Chronic exposure to fulvestrant promotes overexpression of the c-Met receptor in breast cancer cells: implications for tumour–stroma interactions


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Abstract

Our previous investigations using cell models of tamoxifen resistance have shown that the acquisition of an endocrine-insensitive state is accompanied by an invasive in vitro phenotype. In this study, we wished to determine whether this was specifically related to partial oestrogen receptor agonists or whether similar phenomena arise with the newer ‘pure’ anti-oestrogens, exemplified by fulvestrant. Our data demonstrate that the development of fulvestrant resistance in two breast cancer cell lines, MCF7 and T47D, is accompanied by an augmented migratory and invasive phenotype in vitro and overexpression of the HGF/SF receptor, c-Met. Importantly, upregulated c-Met expression in these cells facilitates their stimulation by HGF/SF-secreting stromal fibroblasts, leading to the activation of Src, Akt and ERK1/2 and a profound enhancement of their aggressive phenotype in vitro. These effects could be specifically attributable to activation of the c-Met receptor since the inclusion of neutralising antibodies to c-Met, or siRNA-mediated knockdown of c-Met expression, suppressed both invasion and migration stimulated by either exogenous HGF/SF, fibroblast-conditioned medium or following co-culture with fibroblast cells. Together, these in vitro data suggest that the development of fulvestrant resistance in vivo may confer a metastatic advantage to the cells by allowing their migratory and invasive behaviour to be augmented by surrounding stromal cells.

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Introduction

Breast cancer is the most common female cancer in the Western world. Although administration of anti-oestrogenic compounds, exemplified by tamoxifen, are successful in approximately two-thirds of patients with oestrogen receptor (ER)-positive disease, the majority of patients will develop anti-oestrogen resistance, leading to further disease progression (Osborne & Schiff 2003). In the case of tamoxifen, the phenomenon of resistance may partially be explained through its ability to act both as an ER antagonist, through the suppression of oestrogen-dependent, AF2-mediated gene transcription, and as an agonist of the ER, allowing AF1-mediated ligand-independent transcription. Thus, continued exposure to anti-hormonal agents, which exert a partial ER-agonistic function, may alter the transcriptional profile of the cell, modifying its growth characteristics and ultimately resulting in the development of resistant disease. We have previously demonstrated that endocrine (tamoxifen) resistance in breast cancer is associated with an increase in expression and activation of members of the epidermal growth factor receptor (EGFR) family of growth factor receptors (Knowlden et al. 2003). Moreover, such resistant cells possess a significantly enhanced capacity for migration and invasion in vitro (Hiscox et al. 2004), partially mediated through EGFR signalling.

Fulvestrant (faslodex; ICI 182,780) represents a novel class of endocrine agent for the treatment of breast cancer (Howell et al. 2000, Osborne et al. 2004).
These compounds differ significantly from tamoxifen in their mode of action, promoting the rapid degradation of ER in the cancer cell. Significantly, these compounds also show no oestrogen agonist activity and thus have been regarded as an important improvement in breast cancer therapy. Although we and others have previously demonstrated that acquired resistance to fulvestrant can occur in breast cancer cells following chronic exposure to this agent (McClelland et al. 2001, Sommer et al. 2003), these studies have primarily addressed resistance in terms of cellular growth; it is presently unknown whether such resistance promotes an aggressive cancer cell phenotype in vitro, as is the case with tamoxifen.

In vivo, the tumourigenic potential of cancers is profoundly influenced by their microenvironment. The release of soluble factors from stromal fibroblasts allows paracrine regulation of epithelial cell behaviour including growth, differentiation, migration and invasion; this reciprocal communication between cells is often deregulated in cancer progression (reviewed in Bhowmick et al. 2004, Bhowmick & Moses 2005). Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional cytokine produced by stromal cells and exerts its effects in a paracrine fashion, through activation of the c-Met receptor protein, which is frequently expressed on the cells of epithelial origin (Comoglio & Trusolino 2002, 2005, Kataoka et al. 2003). Activation of the c-Met receptor results in a diverse array of cellular responses both in vitro and in vivo, promoting loss of cell–cell adhesion coupled with enhanced cell migration (cell ‘scattering’) and invasion (Jiang & Hiscox 1997, Parr & Jiang 2001). Therefore, HGF/SF-c-Met signalling has been implicated as playing a central role in promoting tumour progression and metastasis. In breast cancer, a role for HGF/SF-c-Met has been demonstrated with a number of recent studies revealing correlations between high levels of c-Met expression and a significantly reduced survival rate (Ghoussoub et al. 1998, Camp et al. 1999, Edakuni et al. 2001, Elliott et al. 2002, Lee et al. 2005). Furthermore, microarray analysis of breast tumour samples has demonstrated c-Met to be a stronger prognostic indicator than traditional markers, such as Her2/neu and EGFR (Tolgay Ocal et al. 2003, Lengyel et al. 2005). These and other studies have thus highlighted c-Met as a potential prognostic factor for breast cancer patients.

In this report, we provide evidence that c-Met receptor expression is induced in breast cancer cells following their chronic exposure to the anti-oestrogen fulvestrant. Significantly, our data demonstrate that the expression of c-Met in these resistant cells dramatically influences their behaviour when in co-culture with HGF/SF-producing fibroblasts, resulting in cell scattering and enhanced migration and invasion. These observations suggest that the development of resistance to ‘pure’ anti-oestrogens may alter the expression of key growth factor receptors that, in vivo, may confer a metastatic advantage to the tumour by enhancing their interaction with the cells within the surrounding stroma, ultimately aiding tumour progression and spread.

**Materials and methods**

**Cell culture and generation of fulvestrant-resistant breast cancer cells**

Fulvestrant-resistant (FAS-R) breast cancer cells were generated as reported previously (McClelland et al. 2001). Briefly, FAS-R MCF7 cells were generated through continual exposure to $10^{-7}$ M fulvestrant (Faslodex, ICI 182,780; a gift from Dr Alan Wakeling, AstraZeneca Pharmaceuticals, Macclesfield, UK) in experimental medium (phenol-red free RPMI medium containing 5% (v/v) charcoal-stripped (steroid-depleted) FCS supplemented with glutamine, fungizone and penicillin/streptomycin as earlier). After an initial period of growth inhibition (~3 months), the growth rate of these cells increased, suggesting resistance to the growth inhibitory properties of fulvestrant. Following an additional 3 months of culture in the presence of fulvestrant, a fully resistant cell line was established and designated FAS-R. To ensure accurate comparisons, all subsequent treatments of both the parental and the resistant cell lines were carried out in routine medium (supplemented with $10^{-7}$ M fulvestrant in the case of FAS-R cells). FAS-R T47D cells were derived using the same protocol as for MCF7 cells described earlier.

The MRC5 fibroblast cells were obtained from the ATCC and, after initial establishment in Dulbecco’s modified Eagle’s medium (DMEM), were subsequently maintained in experimental medium as earlier. For MRC5-conditioned medium, log-phase cultures were cultured for a period of 4 days after which the medium was removed, filtered through a 0.22 µm filter unit (Millipore Ltd, Watford, UK) and used immediately.

**Antibodies and reagents**

A c-Met (pan) antibody was obtained from Zymed Laboratories Ltd (Cambridge BioScience, Cambridge, UK) whilst antibodies recognising activated c-Met (phosphorylated at Y1234/5 and Y1349/56), along with antibodies against total and activated (Y419) Src,
total and activated (Ser 437) Akt, and total and activated (T202/T204) ERK1/2 were from Cell Signalling Technology (New England Biolabs Ltd, Hitchin, Hertfordshire, UK). A c-Met neutralising antibody was from R&D Systems (Abingdon, Oxford, UK). All other antibodies and reagents were purchased from Sigma, unless otherwise stated.

**Immunocytochemistry**

Cells were seeded onto sterile 3-aminopropyl-triethoxysilane-coated glass coverslips housed in 35 mm petridishes, allowed to grow to log phase and fixed using 3.7% formaldehyde with a post-fixation step in methanol/acetone at −20 °C prior to immunostaining for total c-Met. Detection of bound antibody was by means of an avidin–biotin:horse radish peroxidase (HRP) antibody system in conjunction with a diaminobenzidine tetrahydrochloride (DAB) substrate.

**Cell lysis and western blotting**

Cells were cultured to log phase following, which the medium was replaced with plain medium (as control), medium containing HGF/SF (20 ng/ml unless otherwise indicated) or MRC5 conditioned medium. For co-culture experiments, breast cancer cells were cultured in 12-well plates into which were inserted 2 cm diameter, Transwell chambers containing MRC5 cells growing in log phase on a porous membrane (0.2 μm pore size). Following culture for the times detailed in the text, the breast cancer cell monolayers were washed twice with ice-cold PBS and lysed in 250 μl lysis buffer (50 mM Tris, pH 7.5, 5 mM EGTA, 150 mM NaCl and 1% Triton X 100) containing protease inhibitors (2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenyl-methylsulfonyl fluoride, 20 μM phenylarsine, 10 mM sodium molybdate, 10 μg/ml leupeptin and 8 μg/ml aprotinin). The lysates were placed on ice for 20 min with intermittent mixing and clarified by centrifugation (10 min, 13 000 r.p.m., 4 °C). The concentration of solubilised proteins was then determined using the DC protein assay kit (Bio-Rad).

Fifty micrograms of total protein from these lysates were separated by SDS-PAGE using 8% gels and transferred to nitrocellulose membranes by electrophoretic transfer. Membranes were subsequently blocked with 5% (w/v) milk protein in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TTBS). Blots were then incubated with primary antibodies as indicated, washed in TTBS and incubated with HRP-conjugated secondary antibodies. An enhanced chemiluminescence system (‘West Dura’ reagent, Pierce and Warriner Ltd, Chester, UK) was used for subsequent detection of bound antibodies and the blots exposed to X-ray film (Kodak). Blots shown are representative of a minimum of three separate experiments.

**Cell migration assay**

Porous filters (8 μm pore size) of Transwell chambers (Costar, Cambridge, MA, USA) were pre-coated with fibronectin (10 μg/ml, Sigma) for 2 h at 37 °C and subsequently rinsed in 1× PBS before air drying in a sterile tissue culture hood. The coated inserts were then placed into the lower chamber containing 600 μl medium ± HGF/SF (20 ng/ml) or fibroblast-conditioned medium. In the case of co-culture experiments, 5×10^3 MRC5 cells were seeded into the wells, 3 days prior to the experiments in order to establish fibroblast colonies growing in log phase. Fulvestrant-sensitive or resistant breast cancer cells were subsequently seeded into these inserts at 5×10^4 cells/well and allowed to migrate to the underside of the membrane for a period of 20 h. The non-migratory cells on the upper surface of the membrane were then removed with a cotton swab and the cells that had migrated to the underside of the membrane were fixed in 3.7% formaldehyde in PBS and stained with 0.5% crystal violet. The number of migratory cells per membrane was subsequently counted using an inverted microscope with a (×20 objective. Each determination represents the average of a minimum of three individual experiments, each performed in duplicate with the error bars showing the S.D.

**Matrix invasion assay**

Cell invasion was determined using invasion chambers possessing 8 μm porous membranes (Beckton–Dickenson) coated with Matrigel (0.4 μg/ml). Cells were seeded into the chambers (10^5 cells/well) with or without HGF/SF (20 ng/ml) or in MRC5-conditioned medium whilst 600 μl medium was added to the outside of the well. For co-culture experiments, MRC5 cells were allowed to grow to log phase in the plate wells as described earlier, prior to seeding the epithelial cells into the upper chambers. Inserts were cultured at 37 °C in a tissue culture incubator for 24 h, after which the non-invasive cells and Matrigel were removed from the inside of the wells with a cotton swab. After fixing the invaded cells with 3.7% formaldehyde, the porous membranes were removed from the invasion chamber using a scalpel blade and mounted onto a glass microscope slide using Vectashield (Molecular Probes, Eugene, OR, USA) containing the
nuclear stain, 4',6-diamidino-2-phenylindole (DAPI). Cell invasion was quantified by viewing five separate fields per membrane at ×20 magnification and counting the number of cells in each field. Data were then plotted at mean cells per field ± S.D. for a minimum of three independent experiments, each performed in triplicate.

Cell growth assay
Cells were grown for 7 days in growth-factor free medium in the presence of increasing concentrations (0–80 ng/ml) of HGF/SF. Changes in cell growth were then evaluated by means of trypsin dispersion of the cells and subsequent counting using a Coulter Counter (Beckman, Luton, UK).

Cell scattering assay
wtMCF7 and FAS-R cells were seeded into the wells of a 12-well plate and allowed to reach log-phase growth. Medium was replaced with plain medium (control), MRC5-conditioned medium, medium containing HGF/SF (20 ng/ml) or, in the case of co-culture experiments, a chamber containing MRC5 cells growing on a porous insert (0.2 μm pore size) was introduced into the well so that the two cell populations shared the same medium but had no physical contact. After 24 h, the cells were fixed in 3.7% formaldehyde, and the selected colonies photographed.

Affymetrix analysis of gene expression
RNA for microarray analysis was isolated from triplicate cultures of wtMCF7 and FAS-R cells during log-phase growth as follows: cells were lysed in situ with TriReagent (Sigma), scraped into 1.5 ml micro-centrifuge tubes and stored at −80 °C overnight. Total RNA was isolated as per reagent protocol with subsequent DNase1 treatment and RNA clean-up using RNAeasy Mini Columns (Qiagen). Following quantification and determination of RNA integrity using denaturing agarose gel electrophoresis, samples were used in Affymetrix U133A Genechip analysis (Central Biotechnology Services, Cardiff University, Cardiff, UK). Hybridised arrays were scanned and data output generated using Microarray Suite 5.0 (MAS5.0) software (Affymetrix) and data quality confirmed through analyses of internal control gene expression. Comparative gene expression was performed using median-normalised, log-transformed data using an online software package (www.genesifter.net). Results are presented as the mean signal intensity for each transcript in wtMCF7 and FasR cells.

Wound healing assay
Confluent wtMCF7 or FAS-R cell monolayers were scratched with a pipette tip to create a cell ‘wound’. After 24 h culture in medium containing HGF/SF (20 ng/ml), conditioned medium or in co-culture with MRC5 cells as described earlier, the extent of cell migration into the wound space (‘wound healing’) was determined by fixing the cells with 3.7% formaldehyde in PBS, staining the cells with DAPI and viewing under fluorescence illumination.

siRNA-mediated inhibition of c-Met expression
SMARTpool siRNA against human c-Met was obtained from Dharmacon Ltd (Perbio Science UK Ltd, Northumberland, UK) and used according to the manufacturers instructions. Briefly, FAS-R cells were seeded into 12-well plates at 2 × 10^5 cells/well in antibiotic-free medium. After 24 h culture, the medium was replaced with fresh, antibiotic-free medium or medium containing transfection lipid, 50 nM non-targeting siRNA control or 50 nM SMARTpool siRNA specific for c-Met. Cells were assayed for c-Met gene and protein expression after 48 h by RT-PCR and western blotting. In some samples, cells were exposed to 10 ng/ml HGF/SF or conditioned medium for 5 min prior to lysing. For migration and invasion assays, cells were treated with siRNA and appropriate controls for 24 h following which they were trypsinised, counted and seeded into migration/invasion assay systems as described earlier in the presence of the lipid/siRNAs. After a further 24 h culture in these respective assay systems, migratory/invasive cells were fixed, stained and counted.

RT-PCR amplification
Total RNA was extracted using Qiagen RNeasy kit (Qiagen) and was reverse transcribed to cDNA with Superscript reverse transcriptase (Invitrogen). The forward and reverse PCR primers used to amplify c-Met and HGF-specific gene sequences from the cell cDNA were Met-F (5' ACA TTC TCC TAT GTG GAT CCT GTA A 3') and Met-R (5' ATC TCT GAA TTA GAG CGA TGT TGA C 3'), HGF-F (5' GCC TGA AAG ATA TCC CGA CA 3') and HGF-R (5' GCC TGA AAG ATA TCC CGA CA 3') respectively. The reactions were carried out in 25 μl volumes using 2.5 μL BioTaq Taq polymerase (Bioline, London, UK) with 27 cycles consisting of a 94 °C denaturation step for 30 s followed by a 60 °C annealing step for 60 s and an extension step for 90 s at 70 °C. In order to semi-quantify levels of c-Met and HGF/SF expression,
β-actin was also amplified from the same cDNA using the primers β-actin-F (5' GGA GCA ATG ATC TTG ATC TT 3') and β-actin-R (5' TCC TGA GGT ACG GGT CCT TCC CCT 3') using the same cycling conditions described earlier. The amplified products were then visualised with ethidium bromide following separation on 2% agarose gels.

Results

The development of fulvestrant-resistance in breast cancer cells is accompanied by a gain in migratory and invasive capacity in vitro

We have recently demonstrated that resistance to tamoxifen in poorly-invasive MCF-7 cells promotes an aggressive, invasive phenotype (Hiscox et al. 2004). Here, we show that acquired resistance to the ‘pure’ anti-oestrogen, fulvestrant, in MCF7 cells also results in an augmented migratory (Fig. 1A and B) and invasive (Fig. 1C) capacity in vitro. Previously, we demonstrated that FAS-R cells have an elevated growth rate compared with wtMCF7 cells, an effect that is only apparent after 7 days in culture (McClelland et al. 2001). Although our invasion assays were performed for 36 h, in order to discount any growth effects on the increased cell invasion observed, invasion assays were performed with the inclusion of the growth-inhibitor, aphidicholin (Fig. 1D). These experiments revealed no significant difference between the growth-inhibited cells or control cell invasion.

Fulvestrant-resistant breast cancer cells over-express the c-Met gene and protein


Affymetrix analysis demonstrated that c-Met gene expression was elevated in FAS-R cells compared with wtMCF7 cells.

Figure 1 Fulvestrant resistant cells display increased migration and invasion. (A) FAS-R and fulvestrant-sensitive (wtMCF7) cells were seeded onto fibronectin-coated porous membranes. After 24 h, migratory cells were fixed, stained and counted. (B) Confluent monolayers of wtMCF7 and FAS-R cells were wounded with a pipette tip and, following 24 h culture, fixed, stained with DAPI and visualising with immunofluorescent microscopy to determine the extent of cell migration into the wound space. (C) In vitro cell invasion assays using an artificial basement membrane (Matrigel) were used to assess the invasive capacity of the cells. After 72 h incubation, invasive cells were fixed, stained and counted. (D) Basal FAS-R cell invasion was performed in the presence or absence of the growth inhibitor, aphidicholin. FAS-R cells were significantly more migratory ((A) and (B)) and invasive (C) when compared to their parental cells.*P<0.005 vs wtMCF7 cells.
their parental cells, an observation subsequently confirmed by reverse transcriptase (RT)-PCR (Fig. 2A and B). Immunocytochemical staining of c-Met protein revealed high levels of membraneous c-Met in FAS-R cells, with predominant staining at intercellular junctions, compared with only very weak staining in wtMCF7 cells (Fig. 2C). Western blotting further confirmed these observations, with a prominent band detected at 145 kDa in FAS-R lysates corresponding to the expected size of the c-Met receptor β-unit (Fig. 2D). A lesser signal was also observed at ~190 kDa, representing the mature c-Met heterodimer. c-Met was undetectable in wtMCF7 lysates (Fig. 2D) and subsequent overexposure of these blots produced only a weak signal in wtMCF7 samples (data not shown). Since autocrine c-Met activation has been described in several aggressive cancer phenotypes (Otsuka et al. 1998, Yi & Tsao 2000, Su et al. 2004) and can enhance the invasive and migratory capacity of tumour cells, we examined whether c-Met was constitutively activated in FAS-R MCF7 cells. Immunoprobing of cell lysates using antibodies, which specifically recognise activated (autophosphorylated) c-Met (c-Met phosphorylated at Y1234/5 or Y1349/56) revealed that a weak signal for phosphorylated c-Met could be detected in FAS-R cells, but only at the tyrosine 1234/5 residue (Fig. 2D); no c-Met activity was detectable in wtMCF7 cells.

**Activation of c-Met in FAS-R cells by exogenous ligand greatly enhances their in vitro invasive phenotype**

In the light of a low level of endogenous c-Met activation in FAS-R cells, we next addressed the question of whether the c-Met receptor was fully functional. Following HGF/SF stimulation (0–80 ng/ml), c-Met phosphorylation at both Y1234/5 and Y1349/1356 was readily detectable in FAS-R cells but not wtMCF7 cells (Fig. 3A). With prolonged exposure of the blots to X-ray film, c-Met activity was seen in wtMCF7 cells but only at the highest concentration of ligand tested (80 ng/ml). Since phosphorylation of c-Met facilitates the activation of downstream regulators of cell morphology, migration and invasion (Maulik et al. 2002, Bolanos-Garcia 2005), we assessed the effects of HGF/SF on the activity of a subset of molecular intermediates known to be involved in these pathways. Our data revealed that following c-Met phosphorylation, elevated Src, Akt, ERK1/2, focal adhesion kinase (FAK) and paxillin activities were seen (Fig. 3B). Furthermore, whereas HGF/SF significantly elevated the migratory and invasive capacity of FAS-R cells in vitro, only a small increase was observed for wtMCF7 cells (Fig. 4A and B). Additionally, treatment of FAS-R cell cultures with HGF/SF for 24 h resulted in significant colony scattering, whereas little effect could be seen in wtMCF7 cell colonies (Fig. 4C). Interestingly, although c-Met activation has been demonstrated to promote proliferation in certain cell types (Wang et al. 2003, Taniguchi et al. 2004), HGF/SF did not produce any significant change in the growth of either cell type over a period of 7 days (Fig. 4D).

**Activation of c-Met in FAS-R cells by fibroblasts cells further enhances their invasive phenotype**

The ability of stromal fibroblasts, natural producers of HGF/SF, to modulate the behaviour of c-Met-expressing epithelial cells has been known for some time (Di Renzo et al. 1991, Sonnenberg et al. 1993). We thus tested the hypothesis that the invasive phenotype of c-Met-overexpressing FAS-R cells could be further enhanced by these cells. RT-PCR confirmed presence of HGF/SF gene expression in MRC5 fibroblasts but not in FAS-R or their endocrine-sensitive wtMCF7 counterparts (Fig. 5A). Culture of FAS-R cells in MRC5-conditioned medium or in co-culture with MRC5 cells themselves resulted in activation of the c-Met receptor (Fig. 5B) and subsequent scattering of FAS-R cell colonies (Fig. 5C shows data from co-culture experiments). Furthermore, both MRC5-conditioned medium and co-culture with these cells significantly enhanced the migratory and invasive capacity of FAS-R cells but not wtMCF7 cells (Fig. 5D and E). This behaviour was significantly reduced with inclusion of a neutralising antibody to the c-Met receptor (Fig. 5F and G).

To further ascertain whether these events were specifically attributable to c-Met activation, c-Met expression in FAS-R cells was attenuated by transfection of the cells with c-Met siRNA. siRNA treatment significantly reduced the levels of both c-Met gene (Fig. 6A) and protein (Fig. 6B) whilst the corresponding controls (medium only and medium containing the delivery lipid or non-targeting siRNA) had no significant effect. siRNA knockdown of c-Met prevented subsequent activation of c-Met by either exogenous HGF/SF (Fig. 6C) or following fibroblast co-culture (data not shown), along with preventing both HGF/SF and fibroblast-induced migration and invasion (Fig. 6C–E; data from co-culture experiments shown). Interestingly, these experiments also revealed that siRNA-mediated inhibition of c-Met
Figure 2  FAS-R cells overexpress the c-Met receptor. (A) Pairwise comparative analysis of Met proto-oncogene expression using Affymetrix Probe Set 213807_x (Gene Accession no. X54559) revealed a 3.06-fold higher level of c-Met gene expression in FAS-R cells compared with wtMCF7 controls (*P<0.05 vs wtMCF7 cells). (B) This was subsequently confirmed through RT-PCR (gel shows data from three separate cell pairs; mean signal intensity obtained by densitometry is shown on accompanying graph). Corresponding analysis of c-Met protein expression was determined by both immunohistochemical staining ((C) scale bar represents 20 \( \mu \)m) and western blotting ((D) accompanying graph shows densitometric analysis of three separate experiments). For the western blots, cell lysates were also probed with phospho-specific antibodies which recognise the activated form of the c-Met receptor (c-Met phosphorylated at Y1234/5 and Y1349/56). FAS-R cells demonstrated significantly elevated levels of both c-Met gene and protein compared with their endocrine-sensitive counterparts.
expression also reduced the basal invasive and migratory capacity of FAS-R cells compared with the control treatments (Fig. 6E).

Chronic exposure to fulvestrant induces c-Met expression in additional breast cancer cell lines and greatly enhances their fibroblast-mediated invasive capacity

To explore whether our observations in MCF7 cells were cell-type specific or whether they represented a more generic consequence of Fulvestrant exposure, we generated a FAS-R variant of T47D cells (T47D\textsuperscript{FAS-R}), known to express low levels of the c-Met receptor (Ronen et al. 1999), and examined c-Met receptor expression and function. Chronic exposure of these cells to fulvestrant promoted a marked elevation of both c-Met mRNA and protein expression (Fig. 7A and B) and enhanced both their basal invasive capacity and that stimulated by MRC5 fibroblasts (Fig. 7C). Furthermore, whilst reduction of c-Met protein expression by siRNA promoted a modest, but not significant, decrease in basal T47D\textsuperscript{FAS-R} cell invasion, siRNA treatment prevented almost all T47D\textsuperscript{FAS-R} invasion stimulated by co-culture with MRC5 fibroblast cells (Fig. 7D and E).

Discussion

The aberrant expression of growth factor receptors is a frequent event in human tumours, and can result in a more aggressive cell phenotype. Frequently, altered
growth factor receptor expression accompanies endocrine-resistance in breast cancer both in vitro and in vivo, where it may facilitate the growth of these cells in the absence of hormonal stimulus (Nicholson et al., 1999, 2005). Clinically, the development of resistance to endocrine therapies presents a significant obstacle to the successful treatment of breast cancer patients. Our recent data using cell models of tamoxifen resistance has established a link between the acquisition of an endocrine resistant state and a gain in migratory and invasive behaviour; this is due, in part, to the altered growth factor signalling (Hiscox et al., 2004, 2006). Newer anti-oestrogenic agents, which do not have the agonistic effects observed with tamoxifen, are now being used to treat breast cancers and include the drug fulvestrant (faslodex, ICI 182,780) (Howell et al., 2000). However, recent evidence from our group and others suggests that chronic exposure of breast cancer cells to such agents also results in endocrine resistance (McClelland et al., 2001, Sommer et al., 2003). Despite these studies, it is presently unknown whether fulvestrant-resistance also promotes an aggressive phenotype.

In this report, we show that chronic exposure to fulvestrant enhances the migratory and invasive capacity of breast cancer cells in vitro compared with their parental cells (Figs 1A–C and 7C). In MCF7 cells, we have previously reported that fulvestrant resistance promotes increased expression of the EGFR receptor (McClelland et al., 2001), thus this presented a potential mechanism underlying their enhanced invasive phenotype. However, our investigations have failed to demonstrate EGFR-driven migratory or invasive responses in these cells. Given the well-established role that the c-Met receptor tyrosine kinase plays in promoting such behaviour in tumour cells, we subsequently investigated its expression in these cells. Initial comparisons of gene expression patterns between wtMCF7 and FAS-R cells using Affymetrix analysis revealed a marked increase in c-Met gene expression (Fig. 2A), which was subsequently confirmed by RT-PCR (Fig. 2B). Furthermore, whereas wtMCF7 cells had only barely detectable levels of the receptor, its expression at the protein level was greatly elevated in FAS-R cells, particularly on the cell membrane (Fig. 2C and D). This is interesting in view of previous reports, which have demonstrated a correlation between the high levels of c-Met membrane staining and the lymph node involvement (Lengyel et al., 2005) and thus may be a marker for an aggressive cell phenotype. Furthermore, this appeared not to be a cell-specific effect since exposing an additional breast cancer cell line (T47D) to fulvestrant resulted in increased c-Met gene and protein expression (Fig. 7A and B).

An intriguing question is how chronic exposure to fulvestrant might modulate c-Met expression in breast cancer cells.

Figure 4 c-Met activation promotes FAS-R cell migration, invasion and scattering but not growth. The effect of c-Met activation by exogenous ligand on cell behaviour was assessed using in vitro migration (A), invasion (B) and scattering (C) assays. HGF/SF promoted a dose-dependent stimulation in FAS-R cell motility and invasion but had only a modest effect on wtMCF7 cells and only at the highest concentration. Interest-
ingly, treatment of either cell type with HGF/SF over a period of 7 days did not alter their growth (D). *P<0.01 vs control; †P<0.001 vs control.
cancer cells. As we and others have previously reported, ER protein expression is lost in FAS-R breast cancer cells (McClelland et al. 2001, Sommer et al. 2003); however, whilst c-Met expression may indicate a poor prognosis in breast cancer patients, its expression does not correlate with ER status (Ghoussoub et al. 1998, Lengyel et al. 2005).

c-Met gene transcription has been demonstrated to be regulated by a number of factors, including members of the widely expressed Sp family of transcription factors (Zhang et al. 2003, 2005), whilst Sp1 activity itself may be influence by ER signalling (Kim et al. 2005, Sumi & Ignarro 2005). Furthermore, a recent report by Varshochi et al. (2005) has demonstrated that

**Figure 5** Fibroblasts strongly stimulate c-Met activation and subsequent cell migration and invasion in FAS-R cells. (A) mRNA from endocrine-sensitive (wtMCF7) and -insensitive (FAS-R) cells, along with MRC5 fibroblasts, was assessed for expression of HGF/SF and c-Met transcripts by RT-PCR. Only MRC5 cells expressed HGF/SF mRNA whilst c-Met gene expression was observed only in wtMCF7 and FAS-R cells. (B) Activation of the c-Met receptor in either wtMCF7 or FAS-R cells following addition of exogenous ligand (HGF/SF, 20 ng/ml), MRC5-conditioned medium (c/m) or after co-culture with MRC5 cells as described in Materials and methods, all for 12 h, was assessed using western blotting. Whilst little effect was seen in wtMCF7 cells, c-Met became phosphorylated in FAS-R cells after each treatment. Subsequent co-culture of both cell types with MRC5 fibroblasts promoted FAS-R cell scattering (C), migration (D) and invasion (E). These effects in FAS-R cells were reduced with the inclusion of a neutralising antibody to the c-Met receptor ((F) and (G)). *P < 0.001 vs control; †P < 0.05 vs MRC5 stimulation alone.
fulvestrant treatment of MCF7 cells results in induction of the p21Waf1 gene through Sp1-mediated de-repression of its promoter. Interestingly, we have observed alterations in Sp1 and Sp3 expression in MCF7 cells on exposure to fulvestrant, which may thus represent one mechanism by which c-Met overexpression can be achieved. Studies are presently underway in our laboratory to address these issues further.

Since FAS-R cells display a high degree of intrinsic invasive behaviour, we hypothesised that c-Met might be constitutively activated in these cells as this has been reported for a number of tumour types, arising from either activating mutations within the receptor or from autocrine activation mechanisms (Prat et al. 1991, Follenzi et al. 2000, Jo et al. 2000, Wallenius et al. 2000, Wang et al. 2001). In our studies, we could detect only a very low level of phosphorylation of the receptor in both FAS-R MCF7 and T47D cells under basal conditions (Figs 2D and 7D). Although these cells do not express the c-Met ligand, the basal activity observed in the c-Met receptor may arise from transactivation via other growth factor receptors, as has been demonstrated in other cell types (Jo et al. 2000), a hypothesis which is currently being investigated within our group. Our data suggest that even this low level of c-Met activity may partially contribute to

Figure 6 siRNA-mediated inhibition of c-Met expression inhibits fibroblast-induced FAS-R migration and invasion. FAS-R cells were treated with culture medium (C), lipid (L), 50 nM non-targeting siRNA control (NT) or 50 nM siRNA specific for the c-Met gene (M) for 48 h, prior to analysis of c-Met gene or protein expression using RT-PCR and western blotting respectively. siRNA targeting of c-Met effectively reduced the level of both receptor gene (A) and protein (B) and inhibited both basal and HGF/SF-stimulated c-Met activity (C) arrow indicates position of activated c-Met at 145 kDa; densitometric analysis of western blots shown on accompanying graph). Further analysis of the effects of siRNA treatment was investigated using cell migration and invasion assays: siRNA-mediated reduction in c-Met expression suppressed basal and fibroblast-stimulated FASR migration (D) and invasion (E). *P<0.001 vs FAS-R cells in co-culture with MRC5 cells; †P<0.005 vs control (untreated) FAS-R cells.
the invasive phenotype of FAS-R MCF7 cells, since siRNA-mediated inhibition of c-Met expression reduced their basal invasive capacity (Fig. 6D).

However, in the case of FAS-R T47D cells, siRNA knockdown of c-Met expression had no significant effect on their basal invasion (Fig. 7E), suggesting that other, as yet unidentified, mechanisms mediate the invasive phenotype of these cells in vitro. The lack of strong endogenous c-Met activity was not due to a dysfunctional receptor, since addition of exogenous ligand to FAS-R cells promoted phosphorylation of the receptor with subsequent activation of downstream
signalling components (Fig. 3A and B). In addition to this, we observed a HGF/SF/c-Met-mediated profound increase in their migratory and invasion capacity compared with only a modest change in wtMCF7 cells (Fig. 4A and B). Treatment of FAS-R cell colonies with HGF/SF also promoted their scattering (Fig. 4C), an effect characteristic of c-Met activation. Interestingly, despite its reported ability to promote cell proliferation in a number of systems, c-Met activation did not alter the growth rate of either cell type (Fig. 4D). The effects of HGF/SF on cellular growth rely on c-Met mediated enhanced activation of the GRB2–SOS–RAS pathway and sustained MAPK activation (Trusolino & Comoglio 2002, Bardelli et al. 2005). Although our data demonstrate MAPK activation in response to HGF/SF treatment in FAS-R cells (Fig. 3B), time course studies reveal that these effects are diminished after 1 h treatment, compared with other downstream components, including Src and Akt (data not shown), suggesting that c-Met signalling through the mitogenic pathway in FAS-R cells is not sustained.

In vivo paracrine activation of c-Met, expressed primarily by epithelial cells, can occur through the ability of stromal fibroblasts to secrete HGF/SF. This mechanism has been implicated as a major contributory factor for tumour progression with studies demonstrating the ability of HGF/SF to regulate epithelial-to-mesenchymal transition (EMT) and metastasis (Thiery 2002). Therefore, we wished to investigate whether HGF/SF-secreting fibroblast cells could modulate the invasive phenotype of FAS-R cells which overexpress c-Met. Treatment of FAS-R cells with conditioned medium from MRC5 cells, known producers of HGF/SF (Jiang et al. 2003), greatly enhanced their migratory and invasive nature, an effect also observed following direct co-culture of both the cell types, whereas little effect was seen in their parental, endocrine-sensitive cells (Figs 5D, E and 7C). Since fibroblasts secrete a range of growth factors and cytokines that may modulate epithelial cell behaviour, it was necessary to determine whether these effects were c-Met specific. Our data show that the contribution of other fibroblast-secreted factors to this behaviour could be ruled out since antibody-mediated neutralisation of c-Met function or its suppression by siRNA targeting, both inhibited fibroblast-stimulated migration and invasion in FAS-R MCF7 and T47D cells (Figs 5F and G, 6D and E and 7D and E). Together, these data now provide a rationale for the extension of these studies into three-dimensional culture systems and in vivo models to determine whether similar effects are seen in these complex systems reflective of tumour progression.

The use of endocrine therapy in breast cancer is limited by the development of resistance, which frequently results in tumours of an aggressive phenotype. In this report, we show that chronic exposure of breast cancer cells to fulvestrant results in acquisition of an endocrine-resistant state, over-expression of the c-Met receptor and a marked increase in their migratory and invasive capacity in vitro. Importantly, increased c-Met expression in these cells allows their invasive behaviour to be further enhanced by HGF/SF-producing stromal fibroblasts. Since tumour invasion and spread may be critically influenced by the surrounding stroma, a process in which HGF/SF plays a central role, our data suggest that, in vivo, overexpression of c-Met in anti-hormone-resistant epithelial breast cancer cells may significantly affect tumour progression. Studies are now needed to determine whether c-Met plays a role in breast cancer patients relapsed on anti-hormone therapy.

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