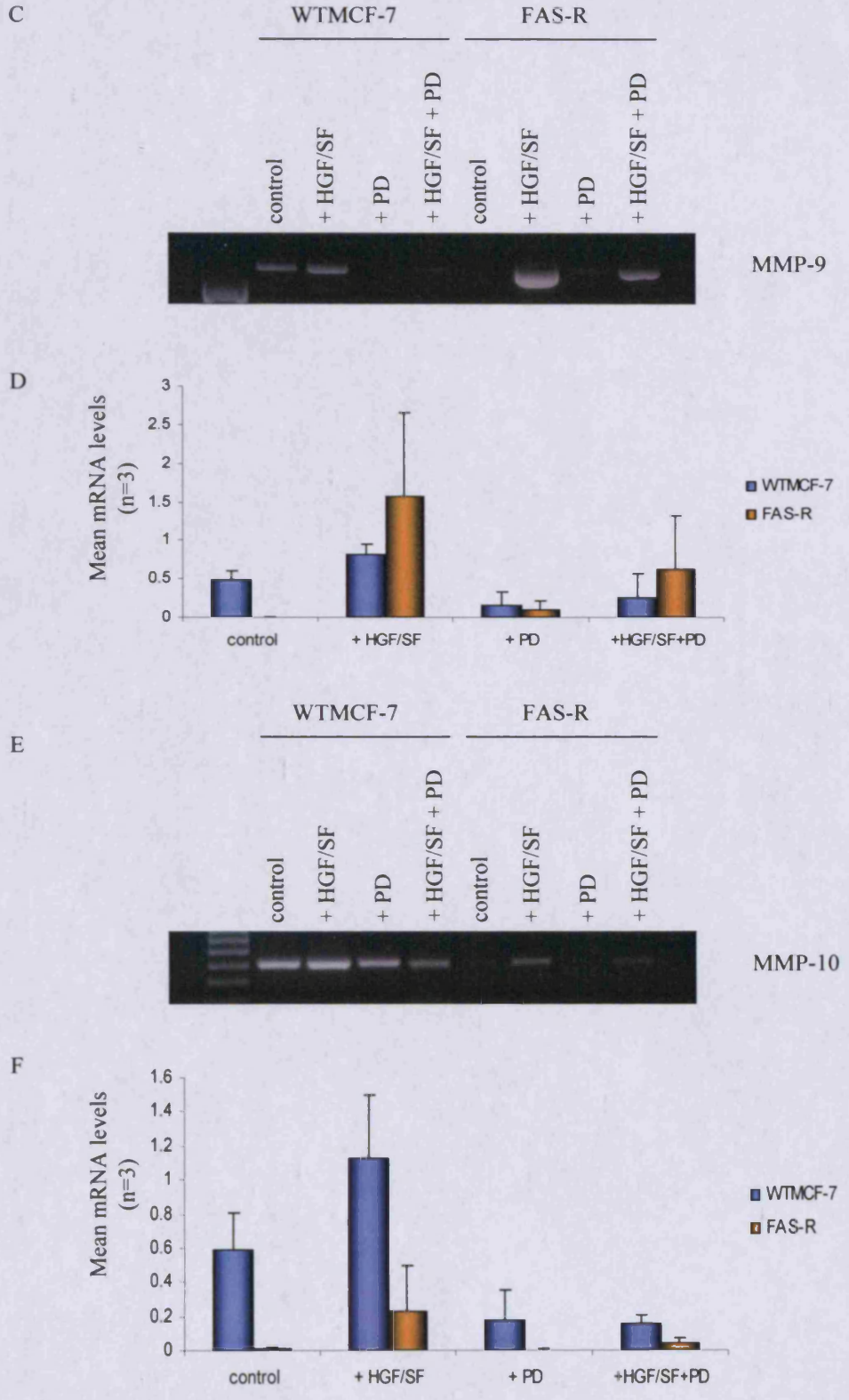


Figure 5.6 Effect of ERK 1/2 inhibition on HGF/SF-modulated MMP mRNA expression levels of WTMCF-7 and FAS-R cells as determined by RT-PCR.
ERK 1/2 inhibition was achieved using the inhibitor PD98059 (50μM). As PD98059 was solubilised in DMSO, a DMSO control was also included. Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) ± PD98059 for 24h. The effect of ERK1/2 inhibition on HGF/SF-modulated MMP-1, MMP-9 and MMP-10 expression was determined using gene expression analysis as outlined in section 2.5. Data was normalised using β-actin and presented as mean expression levels ± S.D. (n=3) (B, D and F). Representative images are shown above and on following pages (A, C, E and G).



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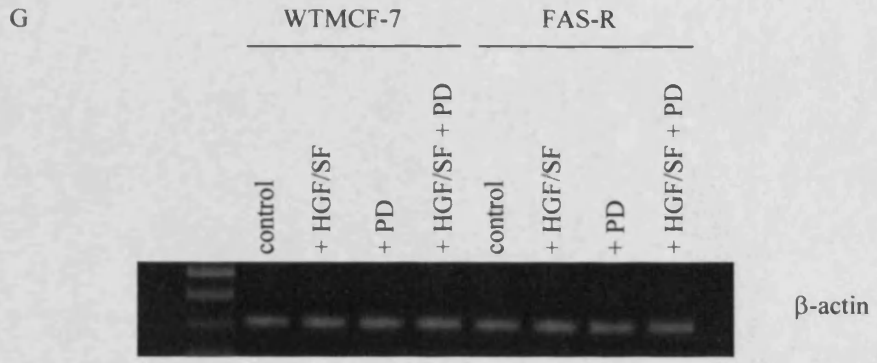

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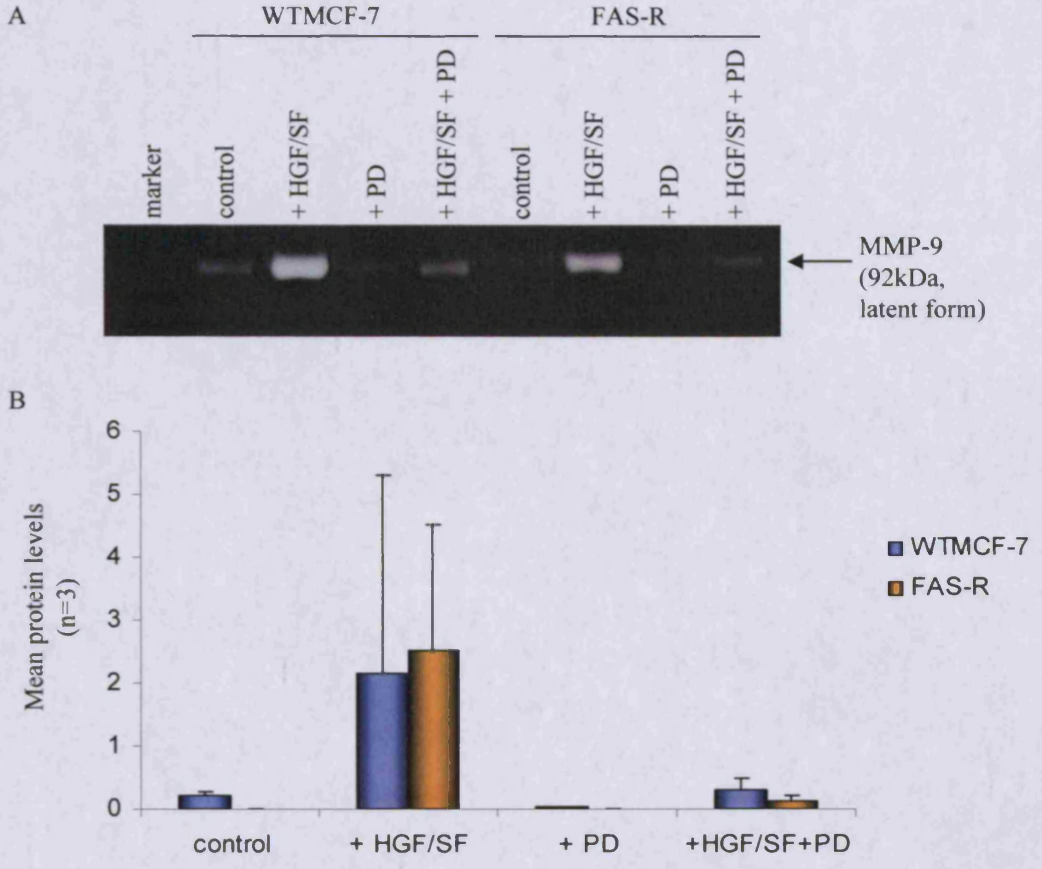


Figure 5.7 Effect of ERK 1/2 inhibition on HGF/SF-modulated MMP-9 protein expression levels of WTMCF-7 and FAS-R cells as determined by zymography. ERK 1/2 inhibition was achieved using the inhibitor PD98059 (50μM). Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) ± PD98059 for 24h. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7 to determine the effect of ERK 1/2 inhibition on HGF/SF-modulated MMP-9 protein expression. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels ± S.D. (n=3) (B). A representative image is shown above (A).

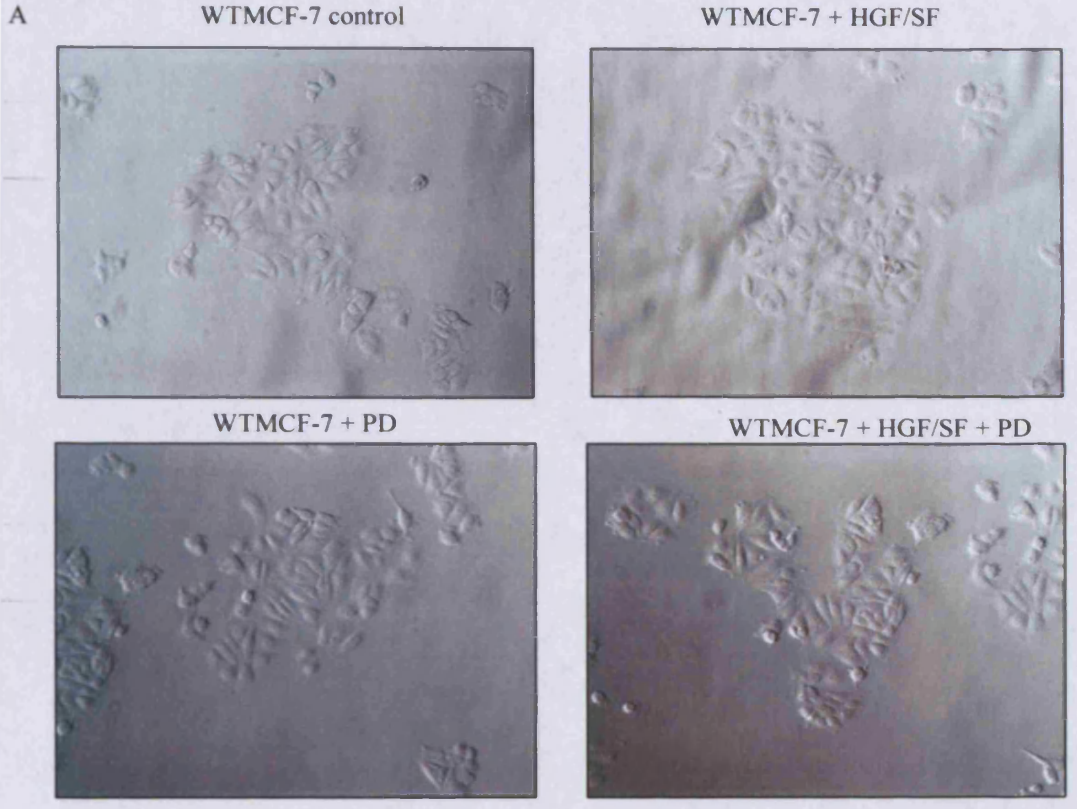
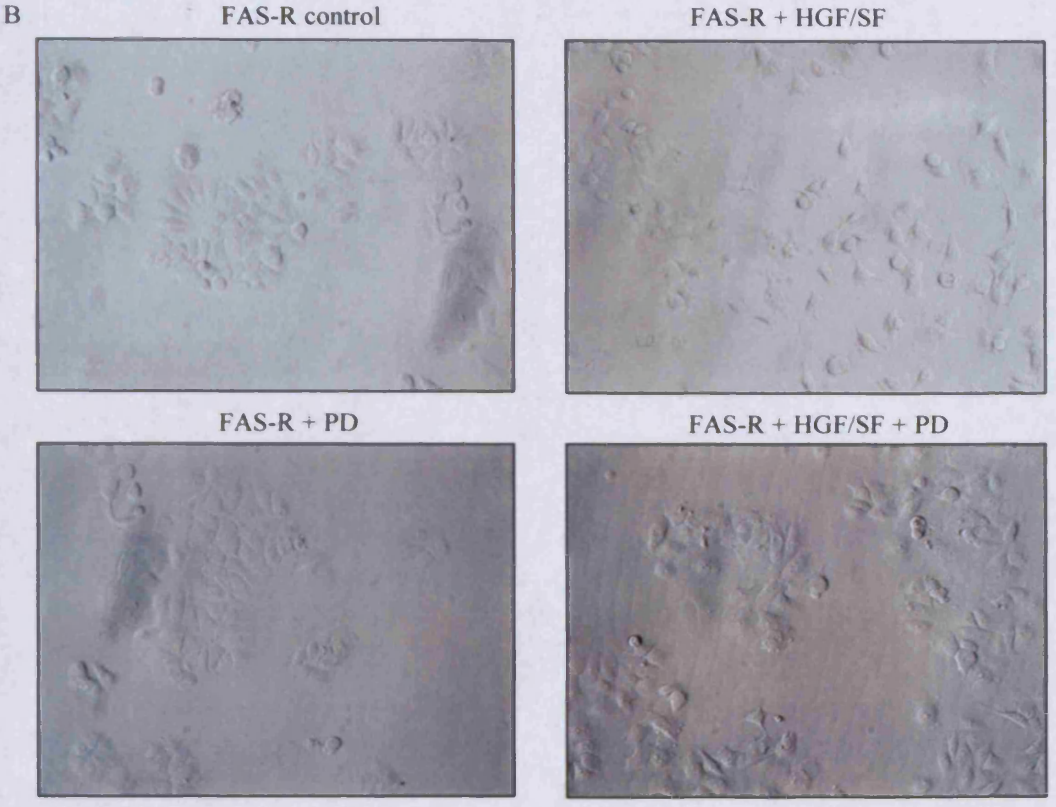


Figure 5.8 The effect of ERK 1/2 inhibition on HGF/SF-induced colony dispersion in WTMCF-7 and FAS-R cells as determined by scatter assays.
ERK 1/2 inhibition was achieved using PD98059 (50μM). WTMCF-7 (A) and FAS-R cells (B) were seeded in EM ± Faslodex (10^{-7}M) and allowed to form discrete colonies prior to 24h treatment with HGF/SF (20ng/ml) ± PD98059 (50μM) in DCCM. The extent to which ERK 1/2 inhibition reduced HGF/SF-induced colony dispersion was determined using cell scatter assays as described in section 2.10. Scatter response was assessed visually and selected colonies were photographed using a Leica DM-IRE2 inverted microscope; representative images are shown above and on the following page. As can be seen ERK 1/2 inhibition reduced HGF/SF-induced colony dispersion in FAS-R cells (B).



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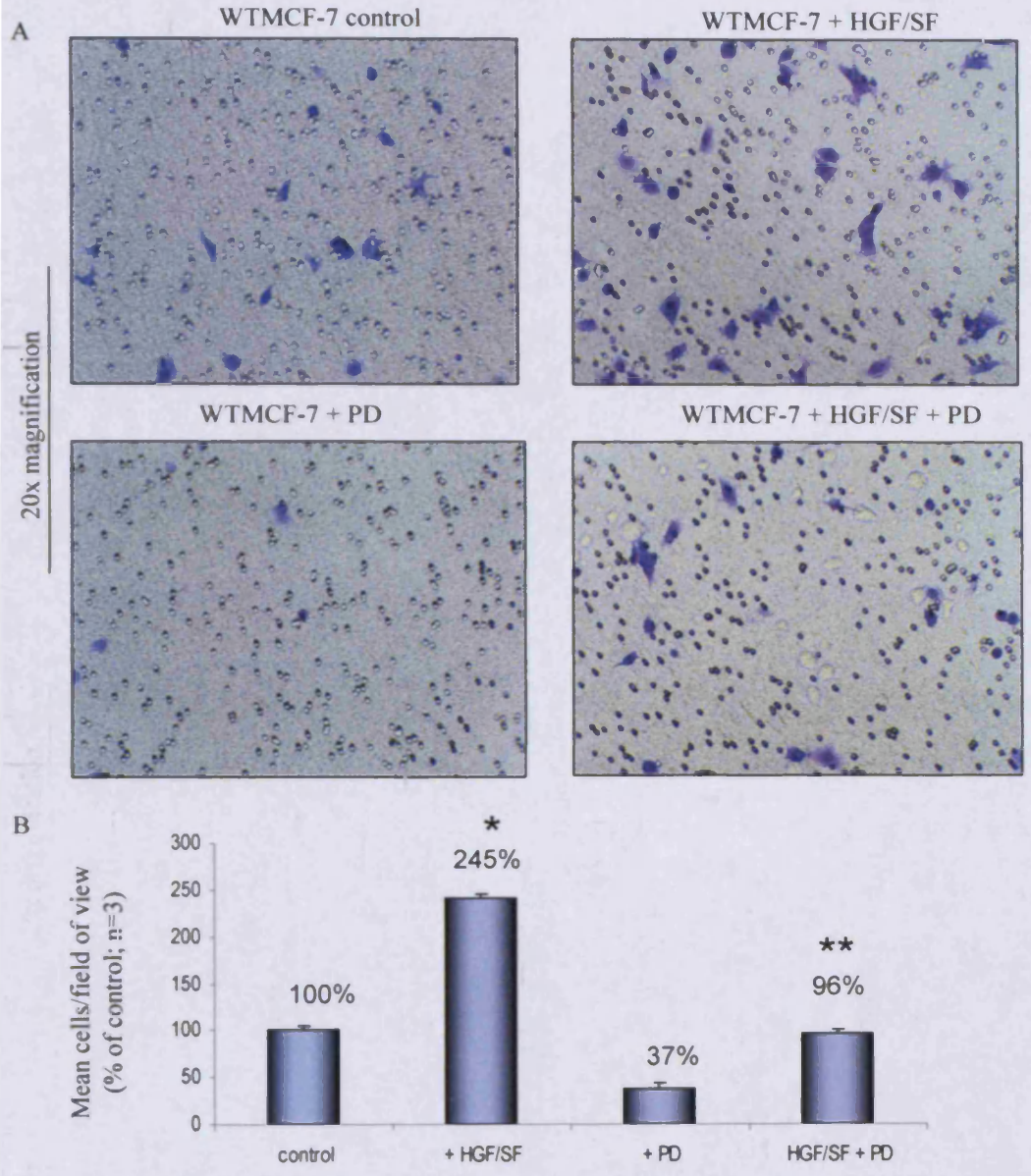
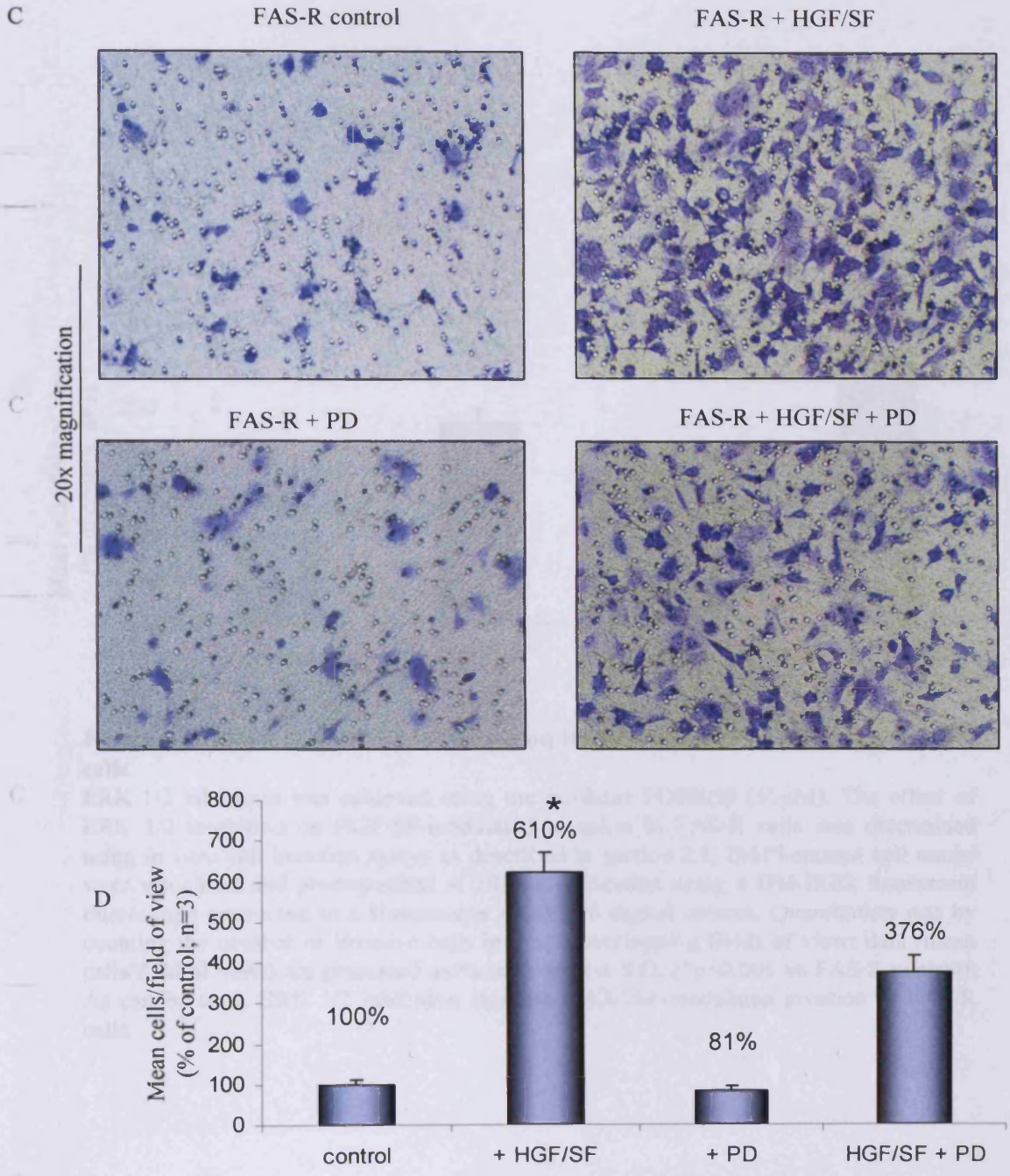


Figure 5.9 Effect of ERK 1/2 inhibition on HGF/SF-modulated migration of WTMCF-7 and FAS-R cells.

ERK 1/2 inhibition was achieved using the inhibitor PD98059 (50μM). The effect of ERK 1/2 inhibition on HGF/SF-modulated migration of WTMCF-7 and FAS-R cells was determined using *in vitro* cell migration assays as described in section 2.9. Crystal-violet-stained cells were photographed at 20x magnification using an Olympus BH-2 phase contrast microscope; representative images are shown above and on the following page (A and C). Quantification was by counting the number of migratory cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of basal WTMCF-7 ± S.D (*$p<0.001$ vs. WTMCF-7 control **$p<0.001$ vs. WTMCF-7 + HGF/SF) and as % of basal FAS-R ± S.D (*$p<0.005$ vs. FAS-R control) (B and D). As can be seen ERK 1/2 inhibition significantly reduced HGF/SF-modulated migration of FAS-R cells (C and D).



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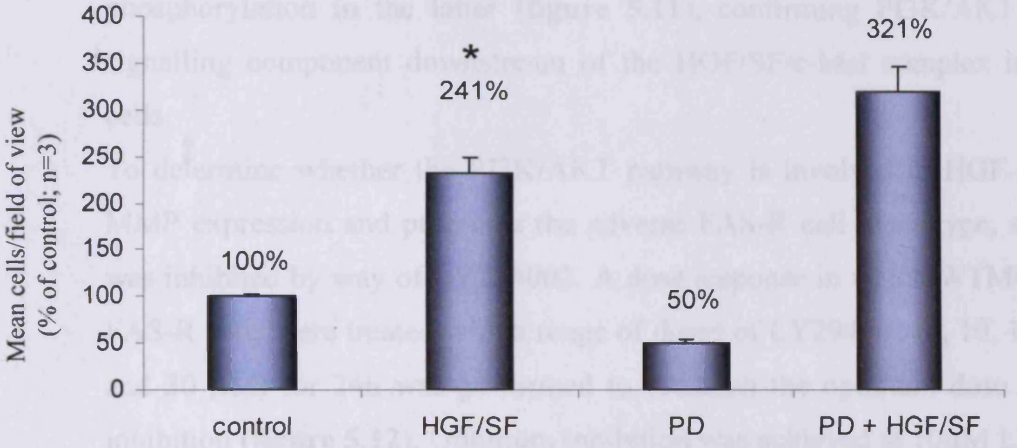


Figure 5.10 Effect of ERK 1/2 inhibition on HGF/SF-modulated invasion of FAS-R cells.
ERK 1/2 inhibition was achieved using the inhibitor PD98059 (50μM). The effect of ERK 1/2 inhibition on HGF/SF-modulated invasion in FAS-R cells was determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera. Quantitation was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of control ± S.D. (*$p<0.001$ vs FAS-R control). As can be seen, ERK 1/2 inhibition increased HGF/SF-modulated invasion of FAS-R cells.

5.2.2 The Role of PI3K/AKT signalling in HGF/SF-mediated MMP Expression and in the aggressive Phenotype of FAS-R Cells

In order to be able to implicate PI3K/AKT signalling in the above processes, the same experiments as described in section 5.2.1 were performed with the AKT-specific inhibitor LY294002, the effectiveness of which is widely acknowledged and has been established in previous in-house studies (Hutcheson *et al.*, 2007, Jordan *et al.*, 2004).

Assessment of AKT protein levels by Western Blotting following HGF/SF stimulation of WTMCF-7 and FAS-R cells revealed a marked increase in AKT phosphorylation in the latter (**figure 5.11**), confirming PI3K/AKT to be a signalling component downstream of the HGF/SF/c-Met complex in FAS-R cells.

To determine whether the PI3K/AKT pathway is involved in HGF-mediated MMP expression and promotes the adverse FAS-R cell phenotype, signalling was inhibited by way of LY294002. A dose response in which WTMCF-7 and FAS-R cells were treated with a range of doses of LY294002 (5, 10, 15, 20, 25 and 30 μM) for 24h was performed to establish the optimum dose for AKT inhibition (**figure 5.12**). Optimum inhibition was achieved at 10μM LY294002 in FAS-R cells. Moreover, higher concentrations proved cytotoxic to both cell models, hence all further studies were carried out using a dose of 10μM.

The next step was to perform a time course of WTMCF-7 and FAS-R cells ± LY294002 ± HGF/SF to assess the stimulatory effect of HGF/SF on phospho-AKT levels and to determine how LY294002 inhibited HGF/SF-augmented PI3K/AKT signalling over time with the aim of identifying the optimum time point to study these events (**figure 5.13**). Samples were set up as described in section 5.2.1 with LY294002 instead of PD98059. Whilst HGF/SF-enhanced AKT phosphorylation had peaked at 60 minutes in FAS-R cells, the largest increase in WTMCF-7 cells was observed after 24h. LY294002-mediated inhibition of this phosphorylation peaked at 5 minutes but was still visible at 24h in both cell types.

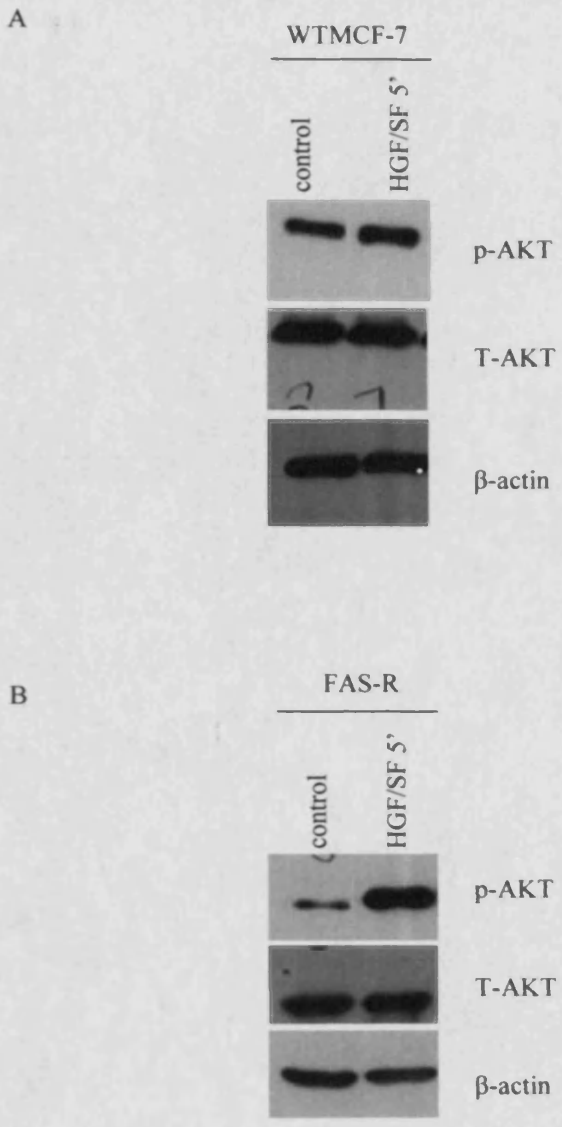


Figure 5.11 HGF/SF enhances AKT signalling in WTMCF-7 and FAS-R cells. Serum-starved subconfluent WTMCF-7 (A) and FAS-R cells (B) were treated HGF/SF (20ng/ml) for 5 minutes. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-AKT and total AKT as outlined in section 2.6. HGF/SF stimulation resulted in enhanced AKT signalling particularly in FAS-R cells.

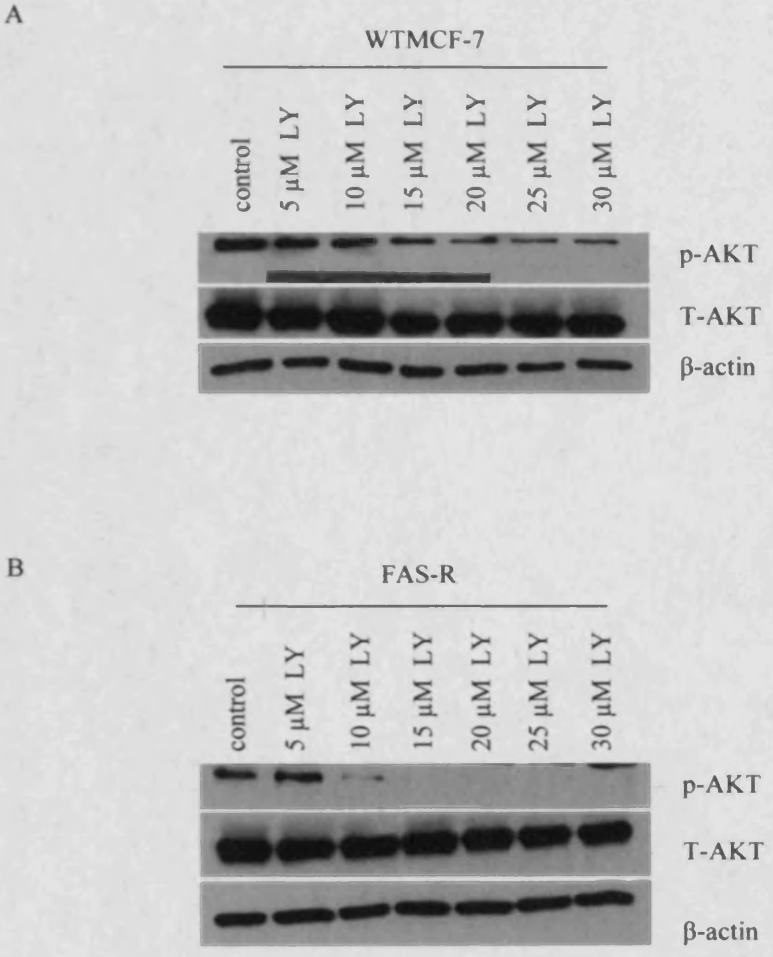


Figure 5.12 LY294002 dose response to establish optimum dose for PI3K inhibition in WTMCF-7 and FAS-R cells.
Serum-starved subconfluent WTMCF-7 (A) and FAS-R cells (B) were treated with a range of doses of LY294002 (5, 10, 15, 20, 25 and 30μM) for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-AKT and total AKT as outlined in section 2.6. As can be seen, the optimum dose for PI3K inhibition was established to be 10μM.

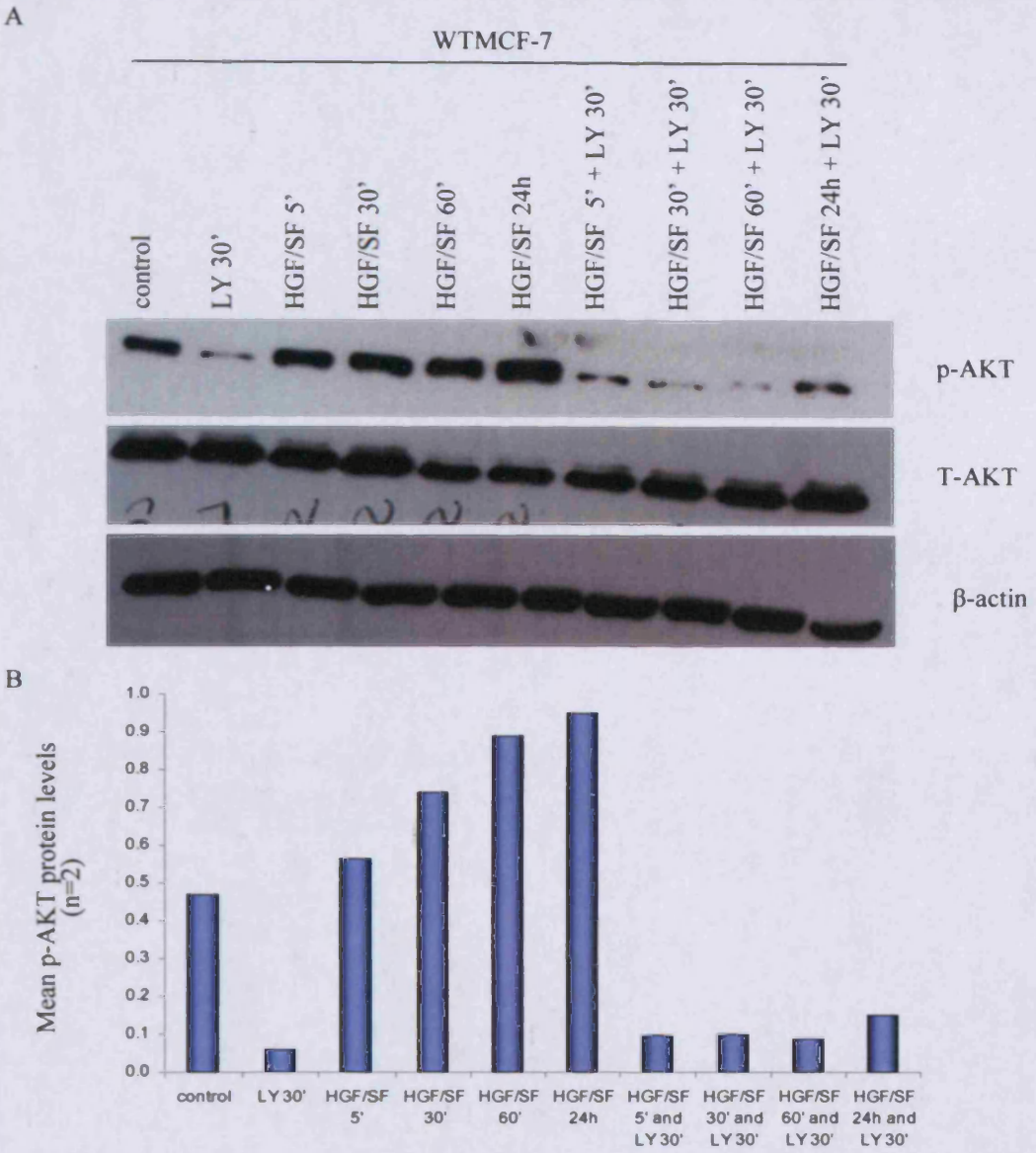
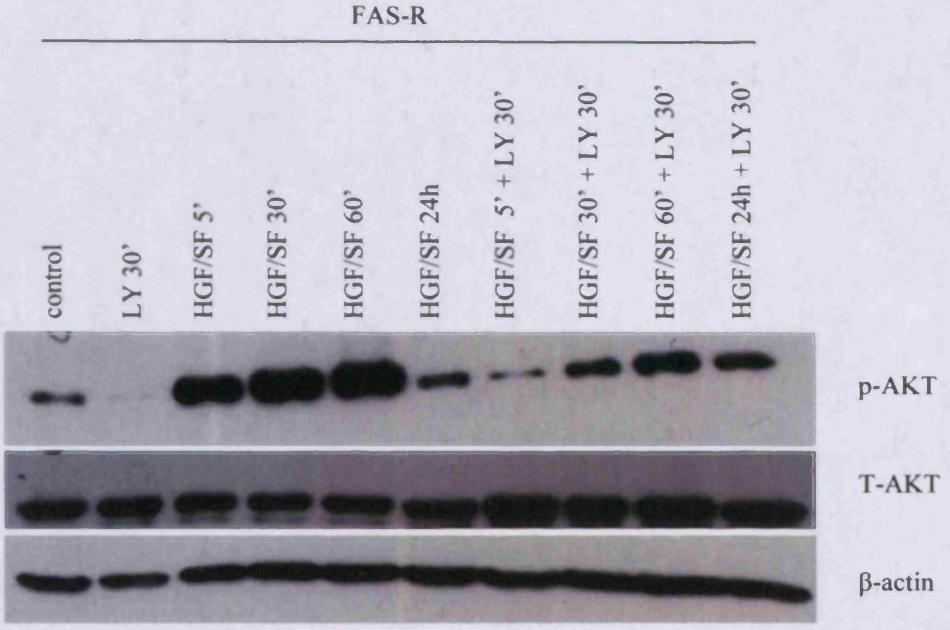


Figure 5.13 Time course with HGF/SF and LY294002 in WTMCF-7 and FAS-R cells.

Subconfluent WTMCF-7 and FAS-R cells were serum-starved 24h prior to treatments. Out of 10 dishes, one set of 5 dishes was pre-treated with LY294002 (10μM) for 30 minutes with the exception of the control sample. Following this pre-treatment the remaining 4 dishes were treated with HGF/SF (20ng/ml) for 5, 30, 60 minutes and 24h respectively. With the exception of another control sample the second set of 5 dishes was also treated with HGF/SF (20ng/ml) for 5, 30, 60 minutes and 24h respectively. At the end of each time point, cell lysates were obtained immediately which were then subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-AKT and total AKT as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels (n=2) (B and D). Representative images are shown above and on the following page (A and C).

C



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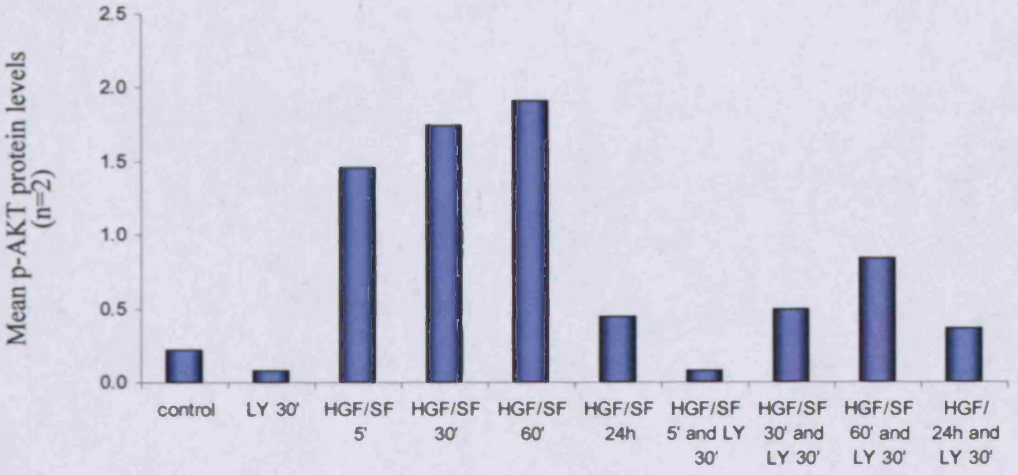


Figure 5.13 continued.

This made it possible to choose the 24h time point for further analysis, which was desirable as it was consistent with the experiments carried out earlier for the ERK1/2 signalling investigation, and also because at a very early time point only insufficient MMP mRNA and protein may have accumulated posing problems for downstream assays.

Upon completion of these preliminary studies, WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± LY294002 (10μM) were grown in parallel for 24h for MMP gene and protein expression analysis by RT-PCR and zymography, respectively, and for AKT and ERK1/2 phosphorylation level determination by Western Blotting.

As observed during the optimisation studies, HGF/SF caused augmented AKT phosphorylation in FAS-R cells (**figure 5.14**) which was effectively inhibited using LY294002; re-confirming PI3K/AKT to be a target of c-Met signalling. As seen before, AKT phosphorylation levels overall were higher in WTMCF-7 cells as compared to FAS-R cells.

To ensure that LY294002 treatment did not impact on ERK1/2 signalling, lysates were simultaneously immunoprobed for phospho-ERK1/2; as expected, ERK1/2 phosphorylation levels were virtually unaltered for both cell models (**figure 5.15**).

RT-PCR was employed to determine the effect of AKT inhibition upon HGF/SF- controlled MMP gene expression. As before, HGF/SF induced or enhanced MMP-1, MMP-9 and MMP-10 expression in FAS-R cells (**figure 5.16**). As expected, LY294002 treatment decreased HGF/SF-augmented MMP-9 gene expression in both cell types. Conversely, LY294002 was seen to increase HGF/SF-augmented MMP-1 and MMP-10 expression in FAS-R cells, suggesting these genes to be negatively controlled by the PI3K/AKT pathway. Overall, however, the data confirmed an involvement of this signalling pathway in HGF/SF/c-Met regulated MMP expression in our faslodex-resistant cell model.

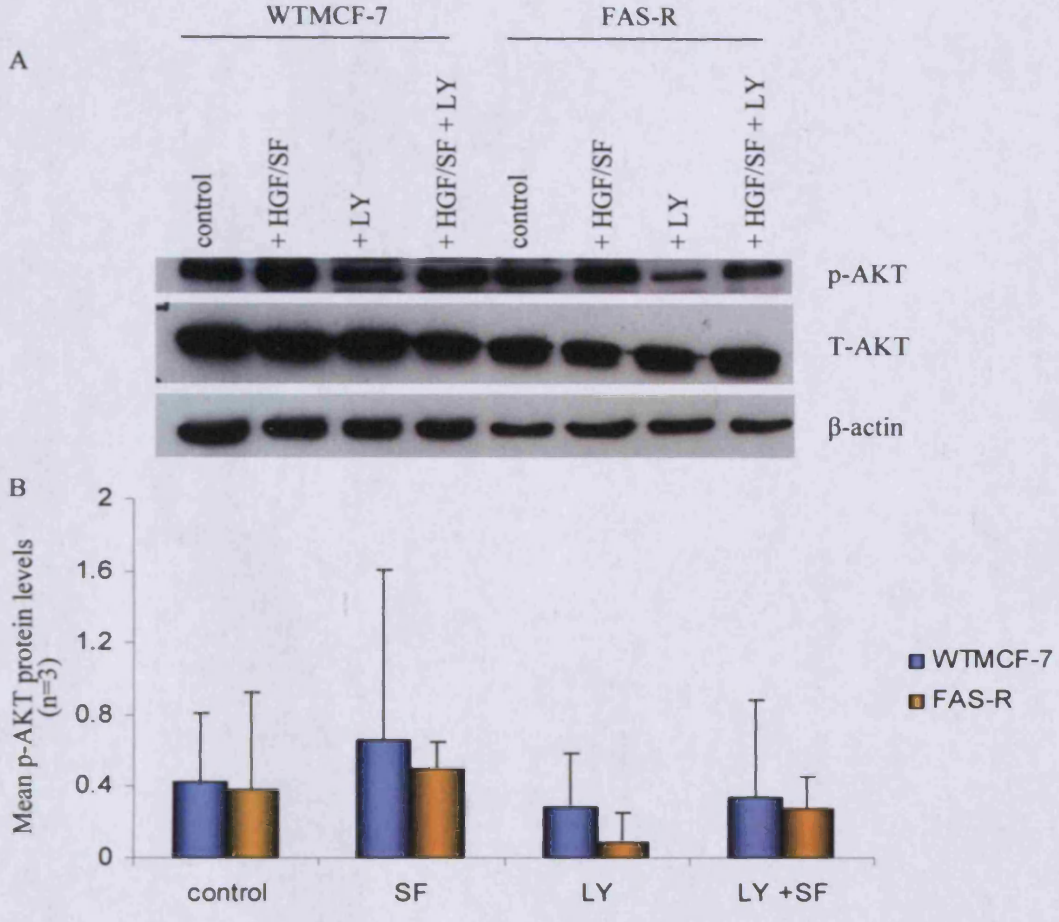


Figure 5.14 The effect of LY294002 on HGF/SF-mediated PI3K phosphorylation in WTMCF-7 and FAS-R cells as determined by Western Blotting.
Serum-starved subconfluent WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± LY294002 (10μM) were incubated for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-AKT and total AKT as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels ± S.D. (n=3) (B). Representative images are shown above (A). As can be seen LY294002 decreased HGF/SF-modulated AKT phosphorylation.

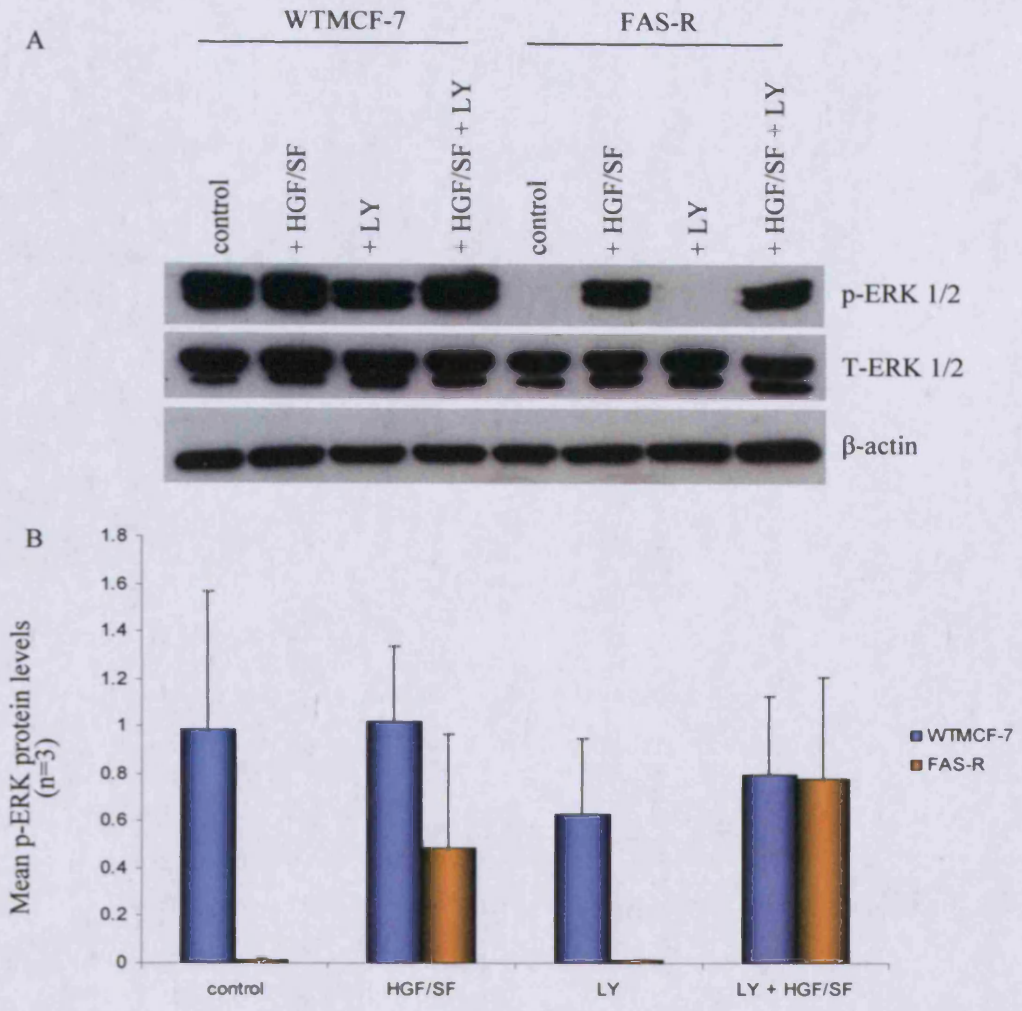


Figure 5.15 Effect of LY294002 on ERK 1/2 phosphorylation in WTMCF-7 and FAS-R cells as determined by Western Blotting.
Serum-starved subconfluent WTMCF-7 and FAS-R cells ± HGF/SF (20ng/ml) ± LY294002 (10μM) were incubated for 24h. Cell lysates were obtained, subjected to SDS-PAGE analysis and probed with antibodies specific for phospho-ERK 1/2 and total ERK as outlined in section 2.6. Densitometry was performed on the resulting signal and all signal values were normalised using β-actin. Data is presented as mean expression levels ± S.D. (n=3) (B). Representative images are shown above (A). As can be seen, LY294002 did not affect ERK 1/2 phosphorylation levels.

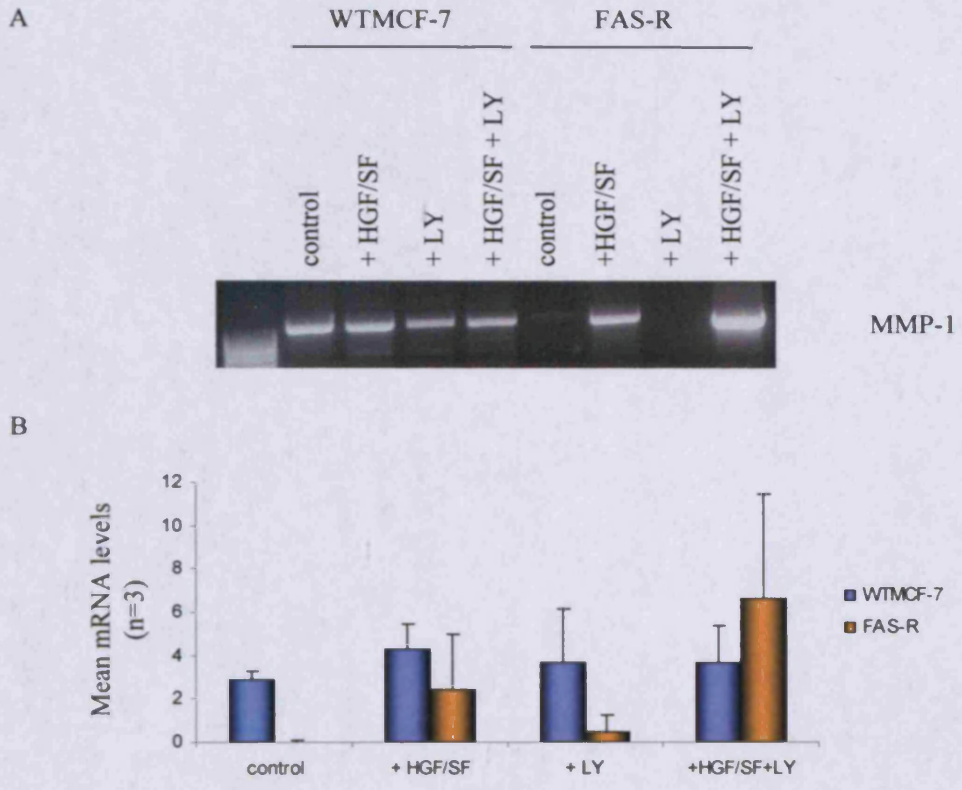
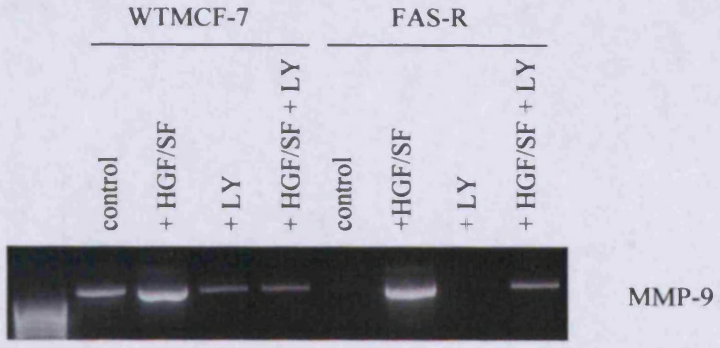


Figure 5.16 Effect of PI3K inhibition on HGF/SF-modulated MMP mRNA expression levels of WTMCF-7 and FAS-R cells as determined by RT-PCR.
PI3K inhibition was achieved using the inhibitor LY294002 (10μM). Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) ± LY294002 for 24h. The effect of PI3K inhibition on HGF/SF-modulated MMP-1, MMP-9 and MMP-10 expression was determined using gene expression analysis as outlined in section 2.5. Data was normalised using β-actin and presented as mean expression levels ± S.D. (n=3) (B, D and F). Representative images are shown above and on following pages (A, C, E and G).

C



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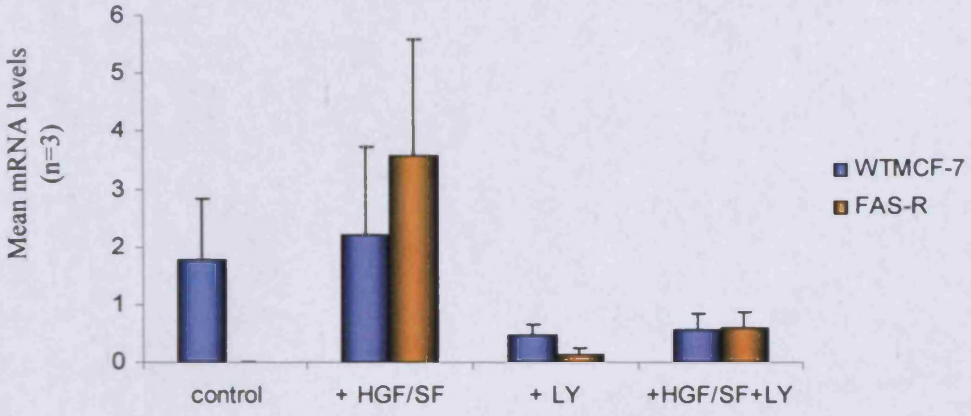

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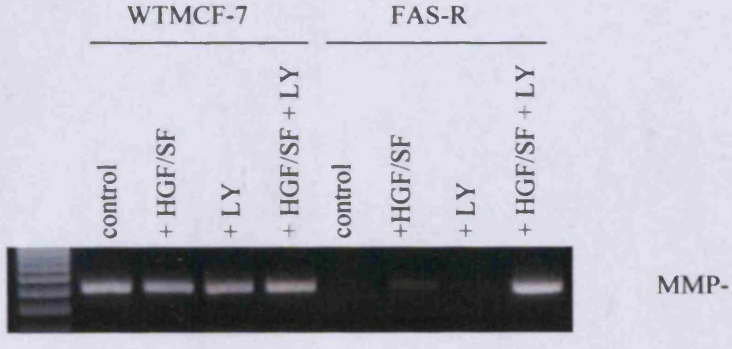
F



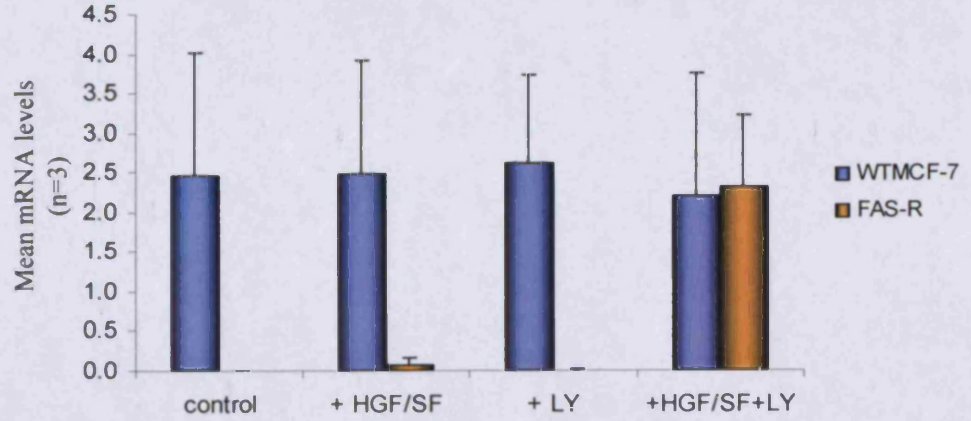


Figure 5.16 continued.

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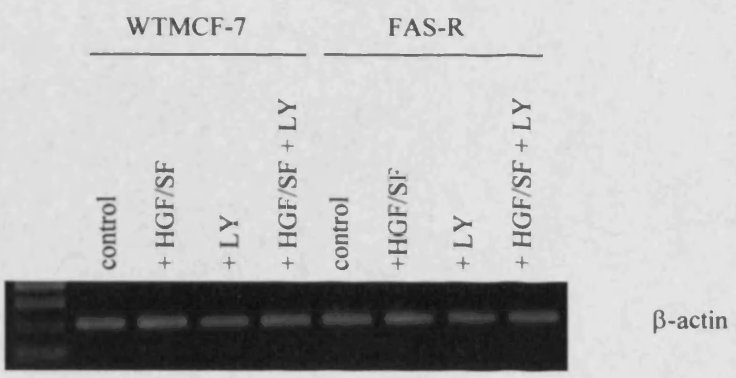


Figure 5.16 continued.

Zymographic analysis demonstrated LY294002 treatment to virtually abolish MMP-9 protein secretion (**figure 5.17**), further validating the role of PI3K/AKT signalling in HGF/SF-mediated MMP expression in FAS-R cells. As before, HGF/SF clearly upregulated MMP-9 protein expression in both cell models, corroborating earlier data.

In order to determine whether PI3K/AKT signalling was also implicated in the adverse phenotype of FAS-R cells, scattering, migration and invasion assays were performed in the presence of LY294002. As seen in earlier assays, HGF/SF greatly enhanced the motile, migrative and invasive capabilities of FAS-R cells (**figures 5.18, 5.19** and **5.20**, respectively) whilst only increasing migration rates in the WTMCF-7 cells and having no impact at all on their motile behaviour (**figures 5.18** and **5.19**); corroborating earlier findings. Excitingly, PI3K/AKT inhibition not only reduced colony dispersion (**figure 5.18**) and migration (**figure 5.19**) but also clearly decreased invasion rates of FAS-R cells (**figure 5.20**); confirming a role for the PI3K/AKT pathway in promoting the aggressive phenotype of FAS-R cells.

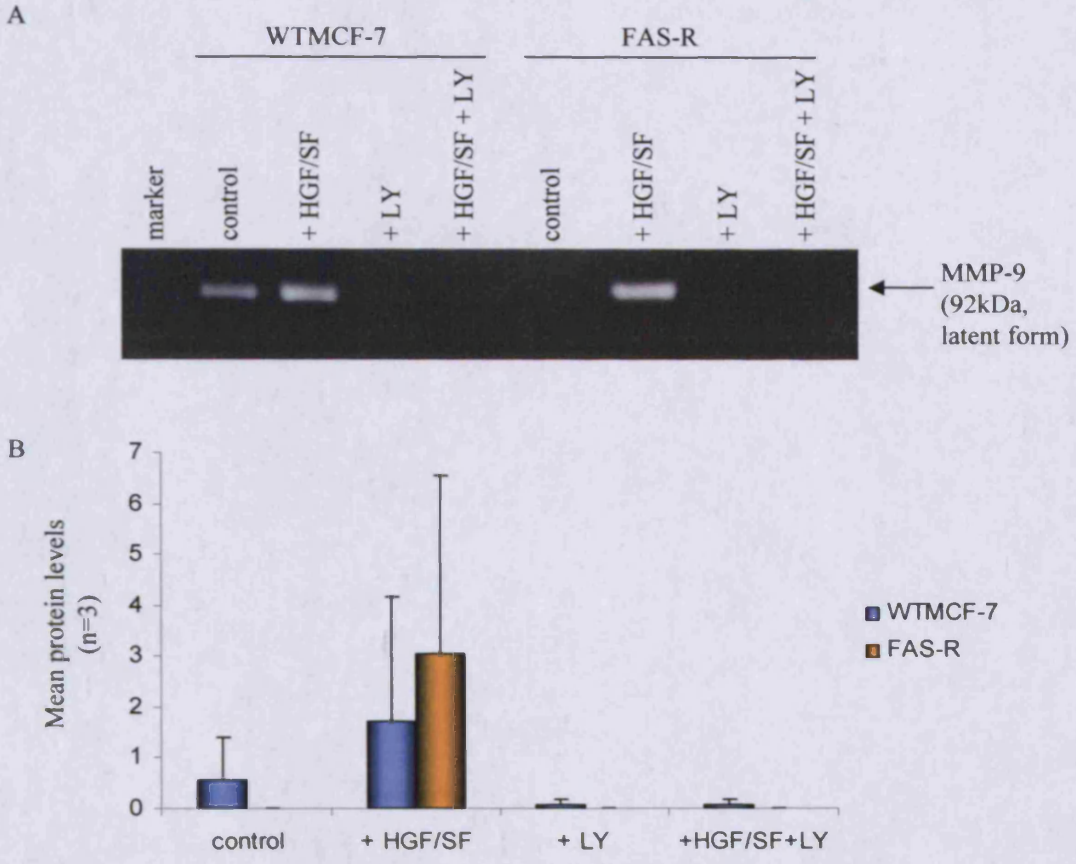


Figure 5.17 Effect of PI3K inhibition on HGF/SF-modulated MMP-9 protein expression levels of WTMCF-7 and FAS-R cells as determined by zymography. PI3K inhibition was achieved using the inhibitor LY294002 (10μM). Serum-starved subconfluent WTMCF-7 and FAS-R cells were treated with HGF/SF (20ng/ml) ± LY294002 for 24h. Cell-conditioned supernatants were collected and processed as described in section 2.6.2. Supernatants were then subjected to zymographic analysis as detailed in section 2.7 to determine the effect of PI3K inhibition on HGF/SF-modulated MMP-9 protein expression. Data was normalised using protein concentration values obtained from respective cell lysates and presented as mean expression levels ± S.D. (n=3) (B). A representative image is shown above (A).

A

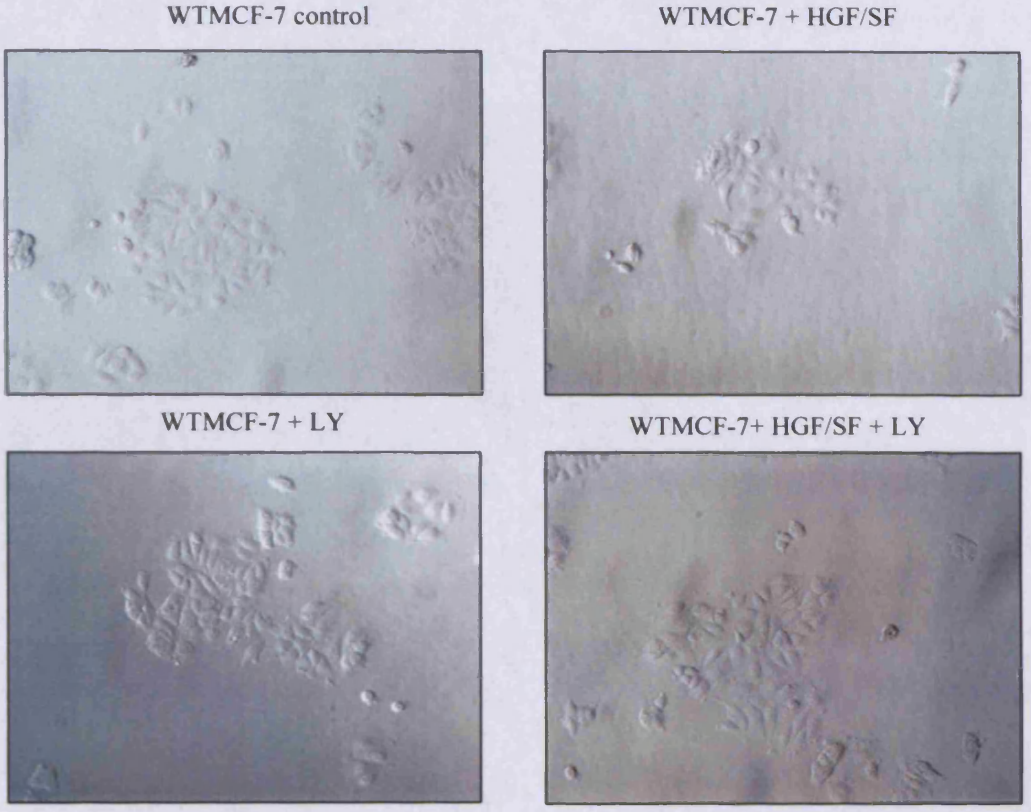
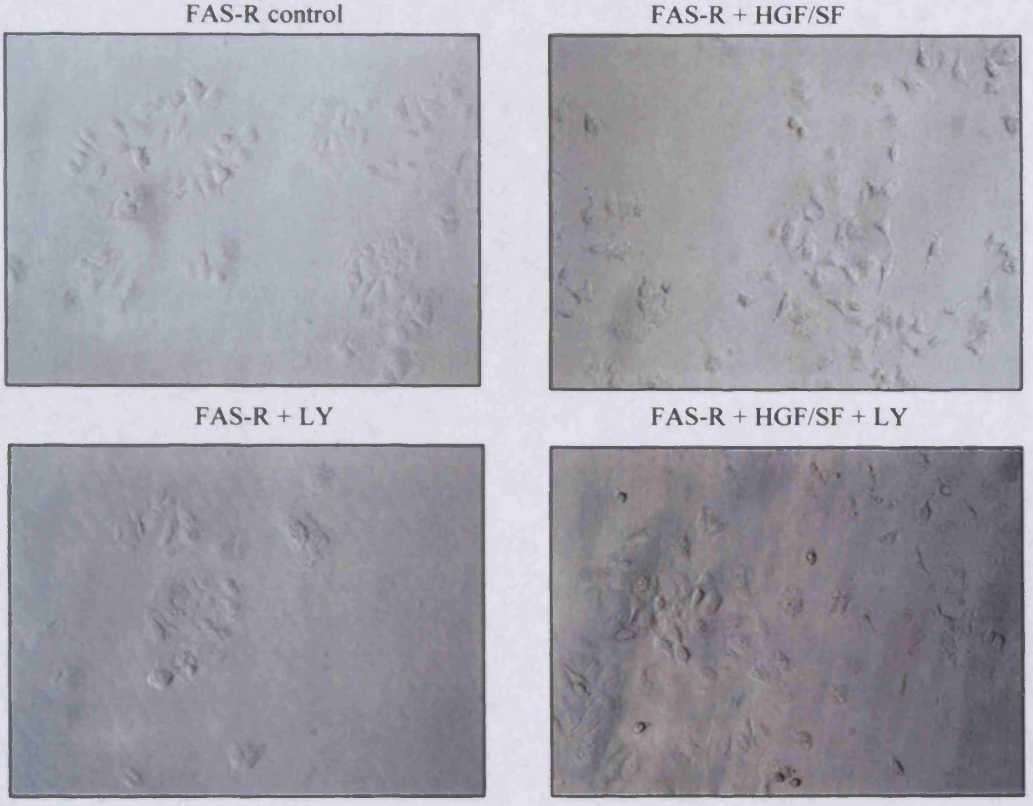


Figure 5.18 The effect of PI3K inhibition on HGF/SF-induced colony dispersion in WTMCF-7 and FAS-R cells as determined by scatter assays.
PI3K inhibition was achieved using the inhibitor LY294002 (10μM). WTMCF-7 (A) and FAS-R cells (B) were seeded in EM ± Faslodex (10^{-7}M) and allowed to form discrete colonies prior to 24h treatment with HGF/SF (20ng/ml) ± LY294002 (10μM) in DCCM. The extent to which PI3K inhibition reduced HGF/SF-induced colony dispersion was determined using cell scatter assays as described in section 2.10. Scatter response was assessed visually and selected colonies were photographed using a Leica DM-IRE2 inverted microscope; representative images are shown above and on the following page. As can be seen, PI3K inhibition caused a modest decrease in HGF/SF-induced colony dispersion in FAS-R cells (B).

B



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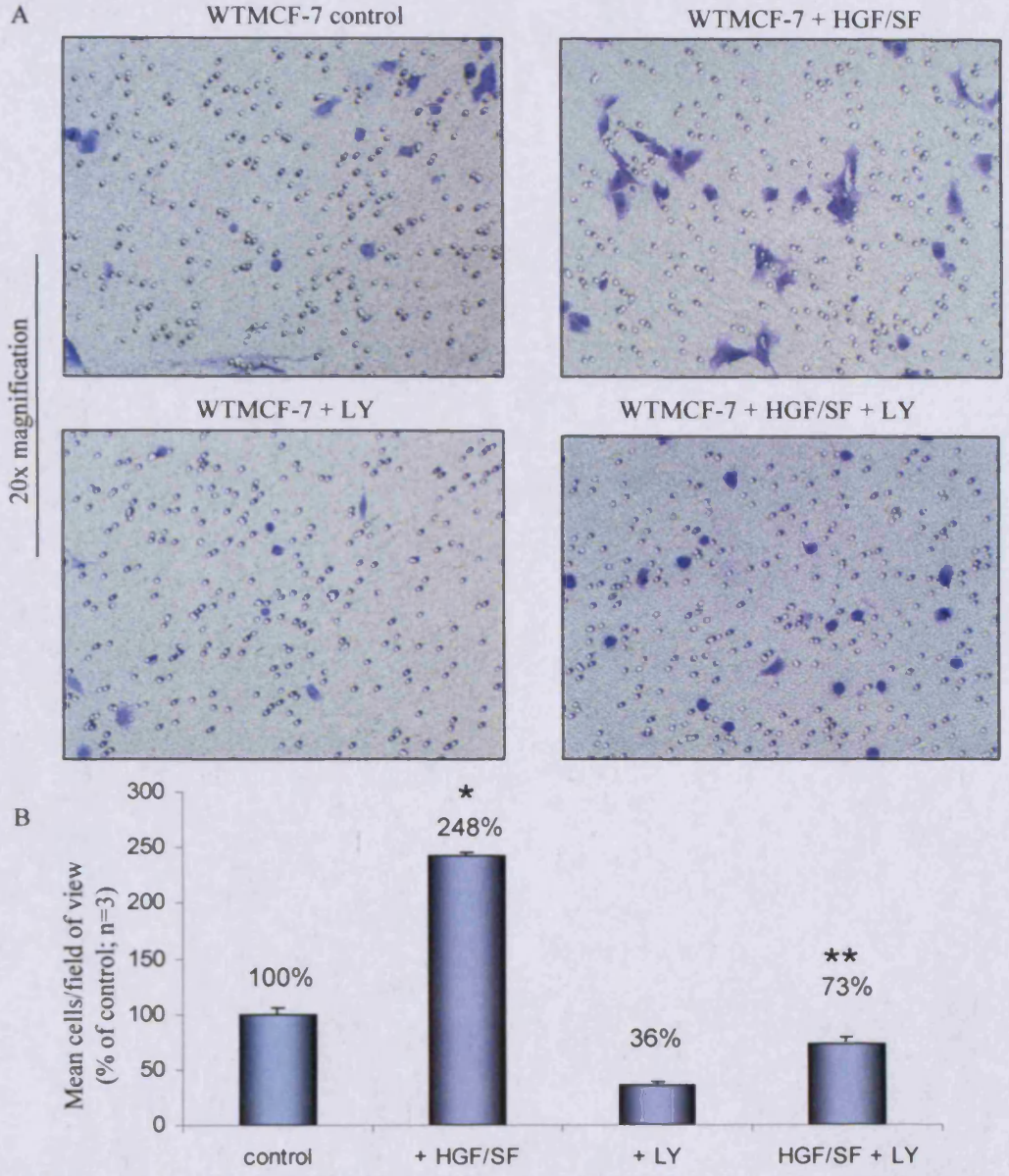
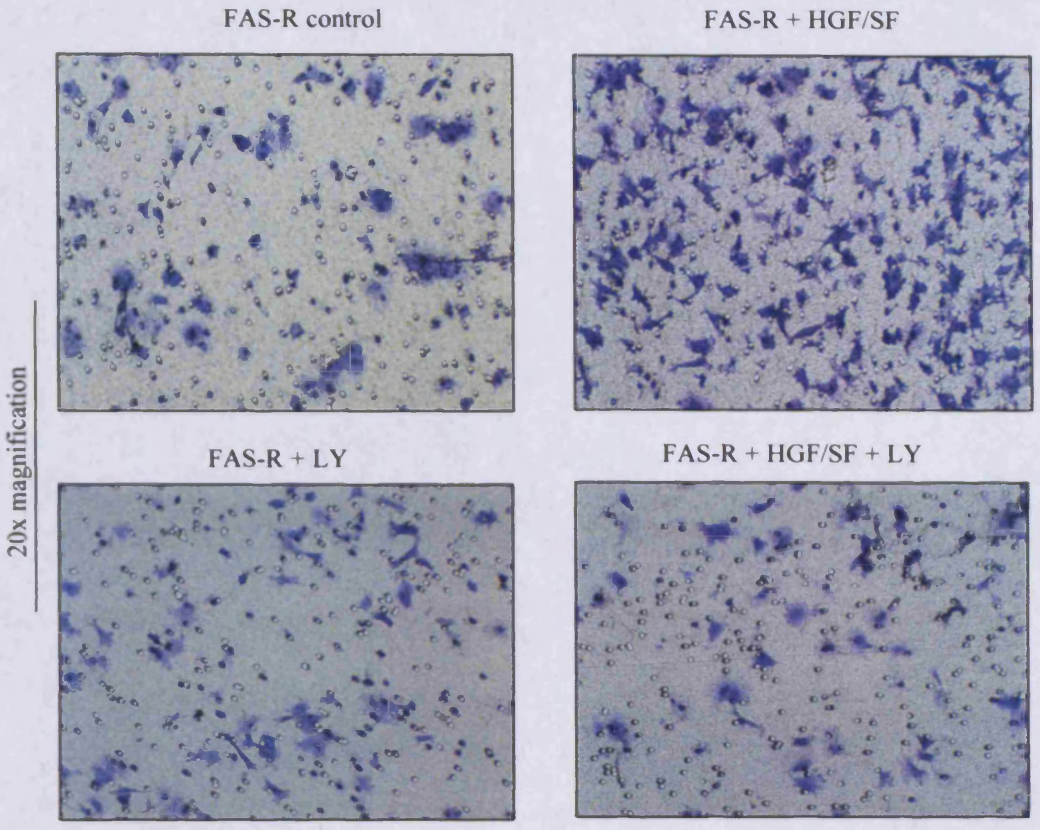


Figure 5.19 Effect of PI3K inhibition on HGF/SF-modulated migration of WTMCF-7 and FAS-R cells.

PI3K inhibition was achieved using the inhibitor LY294002 (10μM). The effect of PI3K inhibition on HGF/SF-modulated migration of WTMCF-7 and FAS-R cells was determined using *in vitro* cell migration assays as described in section 2.9. Crystal-violet-stained cells were photographed at 20x magnification using an Olympus BH-2 phase contrast microscope; representative images are shown above and on the following page (A and C). Quantification was by counting the number of migratory cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of WTMCF-7 ± S.D. (*$p<0.001$ vs. WTMCF-7 control **$p<0.001$ vs. WTMCF-7 vs WTMCF-7 + HGF/SF) and as % of FAS-R cells ± S.D. (*$p<0.005$ vs. FAS-R control) (B and D). As can be seen PI3K inhibition reduced HGF/SF-modulated migration of FAS-R cells (C and D).

C



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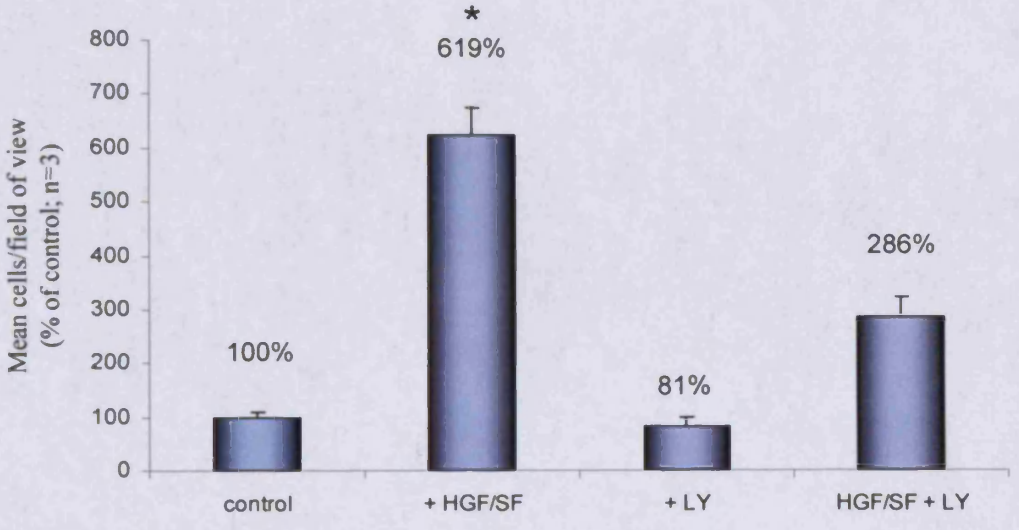

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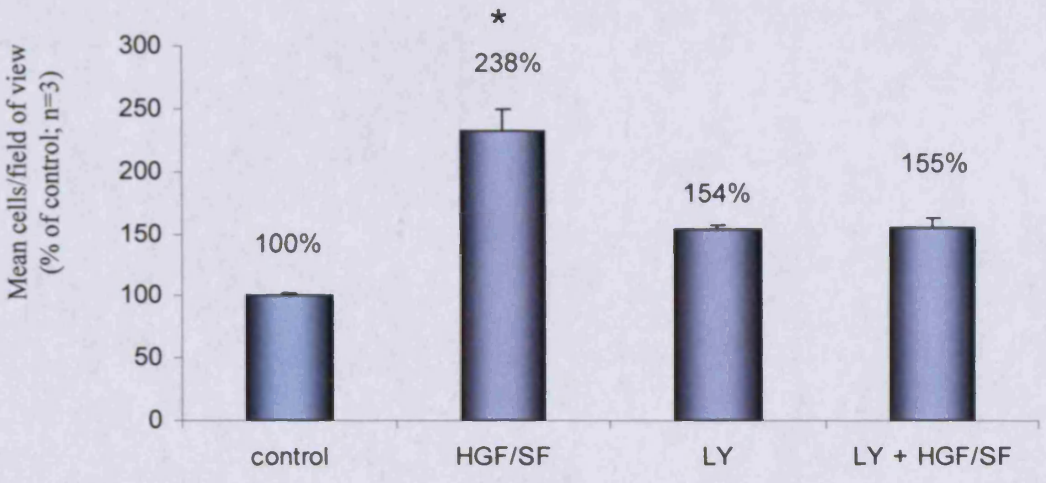

Figure 5.20 Effect of PI3K inhibition on HGF/SF-modulated invasion of FAS-R cells.

PI3K inhibition was achieved using the inhibitor LY294002 (10μM). The effect of PI3K inhibition on HGF/SF-modulated invasion in FAS-R cells was determined using *in vitro* cell invasion assays as described in section 2.8. DAPI-stained cell nuclei were visualised and photographed at 20x magnification using a DM-IRE2 fluorescent microscope connected to a Hamamatsu C4742-96 digital camera. Quantification was by counting the number of invasive cells in 5 non-overlapping fields of view; data (mean cells/field of view) are presented as % of control ± S.D. (*$p<0.001$ vs. FAS-R control). As can be seen PI3K inhibition reduced HGF/SF-modulated invasion of FAS-R cells.

Chapter Six

Discussion

"An expert is a person who has made all the mistakes that can be made in a very narrow field."

Niels Bohr (1885-1962)

6.1 Discussion

The selective oestrogen receptor modulator tamoxifen has been the gold standard endocrine therapy for ER-positive breast cancer for more than 25 years; however, patients with ER-positive disease that are initially responsive to this treatment eventually develop endocrine resistance (Osborne, 1999). Treatment with the selective oestrogen receptor downregulator faslodex has been proved to be effective upon progression on tamoxifen or aromatase inhibitors; and the pure anti-oestrogen is now approved as a second-line agent in postmenopausal women (Howell, 2006). Faslodex displays several superior qualities compared to tamoxifen. It binds the ER with a much higher affinity, downregulating ER protein and suppressing oestrogen-regulated genes more strongly, and thus inhibiting ER-driven cell proliferation more potently. It is furthermore devoid of any agonist activity and hence associated with a far more favourable side effect profile (Howell, 2006). Sadly, faslodex resistance can develop as a result of long-term therapy, presenting a further setback in the pharmacotherapeutic management of breast cancer (Cheung *et al.*, 2006, Robertson *et al.*, 2005, Buzdar, 2004). Consequently, elucidation of the mechanisms underlying resistance to faslodex and rapid identification of novel targets to control faslodex-resistant tumour growth is imperative.

Preliminary studies using our faslodex-resistant cell model have revealed that development of resistance is associated with the acquisition of an adverse cell phenotype *in vitro*, as exemplified by enhanced migratory and invasive capabilities. The objective of this study was to establish the role of MMP proteolytic activity in this process, and further to identify the principal growth factor signalling pathway driving this aggressive behaviour. Confirmation of the involvement of specific MMP family members and identification of a dominant receptor tyrosine kinase would permit their precise targeting using selective MMP and tyrosine kinase inhibitors and may eventually result in successful suppression of FAS-R invasion.

MCF-7 parental cells are ER-positive; and were sensitive to estradiol whilst the anti-oestrogens tamoxifen and faslodex had an inhibitory effect on their growth. In contrast, FAS-R cells derived by chronic exposure of WTMCF-7 cells to faslodex, were insensitive to estradiol, faslodex and tamoxifen. This was due to an apparent loss of the ER. Clinically, loss of ER is associated with poorly differentiated tumours and higher histological grade, limited treatment strategies and hence poor prognosis and decreased overall survival (Putti *et al.*, 2005, Kuukasjarvi *et al.*, 1996, Pichon *et al.*, 1996, Kinne *et al.*, 1987, Parl *et al.*, 1984, Maynard *et al.*, 1978). The main criticism regarding the FAS-R cell model employed in this study is that the cells might stem from an ER-negative outgrowth of the original ER-positive WTMCF-7 population. This would mean the model was *de novo* resistant to faslodex instead of having subsequently acquired resistance to the anti-oestrogen as described in section 2.2.5. However, the status of ER protein expression and endocrine responsiveness in the earlier stages of the FAS-R model are reversible after as little as one week of anti-hormone withdrawal, proving these changes to be of a reversible nature. It is therefore clear that the FAS-R model used does not stem from clonal selection of an ER-negative population.

Numerous faslodex-resistant breast cancer cell models have been established to elucidate the mechanisms underlying resistance to the pure anti-oestrogen. These cell lines vary in their culture conditions and the observations made with respect to ER protein and mRNA expression levels (summarised in **table 6.1**).

Whilst the FAS-R cell model employed in this investigation was established using 10^{-7}M faslodex, varying concentrations of faslodex and additional medium supplementation with E_2 and insulin have been described. Sommer *et al.* (2003), for example, argued that a model cultured in the presence of E_2 was more physiologically relevant as this reflected the E_2 levels present in the human body during anti-oestrogen administration. A more differentiated approach was adopted by Shaw *et al.* (2006) who emphasise that loss of response to oestrogen following oestrogen deprivation is an event distinct from the development of anti-oestrogen resistance. Shaw *et al.* (2006) therefore advocated the

development of cell lines that allow the study of loss of response to faslodex in isolation from loss of response to oestrogen. They hence developed faslodex-resistant cell lines from both the original oestrogen-maintained parental cell line and a long-term oestrogen-deprived MCF-7 cell line which had been established 12 months earlier. The oestrogen-maintained faslodex-resistant cells were considered the most physiologically relevant model. These cells were additionally supplemented with insulin; insulin supplementation was moreover described by Lykkesfeldt *et al.* (1995) and Jensen *et al.* (1999).

Whilst our FAS-R model is characterised by loss of ER protein and reduced ER mRNA levels, this observation was not shared across all faslodex-resistant cell lines (**table 6.1**). ER negativity and decreased ER mRNA expression was reported by Sommer *et al.* (2003) and by Shaw *et al.* (2006) in their oestrogen-maintained model. Dramatic reduction in ER protein levels was observed by Liu *et al.* (2006), whilst a 3-fold reduction was seen by Lykkesfeldt *et al.* (1995) and Jensen *et al.* (1999). In contrast, ER protein was found to be expressed in a model described by Brünner *et al.* (1997) and in the long-term oestrogen-deprived faslodex-resistant cell line developed by Shaw *et al.* (2006).

A 'one size fits all' model seldom stands the test of time when trying to simulate the complex *in vivo* tumour microenvironment. Hence, the existence of several resistant cell lines is of significant advantage, each recreating different scenarios of resistance development to faslodex in the clinic. For example, oestrogen-deprived conditions mirror the post-menopausal state whilst the presence of oestrogen recreates the environment of the pre-menopausal state. Overall, results obtained from the studies using the above models are all equally valid and collectively may throw light onto different facets of the mechanisms underlying the emergence of clinical resistance to faslodex.

In contrast to the studies carried out by other investigators which focused solely on the effect of acquired faslodex resistance and functional ER loss or downregulation on proliferation, the present study addresses the impact of these events on invasion, thus informing on a novel aspect of endocrine resistant cell behaviour.

	Faslodex concentration	E_2 present	Insulin present	ERα protein expression	ERα mRNA expression
Nicholson *et al.* (2005)	10^{-7}M	no	no	absent	reduced
Lykkesfeldt *et al.* (1995)/ Jensen *et al.* (1999)	10^{-7}M	no	yes	reduced by 33%	present*
Brünner *et al.* (1997)	10^{-6}M	no	no	present*	present*
Sommer *et al.* (2003)	10^{-6}M	yes	no	absent	reduced
Shaw *et al.* (2006) a)	10^{-7}M	yes	yes	absent	absent
Shaw *et al.* (2006) b)	10^{-7}M	no	no	reduced by 10.5%	low
Liu *et al.* (2006)	10^{-6}M	no	no	dramatically reduced	reduced by 33%

Table 6.1 Culture conditions and ERα-status of faslodex-resistant breast cancer cell models compared to that of parental cells.
* authors only commented on presence of ERα mRNA and protein; changes in expression levels between resistant and parental cells were not specified

Having characterised FAS-R cells with respect to their aggressive phenotype, the next step was to determine and compare their MMP and TIMP expression profile to that of the parental cells to establish whether differential expression of these proteins may play a role in FAS-R invasion.

Profiling of WTMCF-7 cells revealed both MMP and TIMP family members to be widely expressed at modest levels, with the exception of MMP-7 which was absent. Interestingly, when the mRNA expression pattern for these MMPs and TIMPs observed for WTMCF-7 cells was compared to that reported by other investigators, several differences were observed. Balduyck *et al.* (2000), for example, reported detection of TIMP-1, TIMP-2 and TIMP-3 only. TIMP-1, TIMP-2 and TIMP-3 expression was also described by Kousidou *et al.* (2004) who additionally observed MMP-2, MMP-9 and MMP-11 expression under serum-free conditions. Expression of MMP-1 was observed by Bachmeier *et al.* (2005) and Bartsch *et al.* (2003); the latter also found MMP-11 to be present in WTMCF-7 cells under serum-free conditions. There were also several instances where MMPs and TIMPs detected in MCF-7 cells in this study were not observed by the above investigators. Bartsch *et al.* (2003), for example, could not detect MMP-9 or MMP-10. Similarly, Bachmeier *et al.* (2005) reported absence of MMP-9 mRNA expression. Furthermore, MMP-1 expression was not observed by Kousidou *et al.* (2004). Balduyck *et al.* (2000) did not detect any of the MMPs we found to be expressed in MCF-7 cells; and furthermore only reported presence of TIMP-4 mRNA transcripts.

The observed discrepancies in the MMP and TIMP expression patterns may possibly be attributed to differences in the degree of sensitivity of the RT-PCR systems employed, the passage number of the cells investigated and variations in the culture conditions of each cell line, in particular the presence or absence of serum. Indeed, zymographic analysis of serum alone in preliminary studies revealed considerable MMP-9 expression; hence all further experiments for MMP expression analysis described in this investigation were performed under serum-free conditions (i.e. culture in DCCM) to eliminate false-positive results.

Serum furthermore contains several growth factors, the influence of which on MMP expression is well known (Birkedal-Hansen, 1995); further emphasising the importance of serum-free conditions. Also, since increasing cell density has been demonstrated to downregulate MMP and TIMP expression (Bachmeier *et al.*, 2005), great care was taken to harvest cells as soon as 60-70% confluency had been reached.

Whereas initial profiling included all four TIMP family members, analysis of TIMP expression was discontinued during the course of this study. As indicated previously, the role of these inhibitors in cancer is complex and paradoxical, with not only inhibitory but also stimulatory effects on carcinogenesis and tumour progression demonstrated in cell and animal models which overexpressed TIMPs (Kahlert *et al.*, 2008). Furthermore, TIMPs have been identified as poor prognostic indicators in several human cancers (reviewed by Jiang *et al.*, 2002). Considering that the data arising from early experiments analysing TIMP expression proved inconclusive, and based on the above reports, it seemed appropriate to focus solely on MMP expression for the remainder of the investigation.

With the exception of MMP-2 which was expressed in FAS-R cells only and TIMP-4, mRNA expression of all other MMPs and TIMPs was lower compared to the wildtype or absent altogether. Although MMP-2 had been detected at gene level, FAS-R cells did not secrete MMP-2 protein; and MMP-9 protein was not found to be expressed either. In contrast, latent MMP-9 expression was detected in WTMCF-7 cells.

Considering that FAS-R cells expressed neither MMP at protein level, the observation that FAS-R invasion was reduced in the presence of a broad-spectrum inhibitor seems slightly surprising. However, since gelatinase zymography only permits detection of gelatinases, other MMPs such as MMP-11 and MMP-13 which were expressed in FAS-R cells at mRNA level might, unknown to us, also be expressed at protein level. It is therefore likely that the observed reduction in FAS-R cell invasion following blanket MMP inhibition

was due to abrogation of proteolytic activities of family members not assayed for.

Detection of MMP expression in the conditioned supernatant of matrigel-coated inserts of invasion assays has been described by Balduyck *et al.* (2000). As mentioned earlier, cells to be assayed for MMP expression were cultured under serum-free conditions to avoid detection of MMPs originating from the serum component of the medium. However, when invasion assays were attempted under serum-free conditions, the experiments had to be aborted due to cell death. MMP expression analysis from the upper chamber of transwell inserts from HGF/SF-stimulated invasion could therefore not be performed.

As these findings illustrate, an obvious limitation of the work presented herein was the lack of more detailed MMP protein expression data. Attempts to corroborate observations from zymographic analysis with Western Blotting and immunofluorescence techniques were not successful. Interestingly, the obstacles encountered herein in the protein detection of MMPs were the subject of a comprehensive review by Lombard, Saulnier and Wallach (2005), which suggested that the inherent problems associated with MMP activity analysis have been reported by many investigators. Since the commencement of this study, several highly evolved methods for MMP protein detection have been described, such as solid phase activity assays (Lauer-Fields *et al.*, 2004), energy-based multiplexed assays (Kim *et al.*, 2008) and quantum dot nanosensors (Xia *et al.*, 2008). Application of these novel techniques to future studies of this nature may yield more detailed results on MMP protein expression and activity levels.

The clinical significance of MMP-9 in breast cancer progression is well established. MMP-9 has been correlated with metastatic progression and reduced survival in breast cancer patients by many investigators and, as outlined below, some suggest MMP-9 could even be used as a prognostic marker. As delineated in section 1.6.5, it was not until recently that the host-protective nature of certain MMPs was recognised and that individual family members were accordingly

assigned 'anti-target' or 'target' status. Despite the fact that some of the early studies that implicated MMP-9 in metastatic progression preceded this realisation of the host-protective functions of 'anti-target'- MMPs, their findings are corroborated by several very recent reports. Ranuncolo *et al.* (2003), for example, determined that plasma MMP-9 activity was significantly increased in breast cancer compared to benign mammary pathologies and healthy controls. The investigators therefore concluded that MMP-9 activity might be useful as a marker in the prognosis of breast cancer patients. This study coincided with the recent findings of Quaranta *et al.* (2007) who assessed the prognostic value of MMP-9 amongst other markers. Moreover, the prognostic value of MMP-9 in patients with lymph node-negative breast carcinoma was described by Li *et al.* (2004) who found it to be an unfavourable prognostic factor for relapse-free survival. These observations coincided with a recent study by Wu *et al.* (2008) which confirms the prognostic significance of MMP-9 serum and tissue expression in breast cancer. The investigators found serum MMP-9 levels to be significantly higher in breast cancer compared to in benign breast disease and healthy tissue. Furthermore, high MMP-9 serum levels and tissue expression were associated with higher tumour grade whilst high tissue expression correlated with reduced relapse-free and overall survival. These findings are in accordance with the previous studies and further support the usefulness of MMP-9 as a biomarker for predicting progression and prognosis in breast cancer. Also, as mentioned in the introduction, MMP-9 was one of the 70 genes associated with short interval to distant metastases in a 'poor prognosis' signature of primary breast carcinomas (van 't Veer *et al.*, 2003, van de Vijver *et al.*, 2002). Intriguingly, enhanced expression and activation of MMP-9 amongst other MMPs at the tumour periphery of invasive breast carcinomas was revealed by Bachmeier *et al.* (2005). In addition, Incorvaia *et al.* (2007) suggested that MMP-9 be considered as a target in breast cancer metastasising to bone whilst Thanakit *et al.* (2005) reported that MMP-9 enhanced breast carcinoma cell invasion and was linked to lymph node metastases in high-grade breast carcinoma patients. Finally, a recent study by Shah *et al.* (2009) reported

elevated MMP-9 activity levels in malignant breast tissue compared to adjacent normal tissue, reconfirming the above findings from earlier studies.
The clinical relevance of MMP-9 and the fact that growth factor-stimulated FAS-R cells secrete MMP-9 suggests that development of faslodex resistance in breast cancer patients may be accompanied by elevated MMP-9 expression and activity. It would therefore be of considerable interest to measure MMP-9 proteolytic activity in tissue samples of individuals with faslodex-resistant breast tumours.

Having discovered a potential role for MMP-9 activity in the adverse phenotype of FAS-R cells, the next step was to investigate the possible existence of a dominant receptor tyrosine kinase pathway driving this aggressive behaviour. As delineated in section 4.2.4, FAS-R cells were subsequently found to express elevated c-Met mRNA and protein levels. These observations were particularly interesting in light of the well-established association between clinical c-Met overexpression and poor prognosis and reduced survival rate in breast cancer (Kang and Massague, 2004, Elliott *et al.*, 2002, Edakuni *et al.*, 2001, Camp *et al.*, 1999, Tsarfaty *et al.*, 1999, Ghoussoub *et al.*, 1998). Moreover, some studies have suggested c-Met as a potential prognostic factor for breast cancer patients, replacing more traditional markers such as Her-2 and EGFR (Lengyel *et al.*, 2005, Tolgay Ocal *et al.*, 2003). These findings further underline the potential clinical relevance of dominant c-Met signalling in FAS-R cells which will be considered in more detail later in this chapter.

Whilst HGF/SF treatment of FAS-R cells resulted in MMP-9 secretion, scattering and markedly increased migration and invasion rates, neither scattering nor increased invasion were seen in WTMCF-7 cells. However, WTMCF- cells did display a modest increase in ERK and AKT phosphorylation and an enhanced migratory response and augmented MMP-9 secretion on treatment with HGF/SF which was unexpected considering neither total nor phosphorylated c-Met could be detected in these cells. However,

immunocytochemical analysis had revealed some c-Met expression also in the wildtype. Taken together, this indicated that some albeit very low c-Met activity must exist in these cells after all. In fact, as demonstrated by Hiscox *et al.* (2006), phosphorylated c-Met protein was detectable in WTMCF-7 cells following stimulation with HGF/SF at the much higher concentration of 80ng/ml. Whilst such modest signalling activity is unlikely to account for the response observed, this apparent discrepancy might be accounted for by receptor transactivation events. The fact that c-Met communicates with various secondary receptors supports this hypothesis. Indeed, a recent study in several mammary carcinoma cell lines revealed that EGFR played a significant role in HGF/SF-mediated biological responses (Bonine-Summers *et al.*, 2007); and it is thought that a similar mechanism, possibly involving a different secondary receptor, might potentially be operating in HGF/SF-treated WTMCF-7 cells.

Whereas WTMCF-7 and FAS-R cells treated with HGF/SF both secrete similar levels of MMP-9, HGF/SF only enhanced FAS-R cell invasive rates with WTMCF-7 invasion levels remaining unchanged (data for WTMCF-7 invasion not shown). Whilst these results appear rather paradoxical at first, a closer look at the processes and machinery governing extracellular MMP activation and cell surface localisation seems to offer potential explanations.

The majority of MMPs are secreted as inactive zymogens and subsequently activated extracellularly by proteases including other members of the MMP family and serine proteases. MT1-MMP, for example, has been shown to play a pivotal role in the cell-surface activation of MMP-2 (Sato *et al.*, 1994). Potentially, a similar mechanism might operate in HGF/SF-stimulated FAS-R cell invasion with regard to MMP-9. In an effort to establish a possible link gene expression profiling of MT1-MMP using RT-PCR analysis in HGF/SF-treated FAS-R cells compared to HGF/SF-treated WTMCF-7 cells was attempted but conclusive data could not be obtained. Furthermore, establishment of the gene and protein expression profiles of all potential activating proteases unfortunately lay outside the scope of this project. Nevertheless, it is thought that

the increase in invasion following HGF/SF treatment of FAS-R cells but not of WTMCF-7 cells despite similar MMP-9 secretion may be partially due to differential MMP-9 activation.

Furthermore, in addition to zymogen activation, cell surface localisation of MMPs is critical in order to achieve the localised matrix degradation thought to be central to the invasive process. Molecules such as the hyaluronic acid receptor, CD44, function as cell surface docking receptors that localise secreted MMPs to the invasion front. CD44-mediated cell surface localisation of active MMP-9 has been demonstrated in keratinocytes (Yu and Stamenkovic, 2000), and it is thought that such a mechanism might also operate in FAS-R cells. Intriguingly, FAS-R cells have been shown to express elevated levels of CD44 mRNA and protein, as demonstrated by gene expression analysis using Affymetrix arrays and RT-PCR and immunocytochemistry, respectively (R. McClelland, N. Jordan, M. Harper; unpublished data); these observations lend further weight to the above hypothesis. In summary, HGF/SF-mediated elevation in FAS-R cell invasion only may potentially be due to differential MMP-9 activation and cell surface localisation of MMP-9 via elevated expression of activating proteases and cell surface docking receptors respectively.

The phenomenon of HGF/SF-induced cell scattering, migration and invasion has been firmly established in a variety of cell types. Equally well acknowledged is the fact that HGF/SF-mediated motile and aggressive behaviour is accomplished through stimulation of MMP expression and activity. Indeed, this has been demonstrated for a wide range of epithelial cancer models including colon adenocarcinoma (Tanimura *et al.*, 2003, Kermorgant *et al.*, 2001), prostate carcinoma (Fujiuchi *et al.*, 2003, Tanimura *et al.*, 2003), hepatocellular carcinoma (Abiru *et al.*, 2002, Tanimura *et al.*, 2002), nasopharyngeal cancer (Zhou *et al.*, 2008), oral squamous cell carcinoma (Hanzawa *et al.*, 2000), ovarian cancer (Zhou and Wong, 2006) and, importantly, breast adenocarcinoma (Tanimura *et al.*, 2003), validating the findings of this study. Evidently, HGF/SF-

mediated invasion via MMP proteolytic activity is a mechanism common to all major carcinomas and therefore represents a promising broad-spectrum therapeutic target with considerable potential.

Early investigations into the signalling components mediating HGF-induced motile behaviour downstream of c-Met in MDCK (Madin-Darby Canine Kidney) cells using the pharmacological inhibitors PD98059, LY294002 and wortmannin revealed involvement of both ERK1/2 and PI3K pathways (Potempa and Ridley, 1998, Royal *et al.*, 1997, Royal and Park, 1995). Since then, a distinct role for ERK1/2 and PI3K signalling in HGF/SF-stimulated aggressive behaviour has been observed in and is now widely acknowledged for many cancer cell lines. Importantly, these cancer cell models display differential requirements for ERK1/2 and PI3K activity in their response to HGF/SF. Requirement of ERK1/2 signalling only was detected in the hepatocarcinoma cell line HepG2 (Abiru *et al.*, 2002) and the stomach adenocarcinoma cell line TMK1 (Tanimura *et al.*, 1998). Conversely, sole dependency on the PI3K pathway was seen in the small cell lung cancer cell line NCI-H69 (Maulik *et al.*, 2002), the hepatocarcinoma cell lines Hep3B and Huh-7 (Nakanishi *et al.*, 1999), in ovarian cancer cells (Zhou and Wong, 2006), in the colonic carcinoma cell line Caco-2 (Kermorgant *et al.*, 2001) and in nasopharyngeal cancer cells (Zhou *et al.*, 2008). Significantly, contribution of both pathways to HGF/SF-induced motility, as witnessed in our FAS-R cell model, was reported for the mammary carcinoma cell line 184B5 (Day *et al.*, 1999). Clearly, carcinoma types vary in their dependence upon either pathway for mediating response to HGF/SF stimulation, with an apparent dual signalling activity operational in some breast cancer models.

Furthermore, it appears that HGF/SF-induced invasion in FAS-R cells involves MMP-9, a mechanism also described in colonic cancer (Kermorgant *et al.*, 2001), ovarian cancer (Zhou and Wong, 2006), nasopharyngeal cancer (Zhou *et al.*, 2008) and a murine model of breast carcinoma (Zhang *et al.*, 2004).

The present study is limited by the fact that the observations described were made in one faslodex-resistant breast cancer model only. Repeating these experiments in other ER-positive mammary carcinoma cell lines with acquired resistance to faslodex will inform on whether the mechanism observed for FAS-R cells applies to breast cancer models in general. Targeting the described invasion mechanism using siRNA approaches to both c-Met and MMP-9, neutralising antibodies to c-Met, HGF/SF and MMP-9 or c-Met antagonists could further confirm the role of these targets in the aggressive behaviour arising as a consequence of faslodex resistance. Eventual translation of these 2D cell models into orthotopic mouse models will hopefully corroborate the existence of the invasion strategy proposed in this thesis *in vivo*.

Given that the aggressive behaviour displayed by FAS-R cells is driven by elevated c-Met signalling activity, c-Met may present a potential therapeutic target in such cancers. In direct contrast to the problematic development of MMP inhibitors, the advancement of selective and broad-spectrum c-Met and HGF/SF inhibitors has been a success, with several compounds showing promising activity in clinical trials (see **table 1.5**). For example, the humanised monovalent antagonistic anti-c-Met antibody, one-armed 5D5 (MetMAb, Genentech), was recently evaluated in Phase I clinical studies and found to be safe and well-tolerated (Salgia *et al.*, 2008). Similarly, phase I dose escalation studies with the non-ATP-competitive highly selective c-Met TKI ARQ197 (ArQule) demonstrated the compound to be well-tolerated; moreover, no dose-limiting toxicity was observed (Garcia *et al.*, 2007). Moreover, early clinical trials with the ATP-competitive c-Met TKI XL184 (Exelixis) have indicated promising antitumour activity in the absence of toxicity with a >50% response rate and a 100% disease control rate in a study involving patients with medullary thyroid cancer (Salgia *et al.*, 2007, Salgia *et al.*, 2008).

This is an exciting development particularly in light of the findings presented herein, as follow-on *in vivo* studies further establishing the role of c-Met

signalling in faslodex-resistant breast cancer will be able to directly avail themselves of these agents which were not available at the commencement of this project.

Having identified a role for c-Met signalling in FAS-R cells *in vitro*, it is now necessary to establish whether this mechanism of elevated c-Met activity following acquisition of faslodex resistance can be detected in clinical samples of patients having undergone long-term faslodex therapy, thus ascertaining it is also functional *in vivo*. At present, faslodex is still very much 'the new kid on the block'; thus tumour biopsies of patients relapsed on faslodex are rare and current treatment regimes with faslodex only short-term. However, once the exact positioning of faslodex within the endocrine treatment sequence and an optimal dosing regime has been established, appropriate clinical samples should be more readily available. The outcome of such studies in the future is awaited with great interest.

Excitingly, recent data emerging from in-house studies has shown that elevated c-Met expression is witnessed as early as 2 days after exposure of WTMCF-7 cells to faslodex (S. Hiscox and J.Gee, personal communication) suggesting that switching to c-Met signalling is an extremely early event occurring whilst cells are still in the drug-responsive phase. Studies must now be initiated to determine whether this observation is reflected *in vivo*. If recapitulated in the clinic, this would support the initiation of anti-invasive therapy with c-Met inhibitors right at the outset and in direct conjunction with commencement of faslodex therapy as opposed to later, after establishment of resistance to faslodex has already taken place.

Combined therapy of endocrine agents with signal transduction inhibitors (STIs) is an important concept in the clinical management of breast carcinoma, and is based on the observation that enhanced signal transduction pathways are one of the principal adaptive changes accounting for endocrine-resistant growth (Nicholson and Johnston, 2005).

In vitro data by (Gee *et al.*, 2003) indicated that combined therapy of tamoxifen with the EGFR TKI gefitinib provided greater anti-proliferative and pro-apoptotic effects than tamoxifen monotherapy, leading to the hypothesis that combined endocrine/TKI therapy would prove a more effective strategy than either monotherapy, crucially delaying the emergence of tamoxifen resistance arising from ER/EGFR cross-talk. Recently, several investigators have reported back from clinical trials investigating this hypothesis *in vivo* (Johnston *et al.*, 2009). Significantly, tamoxifen/gefitinib combination as first-line therapy in ER-positive patients was found to improve progression-free survival, confirming the observations made by Gee *et al.* (2003) at *in vitro* level and providing proof-of-concept. Should faslodex combined with c-Met inhibitors be shown to delay faslodex resistance development in WTMCF-7 cells *in vitro* in future studies, clinical trials should then be initiated to investigate whether these synergistic and additive effects are repeated in the clinic, potentially providing the rationale for combined faslodex and c-Met inhibitor treatment. As mentioned in section 1.3.3.3, combinations of fulvestrant with small molecule inhibitors or monoclonal antibodies such as gefitinib or bevacizumab respectively are already showing promising results in phase II trials (Robertson, 2007) (see **table 1.2**), indicating the feasibility of such a combined approach.

Based on the role of MMP-9 activity in the c-Met-driven aggressive behaviour of FAS-R cells, targeting of this particular facet of c-Met signalling may provide additional anti-invasive effects *in vitro*. If such an approach should prove effective *in vitro,* and if the same mechanism should be proven to be functional in patients who progressed on faslodex, this eventually may indicate additional treatment with a specific and highly selective MMP-9 inhibitor.

The observation that emergence of faslodex resistance was accompanied by c-Met overexpression was of particular interest in view of the fact that c-Met signalling has recently been implicated in acquired resistance to other cancer therapeutics. Engelman *et al.* (2007; 2008) and Bean *et al.* (2007), for example, reported that c-Met signalling was involved in the emergence of acquired resistance to the EGFR TKIs gefitinib and erlotinib in non-small-cell lung cancer

patients. Furthermore, Mueller *et al.* (2008) demonstrated a role for combined c-Met/c-src signalling in acquired resistance to gefitinib in the breast cancer cell line SUM 229.

Most significantly, however, c-Met signalling was found to play a role in acquired resistance to the targeted agent trastuzumab (Herceptin^{TM}) in both Her2-overexpressing breast cancer cell lines and Her2-overexpressing tumours (Shattuck *et al.*, 2008). This report provides a second example of the role of c-Met signalling in loss of response to therapy in breast cancer cell lines and is hence in accordance with the findings presented in this thesis. Analysis of a publicly available microarray dataset (http://caarraydb.nci.nih.gov/caarray/) revealed that c-Met receptor transcript levels were elevated in trastuzumab-resistant tumours. In a direct parallel to the observation of rapid c-Met upregulation in faslodex-naïve WTMCF-7 cells after just 48h exposure to the drug, the Her-2 overexpressing breast cancer cell lines SKBR3 and BT474 displayed a significant increase in c-Met protein expression within 48h of trastuzumab treatment (Shattuck *et al.*, 2008). As becomes apparent from these studies, c-Met signalling is emerging as a generic 'escape hatch' for cancer cells facing inhibition of their primary dominant signalling pathway, emphasising the value of c-Met as a therapeutic target in a wide range of malignancies and illustrating the great impact effective c-Met therapeutics will have on delay or even prevention of resistance if given upfront in combination with endocrine agents or STIs.

Whilst the accrual of the advantageous mutation, namely c-Met receptor overexpression, occurs specifically in the epithelial cancer cell, the ligand activating c-Met signalling is derived from the stromal compartment. HGF/SF production by the stroma in turn is stimulated through secretion of HGF/SF-inducers such as interleukin-1β, basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor-α and prostaglandin E2 by the epithelial cancer cells (Matsumoto and Nakamura, 2006). In a perfect example of

tumour-host conspiracy, this paracrine signalling loop emphasises the importance of the tumour microenvironment and identifies the tumour stroma to be a key player in breast carcinoma progression and metastasis rather than an innocent bystander.

Although our FAS-R cell model in which HGF/SF was added exogenously yielded valuable data and made possible the initial discovery of c-Met signalling involvement in the invasive phenotype of these cells, such 2D monolayer cultures of epithelial cells are inadequate for assessing the role of the tumour stroma. Hiscox *et al.* (2006) addressed this issue by performing co-culture studies with HGF/SF-producing MRC5 fibroblasts further highlighting the contributory role of the stroma in the development of the aggressive FAS-R cell phenotype.

While co-culture models do permit the study of the cancer cell-stroma interaction they are only a very basic approximation of the *in vivo* scenario. Thus, although both monolayer and co-culture studies have afforded us considerable insight into the role of c-Met signalling, there is a clear need to develop more complex and physiologically relevant *in vitro* models that reflect the histological complexity of tumours to further investigate the contribution of c-Met signalling to the adverse phenotype of FAS-R cells.

The increasing recognition of the importance of microenvironmental control of tumour cell progression has led to the development of multicellular organotypic assays as a means of dissecting the role of epithelial-stromal cell cross-talk in breast carcinoma progression more accurately (Chioni and Grose, 2008, Holliday *et al.*, 2009, Krause *et al.*, 2008, Kenny *et al.*, 2007, Lee *et al.*, 2007, Kim *et al.*, 2004). Such 3D culture systems allow epithelial cells to organise into structures that mimic their *in vivo* tissue architecture and function and thus reflect the signal transduction and heterotypic cell interactions of the physiological tumour microenvironment (Weigelt and Bissell, 2008). Chioni and Grose (2008) describe an organotypic model in which the stromal component consists of a collagen:matrigel (70:30) matrix enriched with human fibroblasts cultured on a

grid at the air-liquid interface. MDA-MB-231 breast carcinoma cells were then seeded onto this artificial stroma and left to invade for 9 days following which invasive capacity was assessed through Haematoxylin and Eosin staining of sections of the organotypic culture system.

Adoption of such a heterotypic 3D approach in which FAS-R cells are cultured on top of a collagen/matrigel matrix enriched with MRC5 fibroblasts would permit more accurate analysis of c-Met signalling in a physiologically representative context and consequently facilitate the generation of clinically relevant data.

In terms of additional therapeutic options the observation that faslodex-resistant breast cancer is not a tumour cell autonomous condition but rather a pathology involving a dynamic epithelial-stromal cell dialogue reinforces the increasingly popular concept of direct targeting of this interaction as a promising strategy in the endeavour to control metastatic progression (Aharinejad *et al.*, 2009, Anton and Glod, 2009).

As the findings of this thesis demonstrate, ER status correlated inversely with c-Met receptor expression in WTMCF-7 and FAS-R cells. Interestingly, this inverse correlation has been reported for several other breast cancer cell lines (see **table 6.2**).

Breast cancer cell line	ER status	c-Met status	Reference
MCF-7	ER-positive	c-Met-negative	Hiscox *et al.* (2006) Beviglia *et al.*, 1997
T47D			Beviglia *et al.*, 1997
ZR-75-1			Beviglia *et al.*, 1997
SK-BR-3			Beviglia *et al.*, 1997
FAS-R	ER-negative	c-Met-positive	Hiscox *et al.* (2006)
MDA-MB-231			Beviglia *et al.*, 1997 Parr and Jiang (2001)
MDA-MB-435s			Beviglia *et al.*, 1997
BT20			Beviglia *et al.*, 1997

Table 6.2 Inverse correlation of ER and c-Met status in breast cancer cell lines.

Whilst oestrogens stimulate breast cancer cell proliferation, the inhibitory effect of oestrogen signalling on motility and invasion through suppression of transcription of pro-invasive genes ((Hiscox *et al.*, 2007, Platet *et al.*, 2004, Rochefort *et al.*, 1998) and induction of anti-invasive genes such as E-cadherin is well established. Taken together, this indicates that expression of the pro-invasive RTK c-Met may be negatively regulated by the ER in clinical samples. The relationship between c-Met and ER status was queried using the Oncomine clinical transcriptome database (http://www.oncomine.org) which provides publicly available data sets on cancer gene expression (Rhodes *et al.*, 2004). Analysis of 7 separate data sets from the database using t-test confirmed that there was a significant inverse correlation between ER and c-Met mRNA expression in clinical breast cancer samples (J. Gee, personal communication; **table 6.3**).

Oncomine-reported study	Size of test population	p-value
Wang *et al.*, 2005	ER-ve: n = 77 ER+ve: n = 88	p<0.0001
Minn *et al.*, 2005	ER-ve: n = 42 ER+ve: n = 57	p<0.0001
Bittner *et al.*, 2005*	ER-ve: n = 78 ER+ve: n = 154	p<0.0001
Chin *et al.*, 2006	ER-ve: n = 43 ER+ve: n = 75	p<0.0001
Desmedt *et al.*, 2007	ER-ve: n = 64 ER+ve: n = 134	p<0.0001
Saal *et al* , 2007	ER-ve: n = 60 ER+ve: n = 45	p<0.0001
Finak *et al.*, 2008	ER-ve: n = 10 ER+ve: n = 43	p<0.0001

Table 6.3 Results from Oncomine database analysis revealing inverse correlation of ER and c-Met mRNA status (J.Gee, personal communication).
* data base link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109

An inverse correlation between ER status and c-Met mRNA expression was furthermore observed in a published study of patients with invasive ductal carcinoma (Greenberg *et al.*, 2003). Moreover, gene expression and tissue microarray analysis of human breast cancers revealed that high c-Met expression correlated significantly with ER/Her-2 negative tumours and the basal subtype (Graveel *et al.*, 2009). These findings are of considerable interest as they collectively suggest a role for c-Met as an important therapeutic target in ER-negative disease forms which are associated with aggressive disease, poor prognosis and highly limited treatment options. Based on the above observations, inhibition of c-Met signalling may be of significant therapeutic benefit in *de novo* ER-negative disease (possibly including Triple Negative Breast Cancer) and in ER-negative disease arising as a consequence of progression on tamoxifen and eventually faslodex, potentially controlling disease progression and improving patient outlook. The observations arising from this thesis are of potentially of considerable significance since all these disease states represent areas of unmet need with regards to targeted therapy strategies.

6.2 Conclusion

The data presented herein demonstrate that development of faslodex resistance *in vitro* is accompanied by overexpression of c-Met, the activation of which augments the aggressive invasive phenotype of FAS-R cells by a mechanism likely to involve MMPs. If recapitulated *in vivo*, these observations would suggest the importance of c-Met as therapeutic target in faslodex-resistant disease.

Chapter Seven

References

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...' "

Isaac Asimov (1920-1992)

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8 Appendix

8.1 Reverse Transcription and PCR – Buffers and Solutions

8.1.1 10x PCR buffer

Component	Stock concentration	For 20ml	Final concentration
Tris-HCl	0.5M (ph 8.3)	4ml	100mM
KCl	1M	10ml	500mM
$MgCl_2$	1M	0.3ml	15mM
Gelatine	2% (w/v)	0.1ml	0.01% (w/v)
Pure H_2O (Sigma)	-	5.6ml	-

8.1.2 50x Tris-Acetate-EDTA (TAE) Buffer

Component	For 1L	Final concentration
Tris (Base)	242g	2M
Glacial Acetic Acid	57.1ml	1M
EDTA (0.5m; pH 8.0)	100ml	0.05M

- Adjust pH to 8.3 and make up to 1L with distilled H_2O.

8.1.3 Sample Loading Buffer for Agarose Gel Electrophoresis

Component	For 10ml	Final concentration
Sucrose	6g	60% (w/v)
Bromophenol Blue	0.025g	0.25% (w/v)

- Make up to 10 ml with RNAse-free H_2O.

8.2 SDS-PAGE and Western Blotting – Buffers and Solutions

8.2.1 Cell Lysis Buffer

Component	For 100ml	Final concentration
Tris Base	0.61g	50mM
EGTA	0.19g	5mM
NaCl	0.87g	150mM
Triton X-100	1ml	1% (v/v)
H_2O	100ml	-

- Adjust pH to 7.5 with HCl (5M).
- Add protease and phosphatase inhibitors immediately before use.

Chapter Eight

Appendix

8.2.2 Phosphatase and Protease Inhibitors

Inhibitor	Stock concentration	Solvent	Volume added to 10ml cell lysis buffer	Final concentration in cell lysis buffer
Sodium orthovanadate	100mM	H_2O	200µl	2mM
PMSF	100mM	Isopropanol	100µl	1mM
Sodium fluoride	2.5M	H_2O	100µl	25mM
Sodium molybdate	1M	H_2O	100µl	10mM
Phenylarsine	20mM	Chloroform	10µl	20µM
Leupeptin	5mg/ml	H_2O	20µl	10µg/ml
Aprotinin	2mg/ml	H_2O	40µl	8µg/ml

8.2.3 BSA Standard Curve for Bio-Rad D_C Protein Assay (50µl)

BSA concentration (mg/ml)	BSA (µl) (1.45mg/ml stock)	Cell lysis buffer (µl)
0.00	0.0	50.0
0.25	8.5	41.5
0.50	17.5	32.5
0.75	26.0	24.0
1.00	34.5	15.5
1.45	50.0	0.0

8.2.4 SDS-PAGE Running Buffer

Component	For 1L	Final concentration
Tris base	3.03g	0.25M
Glycine	14.4g	1.92M
SDS	1g	0.1% (w/v)
H_2O	1L	-

- Adjust pH to 8.3 with HCl (5M).

8.2.5 Laemmli Sample Loading Buffer

Component	3 x Stock (10ml)	Final concentration
SDS	0.6g	2% (w/v)
Glycerol	3ml	10% (v/v)
Tris base	3.6ml (0.5M)	60mM
H_2O	Make up to 10ml	-
Bromophenol Blue	0.03g	0.01% (w/v)

- Add 23.1 mg di-thiothreitol per ml 3x Laemmli sample loading buffer immediately prior to use.

8.2.6 Western Blot Transfer Buffer

Component	For 1L	Final concentration
Tris base	3.03g	0.25M
Glycine	14.4g	1.92M
Methanol	200ml	20% (v/v)
H_2O	800ml	-

8.2.7 Tris-Buffered Saline (TBS)

Component	For 1L	Final concentration
Tris base	1.21g	10mM
NaCl	5.8g	100mM
H_2O	1L	-

- Adjust to pH 7.6 with HCl (5M).

8.2.8 TBS-Tween (TBS-T)

TBS (see section 8.2.7 above) containing 0.05% (v/v) Tween 20.

8.3 Zymography – Buffers and Solutions

8.3.1 Non-reducing sample loading buffer

Same composition as 3x Laemmli sample loading buffer (section 8.2.5) but without the addition of di-thiothreitol.

8.3.2 Activation buffer

Component	For 1L	Final concentration
Tris base	6g	50mM
Ca_2Cl	1.48g	10mM
H_2O	1L	-

- Adjust to pH 7.6 with HCl (5M).

8.3.3 Stain stock solution

- For a 0.2% stock solution 1 PhastBlue® tablet was dissolved in 80ml of distilled H_2O and stirred.
- 120ml methanol was added and the solution filtered using Whatman filter paper to remove undissolved stain particles.

8.3.4 Destain solution

- 1L destain solution consisted of 100ml glacial acetic acid, 300ml methanol and 600ml distilled H_2O.

8.4 Immunofluorescence microscopy – Buffers and Solutions

8.4.1 Immunofluorescence buffer

PBS containing 1% (w/v) BSA.

8.4.2 Permeabilisation buffer

Immunofluorescence buffer (section 8.4.1) containing 0.2% (v/v) Triton X-100.

8.4.3. Block

Immunofluorescence buffer containing 10% (v/v) normal goat serum.

8.5 Tabulated summary of PCR data shown in figure 4.15

The changes in expression levels of MMPs and TIMPs in WTMCF-7 and FAS-R cells following growth factor challenge shown in **figure 4.15** have been summarised for each single gene below. Within each of the two cell types, expression levels following each treatment were compared to those of the control and accordingly categorised as "increased", "decreased", "no expression" or "no change".

A

MMP-1	TGF-α	EGF	HGF/SF	IGF-II	TPA
WTMCF-7	increased	increased	increased	decreased	increased
FAS-R	decreased	decreased	increased	no expression	increased

B

MMP-2	TGF-α	EGF	HGF/SF	IGF-II	TPA
WTMCF-7	increased	increased	increased	increased	no expression
FAS-R	decreased	increased	decreased	decreased	decreased

C

MMP-9	TGF-α	EGF	HGF/SF	IGF-II	TPA
WTMCF-7	increased	increased	increased	decreased	increased
FAS-R	increased	increased	increased	decreased	increased

D

MMP-10	TGF-α	EGF	HGF/SF	IGF-II	TPA
WTMCF-7	increased	increased	increased	decreased	increased
FAS-R	no expression	no change	increased	decreased	increased

E

MMP-11	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	decreased	decreased	decreased	decreased	decreased
FAS-R	decreased	decreased	decreased	decreased	increased

F

MMP-13	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	decreased	decreased	decreased	decreased	increased
FAS-R	decreased	decreased	decreased	decreased	decreased

G

TIMP-1	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	increased	increased	increased	increased	increased
FAS-R	increased	increased	increased	decreased	increased

H

TIMP-2	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	decreased	increased	decreased	decreased	decreased
FAS-R	increased	increased	no change	decreased	increased

I

TIMP-3	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	decreased	increased	increased	decreased	decreased
FAS-R	decreased	decreased	decreased	decreased	decreased

J

TIMP-4	**TGF-α**	**EGF**	**HGF/SF**	**IGF-II**	**TPA**
WTMCF-7	increased	increased	decreased	increased	increased
FAS-R	increased	increased	increased	increased	increased